Infection, Genetics and Evolution 96 (2021) 105143

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Genetic diversity of *plasmodium falciparum* isolates in Minna, North Central Nigeria inferred by PCR genotyping of Merozoite surface protein 1 and 2

Hadijah Usman-Yamman [a,*], Innocent Omalu C. J [a], Abdulkadir Abubakar [b], Abolarinwa S.O [a], Samuel Eke S. [c], Chidiebere A. Otuu [d]

[a] Department of Animal Biology, Federal University of Technology, Minna, Niger State, Nigeria
[b] Department of Biochemistry, Federal University of Technology, Minna, Niger State, Nigeria
[c] Department of Biology, Air Force Institute of Technology, Kaduna, Nigeria
[d] Parasitology and Public Health Research Unit, Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, Nigeria

ARTICLE INFO

Keywords:
Malaria
Plasmodium falciparum
Polymerase chain reaction (PCR)
Merozoite surface protein (msp)
Monoclonal
Polyclonal

ABSTRACT

North Central Nigeria is one region in Nigeria with a significant incidence of malaria caused majorly by *Plasmodium falciparum*. This study utilizes the *msp1* and *msp2* genes of *P. falciparum* to examine its diversity and multiplicity of infection (MOI). Blood samples were collected from 247 children across selected healthcare facilities in Minna, from infants and children aged 6 months to 17 years. Of the total collection, 143 (58%) of the children were infected with *P. falciparum* with parasite density $\geq$ 1000 µl, and from which fifty (50) samples was randomly selected and presented for PCR for the characterization of *msp1* and *msp2* gene using nested-PCR method. Overall, 57 *msp1* genotypes, including K1, MAD20 and RO33 were identified, ranging from (250-1000 bp), (100-500 bp) and (400-500 bp), respectively. In addition, 54 different *msp2* genotypes of FC27 and 3D7 alleles ranging from (100-900 bp) and (100-800 bp), respectively were selected. A monoclonal infection of 39% and a polyclonal infection of 61% was recorded, however, a particularity about this study is the polyclonal nature of RO33. Determination of gene diversity revealed MAD20 as the predominant allele for *msp1* with a mean MOI of 1.35 and FC27 for *msp2* with 1.72 MOI. The overall MOI recorded for the study was 1.60. There was, however, no statistical significance difference between MOI and age of the child ($P > 0.05$). Meanwhile, findings from this study revealed *P. falciparum* populations were not genetically diverse with Heterozygosity (*He*) index of 0.0636. However, a significant level gene diversity within the antigenic markers of *msp1* and *msp2* was observed with *He* index of 0.714 and 0.830, respectively. This study has demonstrated the potential of gene diversity and MOI of *P. falciparum*, as important markers for assessing differences in malaria transmission intensity. Continuous malaria genetic surveillance is therefore recommended as a fundamental tool for monitoring changes in gene types and for intervention programs' effectiveness.

1. Introduction

Malaria is a major global public health concern, especially in regions where transmission occurs repeatedly. In sub-Saharan Africa, malaria remains one of the contributing causes of morbidity and mortality, with about 24 million children estimated to be infected with *P. falciparum* in 2018. Sub-Saharan Africa and India carried almost 85% of global malaria burden, of which Nigeria alone accounts for 25% of the cases (WHO, World Health Organization, 2019).

P. falciparum parasite has the ability to undergo antigenic switching to evade a developing antibody response; and thus, within the mosquito gut, recombination of these distinct parasite clones occurs during zygote formation (Abukari et al., 2019), resulting in gene diversity of *P. falciparum.* Because of this diversity, the conformation of antimalarial drug targets is altered and then renders the parasites drug resistant (Abukari et al., 2019; Blasco et al., 2017) which hinders the outcome of malaria treatment (Felger et al., 1999; Raj et al., 2004). Yet, the development of an effective vaccine is being hampered by genetic diversity (Healer et al., 2004) even though extensive research on the diversity in the parasite field isolates has been conducted in several studies

* Corresponding author.
E-mail addresses: nnalude@gmail.com (H. Usman-Yamman), omaluicj@futminna.edu.ng (I. Omalu C. J), abukadir2@gmail.com (A. Abubakar), abolarinwa@futminna.edu.ng (Abolarinwa S.O), ekesamuel2012@gmail.com (S. Eke S.), chideberea@gmail.com (C.A. Otuu).

https://doi.org/10.1016/j.meegid.2021.105143
Received 20 February 2021; Received in revised form 1 November 2021; Accepted 13 November 2021
Available online 17 November 2021

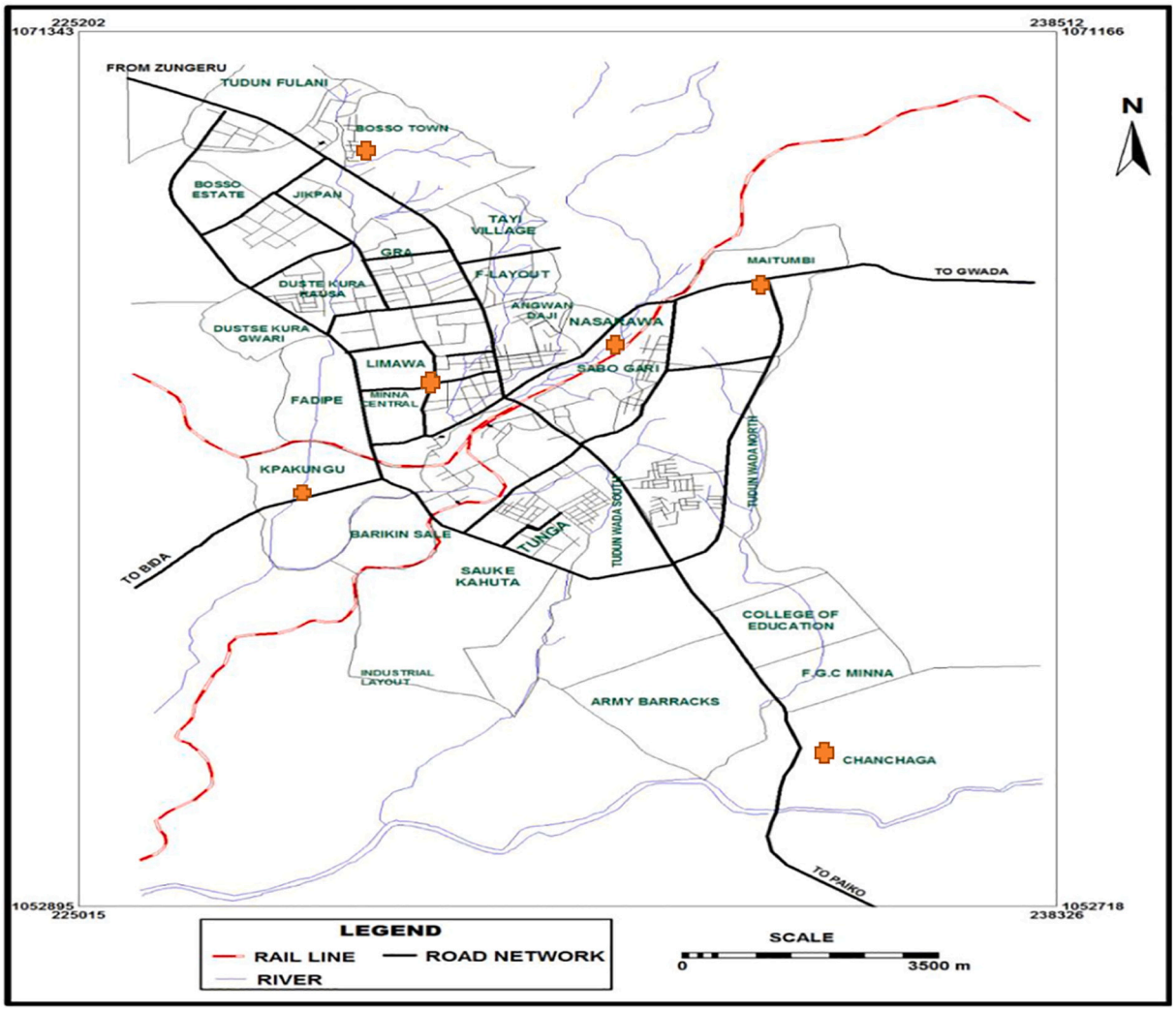

Fig. 1. Locational Map of Minna showing the location of selected Healthcare facilities (Source: Extract from Minna Metric Sheet (2015) as produced by Niger State Geographical Information System (NIGIS)).
Healthcare facilities

(Babiker et al., 2000; Zakeri et al., 2005). Genetic diversity and multiplicity of *P. falciparum* infections are essential parasite indices that could determine the impact of malaria intervention programs as well as endemicity of parasite infections in varying transmission settings (Barry et al., 2013; Nabet et al., 2016; Razak et al., 2016).

Plasmodium parasites, expresses many variant antigens on the cell surfaces. These variants play an important role in virulence and also host immune evasion (Frech and Chen, 2013; Noelle et al., 2010). Severe cases of malaria can occur when parasite infests and then multiplies rapidly within red blood cell. The parasite produces many variant antigenic proteins, encoded by multigene families, which are present on the surface of the infected erythrocyte and play important roles in virulence (Chen et al., 2000). A major surface protein is the merozoite surface protein 1 and 2 (*msp* 1 and 2).

Merozoite surface protein 1 and 2 of *P. falciparum* are major blood-stage malaria vaccine targets (Chaitarra et al., 1999) and are also suitable markers for the identification of genetically distinct *P. falciparum* parasite sub-populations. *Msp1* is a major *P. falciparum* surface protein of approximately 190-kDa size. It plays an important role in the invasion of the red blood cell (Holder et al., 1992) and is a major target of immune responses (Apio et al., 2000). *Msp1* contains 17 blocks of sequence flanked by conserved regions (Takala et al., 2002) Block 2, which is the most polymorphic part of *msp1*, is grouped into three allelic families, namely K1, MAD20, and RO33 type (Takala et al., 2006). *Msp2* is glycoprotein consisting of five blocks where the third block is the most polymorphic (Ferreira and Hart, 2007), and is grouped into two allelic families, FC27 and 3D7/IC1. This study tries to look at the association between gene diversity of merozoite surface protein 1 and 2 (*msp1* and *msp2*) and the transmission intensity of *P. falciparum* infected children in Minna, North Central Nigeria. Therefore, findings from this study will serve as baseline data for future studies on parasite population structure and antimalarial drug resistance surveillance in the region.

2. Materials and methods

2.1. Ethical approval

Ethical approval for the study was obtained from the Niger State Ministry of Health after a research protocol and an application letter was tendered. Following administrative clearance from the ministry, clearance was also obtained from the Director, Primary Healthcare, Chanchaga Local Government. Informed consent was obtained from the parents or guardians of children captured in this study.

Table 1
Demographic and clinical features of study participants.

Age	Total no. of participants (%)	No. positive to *P. falciparum* (%)	P·D ≥ 1000/μl (%)	No. selected for PCR (%)
6 mnths-5 yrs	129 (52.20)	98 (49.20)	61 (42.70)	22/61 (36.10)
6 yrs–11 yrs	76 (30.80)	63 (31.70)	52 (36.40)	19/52 (36.53)
12 yrs–17 yrs	42 (17.00)	38 (19.10)	30 (21.00)	9/30 (30.00)
Total	247	199	143	50

P·D- Parasite density.

2.2. Sample collection

A total of 247 blood samples were collected from infants and children aged 6 months to 17 years with signs of fever attending outpatients Department of selected healthcare facilities (see Fig. 1) during a cross-sectional survey in the months of March to May 2018. Blood samples were screened for malaria by microscopic examination using Giemsa staining technique, of which fifty (50) *P. falciparum* positive samples with parasite density ≥ 1000 μl were randomly selected for molecular characterization.

2.3. Extraction of parasite DNA and molecular identification

P. falciparum DNA was extracted from 100ul blood samples by the use of DNA extraction kit(ExiPrepTMDx Viral DNA/RNA kit version 3.0) following the manufacturer's instructions. Two polymorphic loci, *msp1* and *msp2*, were used for the genotyping of the parasite population in this study. The regions of *msp1* and *msp2*, which vary in repeat number and in adjacent sequence type, with three (MAD20, K1, RO33) and two (FC27, 3D7) allelic families, were analyzed by a nested PCR amplification. For the primary amplifications, outer primer pairs corresponding to the flanking sequence of the conserved regions of *msp1* and *msp2* were used. The second amplification reactions was based on the primary products using allelic-specific primers sets corresponding to K1, RO33 and MAD20 families of msp1 and FC27, 3D7 families of *msp2* as described elsewhere (Soulama et al., 2009).

The first round of PCR reaction (primary PCR) was done with a mixture of forward and reverse reaction for both *msp1* and *msp2* with the following cycling conditions: initial denaturation for 5 min at 95 °C, then followed by 30 cycles of denaturing at 94 °C for 1 min, annealing at 61 °C for 45 s, and extension cycle of 72 °C for 1.5 min, and a final extension at 72 °C for 5 min (Ridzuan et al., 2016).

The cycling conditions for nested PCR (second round) of *msp1* and *msp2* started with a single step of denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing temperature at 57 °C for 45 s for *msp2* allelic families (FC27 and 3D7); while 62 °C and 58 °C annealing for 45 s for (K1, MAD20) and RO33 of *msp1* allelic families, respectively. This was then followed by an extension at 72 °C for 1.5 min, and a final extension at 72 °C for 5 min (Ridzuan et al., 2016). Positive controls and negative controls were incorporated in each set of reaction. The parasite DNA from the P. falciparum isolates was analyzed for *msp1* and *msp2* genes.

PCR amplification was performed on a thermal cycler in 100 ml 1×

Table 2
Number of *P. falciparum* Alleles and Base Pair Ranges Observed per Allelic Family in *msp1* and *msp2*.

Gene/ Allele	No. of Bands (%)	PCR band size (bp) range	Number of observed bands
msp1			
KI	21(46.00)	250–1000	3
MAD20	31 (67.00)	100–500	4
RO33	5(11.00)	400–500	2
msp2			
3D7	11(24.00)	100–800	4
FC27	43(93.00)	100–900	8
Total	111 (100)		

TAE (Tris Acetate) buffer and the PCR product was visualized in 2% agarose powder (Nsobya et al., 2004; Abdel Hamid et al., 2013) by ultraviolet trans-illumination after staining with ethidium bromide. The number and size of the resulting amplified products were analyzed using Gene tool programme (Abdel Hamid et al., 2013). Alleles were considered the same if fragment sizes were within 10 bp (Gosi et al., 2013) to 50 bp (Mwingira et al., 2011) intervals for *msp1* and *msp2*.

2.4. Allele distribution and multiplicity of infection

The number of genotypes per infection, or the multiplicity of the infection (MOI) was estimated by dividing the total number of fragments detected in the individual system by the number of samples positive in the particular system (either *msp1* or *msp2*). It is also defined as an estimate of the average number of PCR fragments per infected individual (Zwetyenga et al., 1998).

2.5. Data analysis

Data was analyzed using SPSS version 23.0 statistical software programs. The correlation between multiplicity of infection (MOI) and age was analyzed by using Spearman's correlation. For this study, frequency of polyclonal infection was calculated using the number of samples with over one amplified fragment out of the total samples. The genetic diversity in each population was assessed by calculating the mean expected heterozygosity (*He*) across loci in each population. Briefly, the allelic diversity (*He*) or antigenic markers was calculated based on the allele frequencies, using the formula $He = n–\sum (1 - p)\ 2 \div (n–1)$, where n is the number of isolates analyzed and p represents the frequency of each different allele at a locus. He has a potential range from 0 (no allele diversity) to 1 (all sampled alleles are different) (Abd Razak et al., 2016). The *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Demographic and clinical features of study participants

From the total of 247 blood samples collected during the period of this study, 199/247 (80.56%) were positive for *P. falciparum* from which 56/199 (28.14%) had parasite density < 1000/μl, while 143/199 (71.86%) recorded parasite density ≥ 1000/μl and from which fifty (50) samples was selected within the various cohorts for the molecular characterization, viz.: 22/61 (36.10%), 19/52 (36.53%) and 9/30 (30.00%) for ages 6 months-5 years, 6 years–11 years and 12 years–17 years, respectively (Table 1).

3.2. Allele diversity of P. falciparum msp1 and msp2 among children in Minna

From the total of 50 *P. falciparum* positive samples presented for PCR, 46 (92.00%) tested positive for at least one allele, which were typed for *msp1* or *msp2* genes, and the remaining 4 (8.00%) tested negative after exposure to PCR. Alleles of *msp1* and *msp2* were classified according to the size of their PCR amplified fragments. A total of 111 alleles were detected for *msp1* and msp2 gene by analysis of PCR products on agarose gels, of which 57/111 (51.35%) and 54/111 (48.64%) of them were

Table 3
Monoclonal and polyclonal infections of Alleles of *msp1* from children in Minna.

Infections	KI (%)	Band size (bp)	MAD20 (%)	Band size (bp)	RO33 (%)	Band size (bp)	Total (%)
Monoclonal infections	6 (50.00)	300/1000	8 (35.00)	100/300/400	0 (0.00)		14 (38.00)
Polyclonal infections	6 (50.00)	250–1000	15 (65.00)	100–500	2 (100.00)	400–500	23 (62.00)
Total	12 (32.43)		23 (62.16)		2 (5.40)		37 (100)

Table 4
Monoclonal and Polyclonal Infections of Alleles of *msp2* from Children in Minna.

Infections	3D7 (%)	Band size (bp)	FC27 (%)	Band size (bp)	Total (%)
Monoclonal infections	3 (43.00)	100/400/450/800	10 (40.00)	100/600/900	13 (41.00)
Polyclonal infections	4 (57.00)	100–800	15 (60.00)	100–900	19 (59.00)
Total	7 (22.00)		25 (78.00)		32 (100)

successfully examined for the presence of *msp1* (MAD20, K1, RO33) and *msp2* (3D7, FC27), respectively. MAD20 was the predominant allele observed in *msp1* with 31/46 isolates (67.00%) and yielded four (4) fragments (within a range of 100-500 bp). Amplification of the allelic family K1 was detected for 21/46 isolates (46.00%) and yielded three (3) fragments (within a range of 250-1000 bp). The lowest allele frequency observed for *msp1* gene was RO33 with only 5/46 isolates (11.00%) and yielded two (2) fragments (within a range of 400-500 bp). *Msp2* gene recorded FC27 as the predominant allele with 43/46 isolates (93.00%) and produced up to eight (8) fragments (within a range of 100-900 bp) and 3D7 produced four (4) fragments (within a range of 100-900 bp) from 11/46 isolates (24.00%) (Table 2).

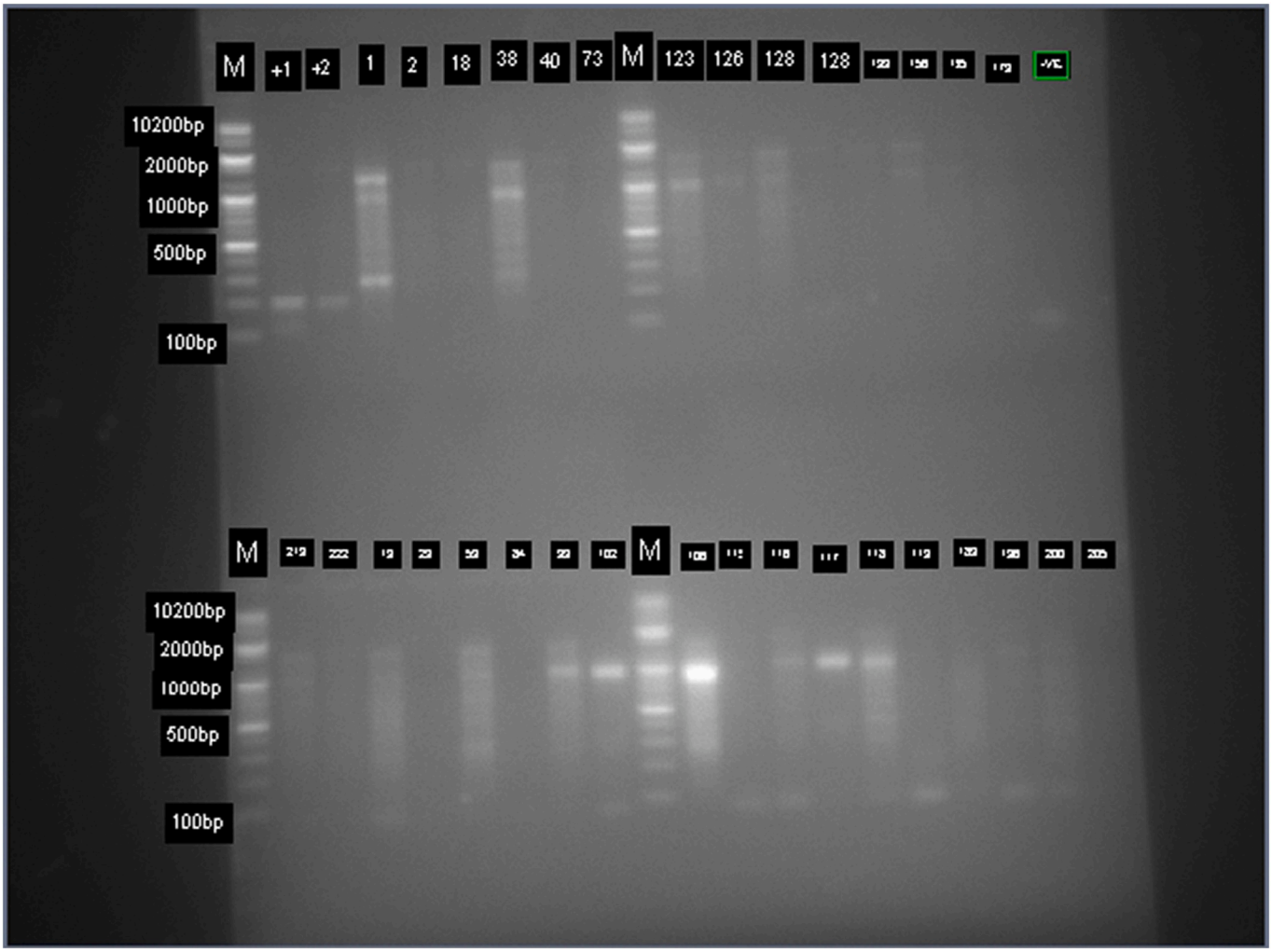

Plate I. PCR Typing of K1 (*msp1*) with Band Size (250–1000 bp).
M- Marker.
+1- Positive control 1.
+2- Positive control 2.
-ve- Negative.
Bp- Base pair.

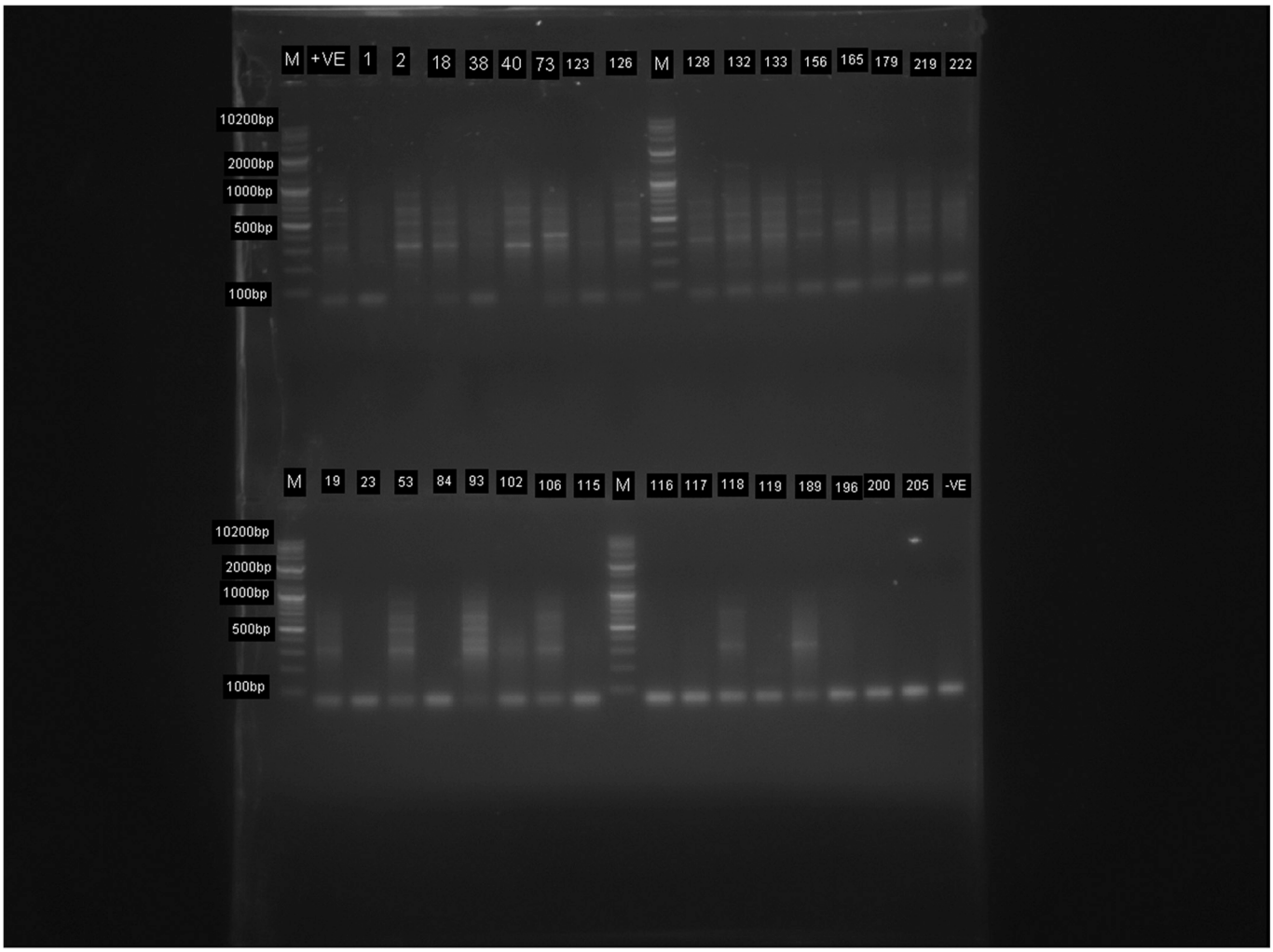

Plate II. PCR Typing of MAD20 (*msp1)* with Band Size (100–500 bp).
M- Marker.
+1- Positive control 1.
+2- Positive control 2.
-ve- Negative.
Bp- Base pair.

3.3. Monoclonal and polyclonal infections of the different allelic families of msp1 and msp2 among children in Minna

A monoclonal infection (single parasite clone per allele) of 14 (38.00%); and a polyclonal infection (multiple parasite clones) of 23 (62.00%) was identified for *msp1* gene. The RO33 is polyclonal with an amplified fragment size of 400-500 bp, whilst MAD20 recorded the highest monoclonal 8 (35.00%) and polyclonal infection 15 (65.00%) in *msp1* gene (Table 3). In the *msp2* gene,13 (41.00%) monoclonal infection and 19 (59.00%) polyclonal infection was detected (Table 4).

Furthermore, of the 46 PCR positive samples, 42 (91.00%) was identified as poly-allelic, i.e. they harboured over one parasite genotype identified by the presence of two or more alleles of one or both genes, and 4 (9.00%) single allele (mono-allelic). However, the combination of the poly-allelic features differs accordingly with a total of 20 (18.00%) with of two (2) allele types in the following order: RO33/FC27 1 (1.00%); MAD20/FC27 15 (14.00%); MAD20/3D7 2 (2.00%) and KI/FC27 2 (2.00%). Whereas, 17 (15.00%) contained three (3) allele combination types with K1/RO33/FC27 2 (2.00%); K1/MAD20/FC27 10 (9.00%); MAD20/3D7/FC27 2 (2.00%) and K1/3D7/FC27 3 (3.00%). In addition, a combination of four (4) allele types was observed: K1/RO33/3D7/FC27 2 (2.00%) and K1/MAD20/3D7/FC27 2 (2.00%) (Plates I-V).

3.4. Genetic diversity analysis of P. falciparum populations and multiplicity of infections (MOIs) of alleles of msp1 and msp2 genes in Minna

The heterozygosity (*He*) index determined for the antigenic markers of *msp1* and *msp2* was 0.714 and 0.830, respectively, which signifies a wide range of allele diversity within the individual genes. However, low levels of allele diversity with *He* levels of 0.085, 0.219 and 0.063 was obtained from the different cohorts of 6 months to 5 years, 6 to 11 years and 12 to 17 years, respectively. In the overall, a very low level of genetic diversity was detected in Minna with expected heterozygosity (*He*) value of 0.0636.

Children within the age of 6 months to 5 years recorded the highest mean multiplicity of infection (MOI) for *msp1* and *msp2* (2.52), followed by age groups 6 to 11 years, and then 12 to 17 years with 2.47 and 2.42, respectively. It was observed that MOI decreases with age, though there was no statistical significance difference ($P > 0.05$) between MOI and age. The mean MOI for *msp2* loci was higher (1.68) than *msp1* (1.54)

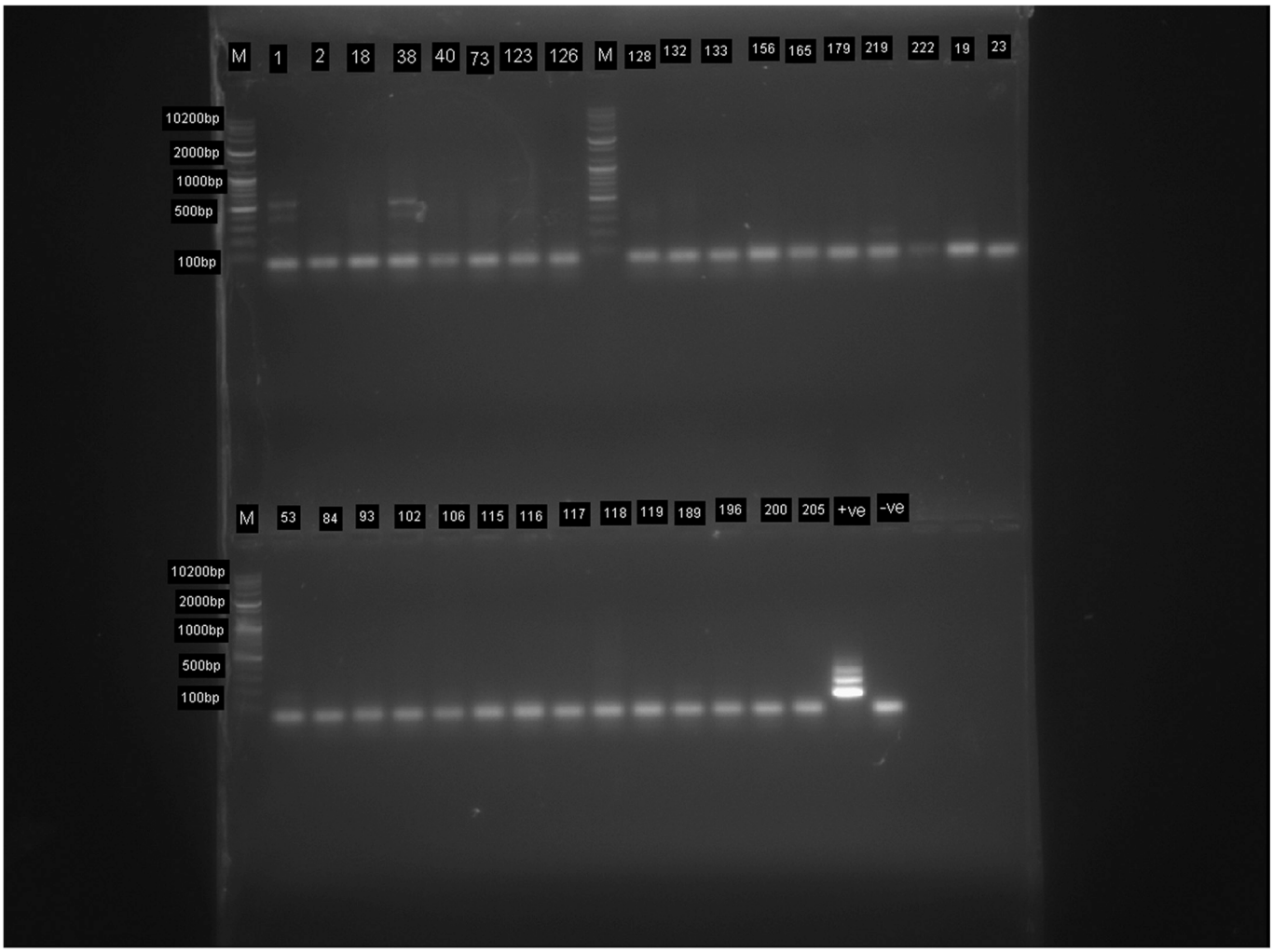

Plate III. PCR Typing of RO33 (*msp1)* with Band Size (400–500 bp).
M- Marker.
+1- Positive control 1.
+2- Positive control 2.
-ve- Negative.
Bp- Base pair.

with the overall mean MOIs for both *msp1* and *msp2* gene as 1.60. In addition, RO33 was identified with the highest mean MOI for individual alleles (2.50). Both K1 and MAD20 detected a MOI of less than 2 (1.75 and 1.35, respectively) for *msp1* loci. 3D7 and FC27 of *msp2* recorded 1.60 and 1.72, respectively. The mean MOI values calculated for the individual antigenic markers of *msp1* and *msp2* were similar, but substantially different from RO33 for *msp1*.

4. Discussion

The parasite population structure of *P. falciparum* isolates from Minna, North-Central Nigeria, was determined using the diversity in *msp1* and *msp2* antigenic markers. These markers were recommended for genotyping parasites in antimalarial drug efficacy trials and parasite population structure analysis by the World Health Organisation (WHO, 2007a; WHO, 2007b). Findings from this study revealed MAD20 to have the highest frequency of alleles in the *msp1* gene, with 31 (67%) and yielded four fragments within the range of 100-500 bp. Amplification of the allelic family K1 was positive for 21 isolates (46%) and yielded three fragments within the range of 250-1000 bp. Allelic family RO33 which recorded the lowest frequency in *msp1* gene had only 5 isolates (11%) and yielded two fragments 400 and 500 bp. However, there seems to be a slight similitude from the previous study conducted in South-western, Nigeria (Olasehinde et al., 2012), it detected proportion of isolates with K1 family as 68% with 4 alleles within the range of 100 to 300 bp. Proportion of isolates with MAD20 family as 40% and a total of 3 alleles were observed within 100 to 300 bp. RO33 proportion was 20% and the family was observed to be monomorphic with an allele size of 200 bp.

The predominant allele types for this study were MAD20 (*msp1*) and FC27 (*msp2*) with more than half of the study population infected. This observation was not in conformity with previous works conducted in South-west Ethiopia, Sudan and Malaysia, which detected the K1, and RO33 allelic family as predominant (Abdel Hamid et al., 2013; Atroosh et al., 2011; Mohammed et al., 2015). However, this was in conformity with a previous work by Soe et al. (Soe et al., 2017) who observed MAD20 type as the most prevalent for *msp1*. Meanwhile, previous works from Ehiopia, Kenya, Congo Brazzaville Myanmar and other sub-Saharan-African countries detected 3D7 as the predominant allele in the *msp2* gene (Mwingira et al., 2011; Soe et al., 2017; Mayengue et al., 2011), which was contrary to what was observed from this study with FC27 as the predominant allele.

More than 90% of the population harboured multiple genotypes of 2,

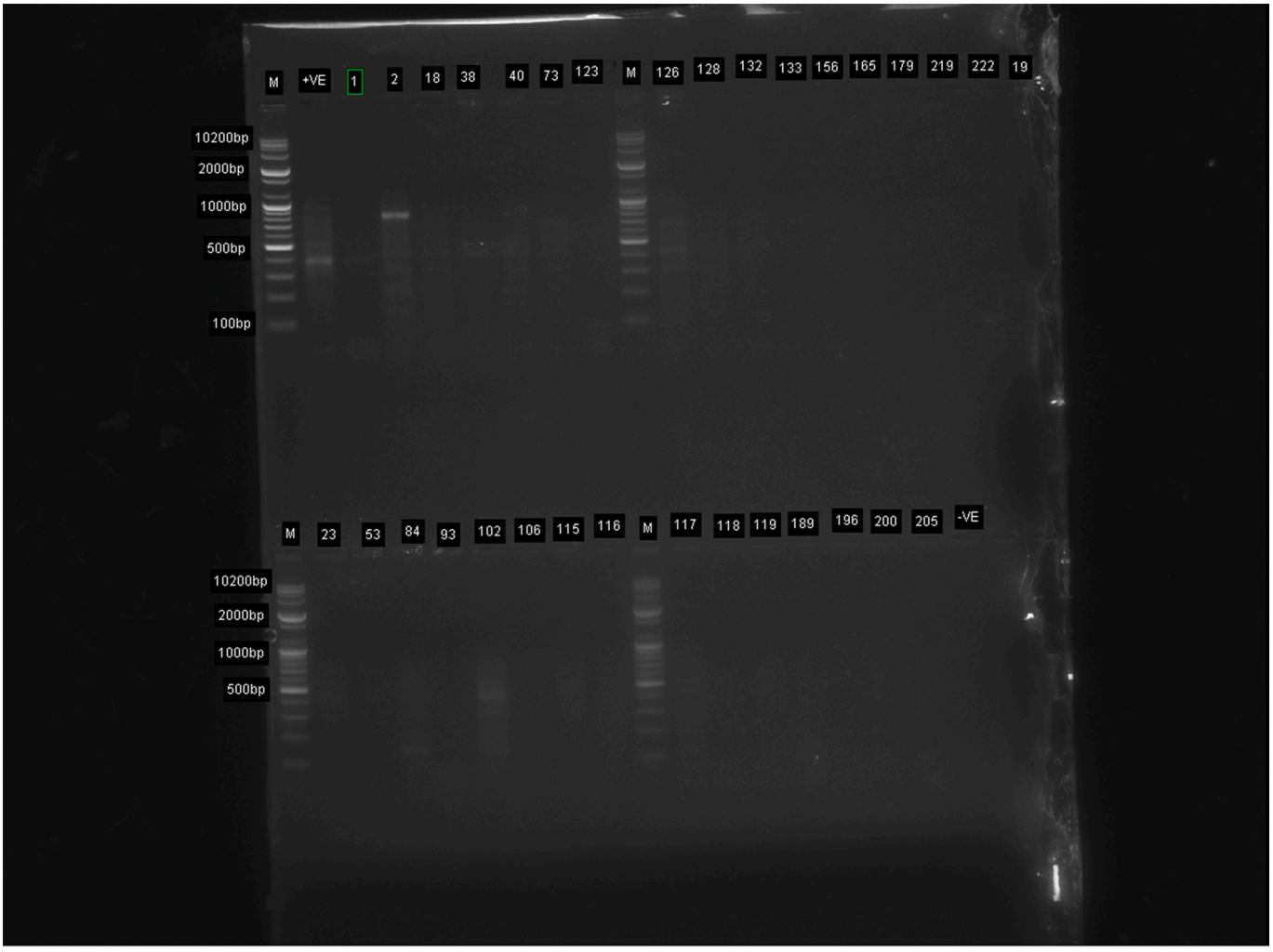

Plate IV. PCR Typing of 3D7 (*msp2)* with Band Size (100–800 bp).
M- Marker.
+1- Positive control 1.
+2- Positive control 2.
-ve- Negative.
Bp- Base pair.

3 or 4 from a single isolate of either or both *msp1* and *msp2* alleles; and about 60% are polyclonal. The high level of mixed infections indicates a high transmission intensity in Minna. The frequency was quite higher than what was obtained from a previous study by Abdel Hamid et al. (Abdel Hamid et al., 2013), and Mohammed et al. (Mohammed et al., 2015) which reported that almost two-third of the samples (62%) and (59%) harboured multiple genotypes, respectively.

The overall mean multiplicity of infection observed from this study was 1.54 and 1.68 for the genetic markers *msp1* and *msp2*, respectively which were quite similar to what was observed by Mohammed et al. (Mohammed et al., 2015) with 1.37 and 1.20 for *msp1* and *msp2*, respectively. In another similar study in Côte d'Ivoire, a high MOI of 2.88 was recorded for *msp2* (Silue et al., 2006). This discrepancy may be due to differences in geographical areas and their transmission patterns and may also be because of differences in sample population. Generally, the higher the malaria transmission level, the greater becomes the tendency to get a higher MOI (Mohammed et al., 2015). Epidemiologic data from some studies in Africa suggest that the multiplicity of *P. falciparum* infection could be directly related to the intensity of transmission.

The present study found a non-significant association between MOI and age. In a similar study conducted by Abdel Hamid et al. (2013), they unfolded that the association between age and multiplicity of infection was not well understood.

Findings from this study, revealed that *P. falciparum* populations were not genetically diverse (0.0636), although, a high level gene diversity within the individual antigenic markers- *msp1* and *msp2* (with 0.714 and 0.830, respectively) indicates a larger genotype diversity within the *msp1* and *msp2* loci. This was common to the antigenic marker genotyping carried out in African regions like Malaysia, Burkina Faso, Sao Tome, Malawi, Uganda and Tanzania which have identified *P. falciparum* populations with alleles occurring at a very high *He* level (0.78 to 0.99) (Mwingira et al., 2011; Abd Razak et al., 2016). Though, Mohammed et al. (Mohammed et al., 2015), also observed a high *He* level of 0.79 for *msp1*, this observation differs for *msp2* (0.54) with moderate *He* levels. Meanwhile, low level genetic diversity of less than 0.2 was observed in all cohorts of children. However, the lack of major differences in parasite variants across Minna may be as a result of malaria control program across Minna and the State as a whole by the distribution of long lasting insecticide treated nets LLINs across the health facilities and improved service delivery to control and treat malaria which may cause the genetic drift and decrease the level of *He* and MOI.

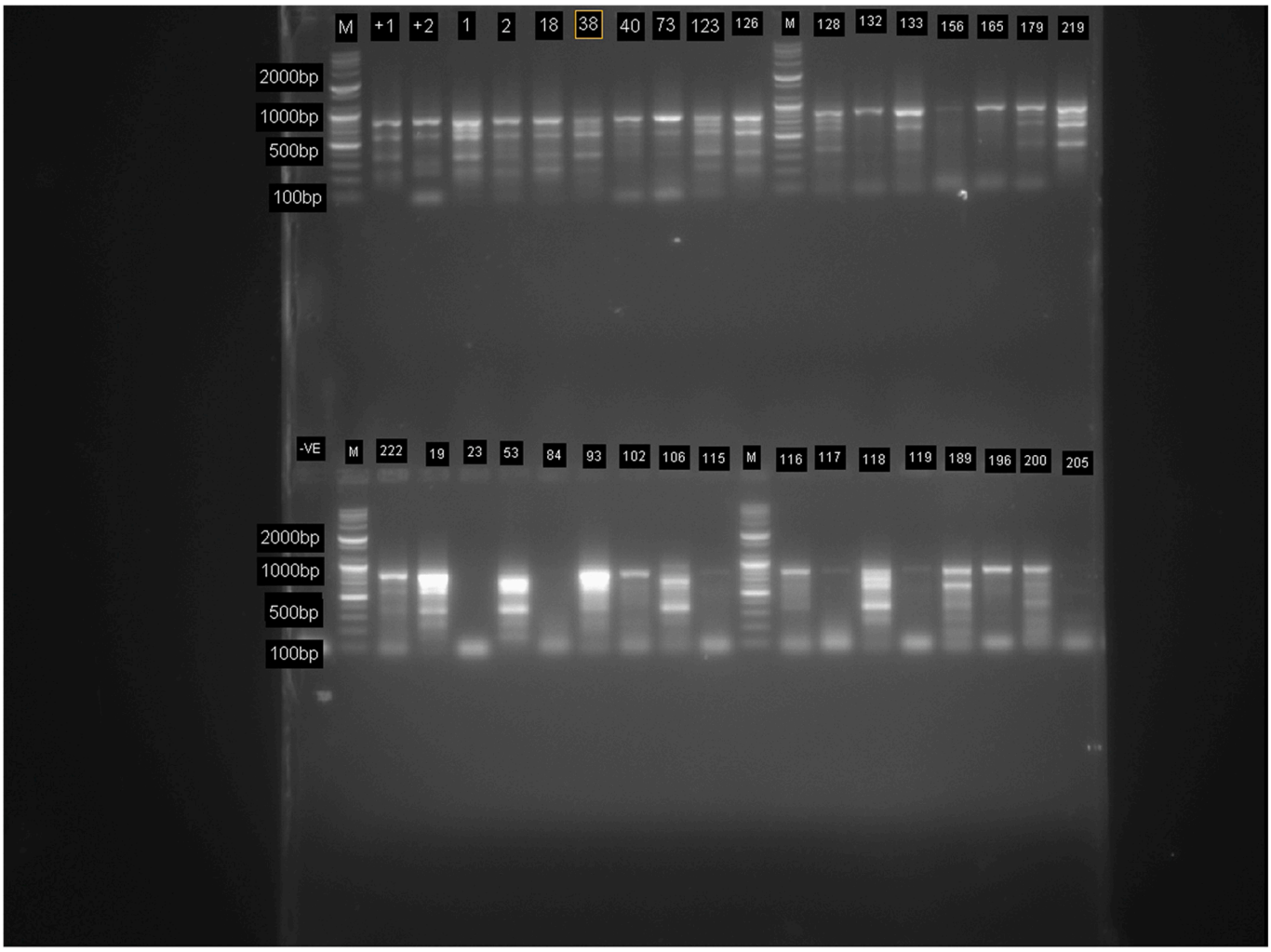

Plate V. PCR Typing of FC27 (*msp2)* with Band Size (100–900 bp).
M- Marker.
+1- Positive control 1.
+2- Positive control 2.
-ve- Negative.
Bp- Base pair.

5. Conclusions

P. falciparum populations in Minna were not genetically diverse, however, a high level of gene mix was observed within the individual antigenic markers of *msp1* and *msp2*. More so, there was a non-significant association between MOI and age, with generally a low transmission intensity. This study has demonstrated the potential of malarial gene diversity and MOI, as important markers for assessing differences in malaria transmission intensity. The determination of gene diversity paved way to compare prevalence across different cohorts and confirm the heterogeneity in malaria transmission. This can provide promising information for future research, particularly in evaluating control interventions for malaria elimination in high transmission settings where reductions are most difficult to achieve and sustain. This study, warrants further investigation in a wider population and also in asymptomatic individuals in order to have an inclusive picture of the parasite diversity.

This study has several limitations: the study is a cross-sectional, and it provides limited information about the subject. Meanwhile, the convenience sampling method used in this study has inherent selection bias. The survey was conducted in healthcare facilities, meaning that children out of the healthcare facilities were not represented, though this approach is valid in obtaining an estimate of *P. falciparum* gene diversity in children infected with the parasite. The outpatient department of the healthcare facilities were visited by mostly children under the age of 5, so older children were under sampled which could have led to an underestimation of the MOI.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

We thank the Director Chanchaga Primary Healthcare Hajiya Fatima Garba, and her staff, Aishatu Mustapha (of Tunga PHC), Mr. Hamza (Oldairport PHC), Mr. Salihu (Limawa FSP), Mr. Habib (Gwari-road PHC); community leader of MAWIS Sheikh (Dr.) Isah Yahya; and the study participants and their parents/guardians. We sincerely thank Mr. Zakari Jibrin for his moral and spiritual support. We really appreciate the team at General Hospital Minna, Mr. Abdullahi Habib Etsugaie, Mr. Abel Nsude Okechukwu and Mr. Abdullahi; and also DNA Lab, Kaduna.

References

Abd Razak, M.M.R., Sastu, U.R., Norahmad, N.A., Abdul-Karim, A., Muhammad, A., Muniandy, P.K., 2016. Genetic diversity of *plasmodium falciparum* populations in malaria declining areas of Sabah, East Malaysia. PLoS One 11 (3), e0152415.

Abdel Hamid, M.M., Mohammed, S.B., El Hassan, I.M., 2013. Genetic diversity of *plasmodium falciparum* field isolates in Central Sudan inferred by PCR genotyping of Merozoite surface protein 1 and 2. N. Am. J. Med. Sci. 5 (2), 95–101.

Abukari, Z., Okonu, R., Nyarko, S.B., Lo, A.C., Dieng, C.C., Salifu, S.P., Gyan, B.A., Lo, E., Amoah, L.E., 2019. The diversity, multiplicity of infection and population structure of *P. falciparum* parasites circulating in asymptomatic carriers living in high and low malaria transmission settings of Ghana. Genes. 10 (6), 434.

Apio, B., Nalunkuma, A., Okello, D., Riley, E., Egwang, T.G., 2000. Human IgG subclass antibodies to the 19 kilo Dalton carboxy terminal fragment of *plasmodium falciparum* merozoite surface protein 1 (MSP-119) and predominance of the MAD20 allelic type of MSP-1 in Uganda. East Afr. Med. J. 77, 189–193.

Atroosh, W.M., Al-Mekhlafi, H.M., Mahdy, M.A., Saif-Ali, R., Al-Mekhlafi, A.M., Surin, J., 2011. Genetic diversity of *plasmodium falciparum* isolates from Pahang, Malaysia based on *MSP-1* and *MSP-2* genes. Parasit. Vectors 4, 233.

Babiker, H.A., Abdel-Muhsin, A.A., Hamad, A., Mackinnon, M.J., Hill, W.G., Walliker, D., 2000. Population dynamics of *plasmodium falciparum* in an unstable malaria area of eastern Sudan. Parasitology 120, 105–111.

Barry, A.E., Schultz, L., Senn, N., Nale, J., Kiniboro, B., Siba, P.M., Mueller, I., Reeder, J. C., 2013. High levels of genetic diversity of plasmodium falciparum populations in Papua New Guinea despite variable infection prevalence. Am. J. Trop. Med. Hyg. 88 (4), 718–725.

Blasco, B., Leroy, D., Fidock, D.A., 2017. Antimalarial drug resistance: linking *plasmodium falciparum* parasite biology to the clinic. Nat. Med. 23, 917.

Chaitarra, V., Holm, I., Bentley, G.A., Petres, S., Longacre, S., 1999. The crystal structure of C-terminal merozoite surface protein 1 at 1.8 resolution, a high protective malaria vaccine candidate. Mol. Cell 3, 457–464.

Chen, Q., Schlichtherle, M., Wahlgren, M., 2000. Molecular aspects of severe malaria. Clin. Microbiol. Rev. 13 (3), 439–450.

Felger, I., Irion, A., Steiger, S., Beck, H.-P., 2., 1999. Genotypes of Merozoite surface protein 2 of *plasmodium falciparum* in Tanzania. Trans. R. Soc. Trop. Med. Hyg. 93, 3–9.

Ferreira, M.U., Hart, D.L., 2007. *Plasmodium falciparum*: worldwide sequence diversity and evolution of the malaria vaccine candidate merozoite surface protein-2 (MSP-2). Exp. Parasitol. 115, 32–40.

Frech, C., Chen, N., 2013. Variant surface antigens of malaria parasites: functional and evolutionary insights from comparative gene family classification and analysis. BMC Genomics 14, 427.

Gosi, P., Lanteri, C.A., Tyner, S.D., Se, Y., Lon, C… & Spring, M., 2013. Evaluation of parasite subpopulations and genetic diversity of the msp1, msp2 and glurp genes during and following artesunate monotherapy treatment of *plasmodium falciparum* malaria in Western Cambodia. Malar. J. 12, 403.

Healer, J., Murphy, V., Hodder, A.N., Masciantonio, R., Gemmill, A.W., Anders, R.F., et al., 2004. Allelic polymorphisms in apical membrane antigen-1 are responsible for evasion of antibody-mediated inhibition in *plasmodium falciparum*. Mol. Microbiol. 52, 159–168.

Holder, A.A., Blackman, M.J., Burghaus, P.A., Chappel, J.A., Ling, I.T., McCallum-Deighton, N., Shai, S., 1992. A malaria merozoite surface protein (MSP1)-structure, processing and function. Mem. Inst. Oswaldo Cruz 87 (3), 37–42.

Mayengue, P.L., Ndounga, M., Malonga, F.V., Bitemo, M., Ntoumi, F., 2011. Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in *plasmodium falciparum* isolates from Brazzaville, Republic of Congo. Malar. J. 10, 276.

Mohammed, H., Mindaye, T., Belayneh, M., Kassa, M., Assefa, A., Tadesse, M., Woyessa, A., Mengesha, T., Kebede, A., 2015. Genetic diversity of *plasmodium falciparum* isolates based on MSP-1 and MSP-2 genes from Kolla-Shele area, Arbaminch Zuria District, Southwest Ethiopia. Malar. J. 14, 73.

Mwingira, F., Nkwengulila, G., Schoepflin, S., Sumari, D., Beck, H.P., Snounou, G., Felger, I., Olliaro, P., Mugittu, K., 2011. *Plasmodium falciparum msp1, msp2* and *glurp* allele frequency and diversity in sub-Saharan Africa. Malar. J. 10, 79.

Nabet, C., Doumbo, S., Jeddi, F., Konaté, S., Manciulli, T., Fofana, B., L'Ollivier, C., Camara, A., Moore, S., Ranque, S., 2016. Genetic diversity of plasmodium falciparum in human malaria cases in Mali. Malar. J. 15, 353.

Noelle, Y.J., Makhtar, N., Peter, R.P., 2010. Small variant surface antigens and *plasmodium* evasion of immunity. Future Microbiol. 5 (4), 663–682.

Nsobya, S.L., Parikh, S., Kironde, F., Lubega, G., Kamya, M.R., Rosenthal, P.J., Dorsey, G., 2004. Molecular evaluation of the natural history of asymptomatic Parasitemia in Ugandan children. J. Infect. Dis. 189 (12), 2220–2226.

Olasehinde, G.I., Yah, C.S., Singh, R., Ojuronbge, O., Ajayi, A.A., Valecha, N., Abolaji, A. O., Adeyeba, A.O., 2012. Genetic diversity of *plasmodium falciparum* field isolates from south western Nigeria. Afr. Health Sci. 12 (3), 355–361.

Raj, D., Das, A., Supakar, P., 2004. Genetic diversity in the merozoite surface protein 1 gene of P. falciparum in different malaria-endemic localities. Am. J. Trop. Med. Hyg. 71 (3), 285–289.

Razak, M.R.M.A., Sastu, U.R., Norahmad, N.A., Abdul-Karim, A., Muhammad, A., Muniandy, P.K., Jelip, J., Rundi, C., Imwong, M., Mudin, R.N., 2016. Genetic diversity of *plasmodium falciparum* populations in malaria declining areas of Sabah, East Malaysia. PLoS One 11, e0152415.

Ridzuan, M.M.A., Sastu, U.R., Norahmad, N.A., Abdul-Karim, A., Muhammad, A., Muniandy, P.K., Jelip, J., Rundi, C., Imwong, M., Mudin, R.N., Abdullah, N.R., 2016. Genetic diversity of *plasmodium falciparum* populations in malaria declining areas of Sabah, East Malaysia. PLos J. 11 (3), e0152415.

Silue, K.D., Felger, I., Utzinger, J., Beck, H.P., Smith, T.A., Tanner, M., N'goran, E. K., 2006. Prevalence, genetic diversity and multiplicity of *plasmodium falciparum* infection in school children in central cote d'Ivoire. Med. Trop. 66, 149–156.

Soe, T.N., Wu, Y., Tun, M.W., Xu, X., Hu, Y., Ruan, Y., Win, A.Y.N., Nyunt, M.H., Nwe Mon, N.C., Han, K.T., Aye, K.M., Morris, J., Su, P., Yang, Z., Kyaw, M.P., Cui, L., 2017. Genetic diversity of *plasmodium falciparum* populations in southeast and western Myanmar. Parasit. Vectors 10, 322.

Soulama, I., Nébié, I., Ouédraogo, A., Gansane, A., Diarra, A., Tiono, B.A., Bougouma, C. E., Konaté, T.A., Kabré, B.G., Taylor, R.J.W., 2009. Sirima *plasmodium falciparum* genotype diversity in symptomatic malaria of children living in an urban and a rural setting in Burkina Faso. Malar. J. 8, 135.

Takala, S., Branch, O., Escalante, A.A., Kariuki, S., Wootton, J., Lal, A.A., 2002. Evidence for intragenic recombination in *plasmodium falciparum*: identification of a novel allele family in block 2 of merozoite surface protein-1: Asembo Bay Area cohort project XIV. Mol. Biochem. Parasitol. 125, 163–171.

Takala, S.L., Escalante, A.A., Branch, O.H., Kariuki, S., Biswas, S., Chaiyaroj, S.C., Lal, A. A., 2006. Genetic diversity in the block 2 region of the merozoite surface protein 1 (MSP-1) of *plasmodium falciparum:* additional complexity and selection and convergence in fragment size polymorphism. Infect. Genet. Evol. 6, 417–424.

WHO, 2007a. Recommended Genotyping Procedures (RGPs) to Identify Parasite Populations. Medicines for Malaria Venture and World Health Organization, Amsterdam. www.who.int/malaria/publications/atoz/rgptext_stipdf. Accessed 2 Jun 2014.

WHO, 2007b. Methods and Techniques for Clinical Trials on Antimalarial Drug Efficacy: Genotyping to Identify Parasite Populations: Informal Consultation Organized by the Medicines for Malaria Venture and Cosponsored by the World Health Organization, 29–31 May 2007. WHO, Amsterdam.

WHO, World Health Organization, 2019. The "World Malaria Report 2019" (at a glance).

Zakeri, S., Bereczky, S., Naimi, P., Pedro Gil, J., Djadid, N.D., Farnert, A., et al., 2005. Multiple genotypes of the merozoite surface proteins 1 and 2 in *plasmodium falciparum* infections in a hypoendemic area in Iran. Tropical Med. Int. Health 10, 1060–1064.

Zwetyenga, J., Rogier, C., Tall, A., Fontenille, D., Snounou, G., Trape, J.F., Puijalon, O. M., 1998. No influence of age on infection complexity and allelic distribution in *plasmodium falciparum* infections in Ndiop, a Senegalese village with seasonal, mesoendemic malaria. Am. J. Trop. Med. Hyg. 59, 726–735.