ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Research Article

Genetic Polymorphisms of the Merozoite Surface Protein-2 Block-3 in Plasmodium falciparum Field Isolates in Cameroonian Asymptomatic Children: Relation to Multiplicity of Infection, Parasitaemia and Age

Dongang Nana Rodrigue Roman[1,2,3], Ngono Ngane Rosalie Anne[1], Vineeta Singh[3]*, Koanga Mogtomo Martin Luther[1], Pooja Thathola[3], Aditi Arya[3], Moyou-Somo Roger[2] and Mouelle Sone Albert[4]

[1]*Department of Biochemistry, Faculty of Sciences, University of Douala, Cameroon*

[2]*Institute of Medical Research and Medicinal Plants Studies (IMPM), Yaoundé, Cameroon*

[3]*Cell Biology Laboratory and Malaria Parasite Bank, National Institute of Malaria Research, (ICMR), Dwarka, New Delhi, India*

[4]*Department of Clinical Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, Cameroon*

***Corresponding Author**: Vineeta Singh, Cell Biology Laboratory and Malaria Parasite Bank, National Institute of Malaria Research, (ICMR), Dwarka, New Delhi, India.

Received: September 28, 2017; **Published:** November 02, 2017

Abstract

Background: Analyzing the genetic diversity of malaria parasites is an essential step in providing valuable information for control strategies used to achieve elimination targets.

Objectives: This cross-sectional study examined *Plasmodium falciparum* merozoite surface protein 2 (*msp-2*) genotypes with multiplicity of infection (MOI) and its relationship with hemoglobin level, age and parasitaemia in asymptomatic children.

Methods: Blood samples were collected from 233 children (aged 3 - 14 years) for parasite density, hemoglobin level determination and *msp*-2 genotyping.

Results: In asymptomatic samples the mean (SEM) parasite density was 6,847 (2,789) parasites/µl. *Msp*-2 allelic families were successfully identified in 76 samples out of 83 *P. falciparum* positive samples. The FC27 and IC/3D7 allelic families were detected in 39.5% and 85.5% of samples respectively and 75.0% of infections were polyclonal. The estimated number of genotypes was 44 (23 for *FC27* and 21 for *IC/3D7*). Heterozygosity for *IC/3D7* and *FC27* were 0.92 and 0.93 respectively. Overall mean (SEM) multiplicity of *msp-2* genotypes per infection was 2.45 (0.15) and MOI was significantly associated with parasitaemia ($P = 0.01$) but not with age and sex ($P > 0.05$).

Conclusion: The MOI specific *msp*-2 alleles seem to increase with parasitaemia; suggesting an association between the number of clones per infection and the progression of asymptomatic to symptomatic malaria.

Keywords: *P. falciparum; msp-2; Genotyping; MOI; Asymptomatic Samples; Cameroon*

Introduction

Human malaria is caused by parasitic protozoa in the genus *Plasmodium*. Of the five species of malaria parasite that infect humans, *P. falciparum* is responsible for the highest burden of disease with severe and lethal cases causing a major public health concern [1,2]. Globally, around 3.2 billion people in 97 countries are at risk of being infected with malaria. In 2015, 214 million cases of malaria occur causing 438,000 deaths, especially in sub-Saharan African countries, where children under five years were the most affected population group [3]. *P. falciparum* disease exhibits a wide range of clinical manifestations ranging from asymptomatic parasitaemia, uncomplicated (mild)

to complicated (severe) disease [4]. In endemic areas where individuals are often simultaneously infected by multiple parasite clones, genetic diversity and multiplicity of infection (MOI) of parasites represents a major issue in understanding several aspects of malaria infection and disease-transmission dynamics and are described as a factor determining the host immune status [5-11]. To control and eventually eradicate malaria, an effective vaccine is needed in addition to the existing strategies such as artemisinin-based combination therapy (ACT), insecticides treated bed nets (ITNs), indoor residual spraying (IRS) and intermittent preventive treatment [12]. However, extensive genetic polymorphism in natural malaria parasite populations is a major obstacle for the development of an effective vaccine against these parasites because antigenic diversity limits the efficiency of acquired protective immunity to malaria [5,13-15].

Many malaria parasite proteins have been proposed as vaccine candidate antigens and *msp-2* (a glycoprotein anchored in the plasma membrane of the merozoite) is one of these with an approximate molecular size of ~30 kD and is thought to be involved in erythrocyte invasion [16,17]. The *msp-2* gene, located on chromosome 2, contains a single open reading frame with conserved, semi conserved and variable sequences and has been divided into five blocks (1-5) [18]. The N and C-terminal sequences (block 1 and 5) are highly conserved while block 2 and 4 are semi-conserved. Block 3 contains variable non-repetitive sequences flanking repetitive units that differ in length and copy number and these non-repetitive sequences define the two allelic types *viz* the *FC27* and the *IC/3D7* [19,20].

In Cameroon, malaria remains one of the most important public health problems especially in rural and remote areas with the most virulent species i.e. *P. falciparum* [21]. Despite policy change, there is still need to collect more data on the epidemiology of malaria in the country to facilitate the development and implementation of malaria control interventions such as the search for alternative drugs or vaccine trials. In this regards, characterization of malaria parasite populations circulating in an area is part of site characterization before any intervention, as a basis for evaluating the impact of the intervention on genetic diversity, parasite species, and multiplicity of infection (MOI) in the human [13]. Information about the genetic diversity based on the *msp-2* gene in children is lacking in Cameroon, although these are necessary to implement malaria control strategies in the affected areas. This study presents the allelic types and distribution patterns of the *P. falciparum msp-2* gene in asymptomatically infected children. Genotyping of the polymorphic loci of the *msp-2* was used to examine the genetic diversity and MOI as well as their relationship with age and parasitaemia.

Materials and Methods

Study sites

Cameroon is divided into three main malaria epidemiological facies linked to geo-climatic variations, level of transmission and disease vector prevalence [21,22] (Figure 1). For this cross-sectional survey, participants were recruited in the Mvan neighborhood located at the periphery of the Yaoundé city (capital of Cameroon); that belongs to the equatorial epidemiological facies where malaria is present throughout the year with a marked increase during the raining season. The geographical coordinates of the area are 7° 80' N, 4° 23' E. The climate of the locality is equatorial Guinean type. The area experiences four seasons: a long dry season (mid-November to mid-March); a small rainy season (mid-March through June); a short dry season (from July to August) and a long rainy season (from September to mid-November). The annual average temperature is 24.2°C with an annual average precipitation of 1, 541.1 mm and relative humidity of 82.8%.

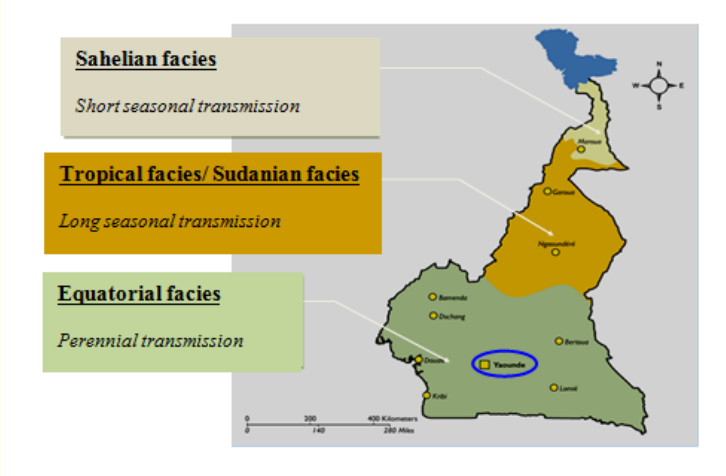

Figure 1: *Map of Cameroon showing Yaoundé and malaria epidemiological facies.*

Patients, sample collection and malaria definition

Children between the ages of 3 - 14 years with an axillary temperature of < 37.5°C with no recent malaria treatment (at least in the last seven days) were eligible for enrollment in the study. All eligible children were referred to study personnel for further screening. The children were examined and axillary temperature was measured by a well-trained team member. Blood samples were collected into EDTA microcontainers in accordance with routine clinical practice and transported to the laboratory where hemoglobin (Hb) levels were immediately assessed by using a haemoglobinometer (URIT Medical Electronic Co LTD, China). Thick and thin blood smears were obtained and blood blot filter mats (Whatman grade 1 filter paper, Whatman, Maidstone, UK) were prepared and labeled for each study participant. The study was conducted in accordance with the latest version of the Declaration of Helsinki and Good Clinical Practice [23]. Informed consent was obtained from all parents/legal guardians of children. Children with detectable parasitaemia were treated free of charge according to national guidelines. Study was approved by the Ethical Review Committee of Institute of Medical Research and Medicinal Plants Studies, Yaoundé.

Malaria parasitaemia examination

Thick blood smears were stained with 10% Giemsa for 20 minutes and examined for *P. falciparum* parasites status and density. Parasitaemia was determined as parasite numbers per microlitre (parasites/µL) of blood by counting parasites against white blood cells (WBCs). Using oil microscope objective (x100), 200 WBCs were counted systematically, estimating at the same time the numbers of parasites (asexual forms only) in each field covered, with two tally counters [24].

Molecular identification of *P. falciparum*

Parasite DNA was extracted from blood spots collected on filter papers using the QIAamp® blood minikit (QIAGEN®, Germany) following the manufacturer's instructions. The prepared DNA was then stored at -20°C until used for PCR. *Plasmodium* spp were identified by 18S rRNA based nested PCR method using genus and species-specific primers as mentioned previously [25].

Allelic typing of *P. falciparum msp-2*

The highly polymorphic regions of *msp-2* (Block 3) gene were further analyzed by Nested PCR amplification. The oligonucleotide primers sets used for detecting the different families of *msp-2* (*FC27* and *3D7*) were described previously (Table 1) [26]. All PCR reactions were performed using Eppendorf Mastercycler Gradient (Eppendorf, Germany). Each PCR was done in a 25 µl PCR mixture containing 2.0 µl

genomic DNA, 1x Taq buffer with 15 mM $MgCl_2$; 10 mM of dNTPs mix (2.5 mM each), 0.4 µM of each primer (forward and reverse) and 1.6 U of Taq polymerase enzyme. Cycling conditions for primary PCR reactions were as follows; initial denaturation step at 95°C for 5 minutes, then followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 2 minutes; and a final extension was added at 72°C for 5 minutes. The nested PCR cycling parameters included a single step of initial denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 61°C for 1 minute, extension at 72°C for 2 minutes and a final extension at 72°C for 5 minutes.

Gene/Family	Primer Name	PCR round	Primers sequence (5' 3')
MSP-2	M2-OF	Primary	5' ATGAAGGTAATTAAAACATTGTCTATTATA 3'
	M2-OR		5' CTTTGTTACCATCGGTACATTCTT 3'
FC27	M2-FCF	Nested	5' AATACTAAGAGT GTAGGTGCARAT GCTCCA 3'
	M2-FCR		5' TTTTATTTGGTGCATTGCCAGAACTTGAAC 3'
IC/3D7	M2-ICF		5' AGAAGTATGGCAGAAAGTAAkCCTYCTACT 3'
	M2-ICR		5' GATTGTAATTCGGGGGATTCAGTTTGTTCG 3'

Table 1*: Sequences of primers used to genotype the msp-2 polymorphic regions of P. falciparum isolates recovered from asymptomatic children in Yaoundé, Cameroon.*

Detection of alleles

Following amplification, secondary PCR products were resolved by electrophoresis on 1.5% ethidium-bromide stained agarose gel, visualized by ultraviolet and photographed using the Alpha Innotech system (Alpha Innotech-Genetic Technologies, Inc, USA). The number and size of DNA fragments was estimated using 100 bp DNA ladder (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA).

Multiplicity of infection and heterozygosity

The multiplicity of infection (MOI) was defined as the number of different *P. falciparum* genotypes (alleles) co-infecting a single individual. The MOI was estimated by dividing the total number of distinct *msp-2* genotypes detected by the number of PCR positive isolates. Isolates with more than one genotype were considered as a polyclonal infection while the presence of a single allele was considered as monoclonal infection. Expected heterozygosity index (H_E) which measures locus diversity was calculated using the formulae $H_E = [n/(n-1)] [(1- \Sigma Pi^2)]$, where n = sample size, Pi = allelic frequency [27].

Statistical analysis

Demographics, clinical features and bed net use data were collected using hard copy study case record forms while laboratory results were transcribed into study laboratory registers. Both datasets were double-entered into Microsoft office Excel 2007 (Microsoft Corporation) database and later transferred into SPSS (version 16.0, SPSS Inc., Chicago, USA) and GraphPad Prism demo (version 6.05, GraphPad Software Inc) for analysis. Parasitaemia defined as the number of parasites/µL of blood was graded as low (< 1000), moderate (1000 - 4999) and high (≥ 5000) [28]. Descriptive statistics of proportions and means were used to summarize distributions of allelic families, baseline demographics, MOI and other covariate data. Chi square tests were used to compare distributions between variables sub-groups. Independent t test, Mann Whitney and one-way ANOVA tests was used to compare mean MOI outcome by independent factors of age group, sex, bed net use, parasitaemia and haemoglobinaemia. Statistical significance was defined as p value ≤ 0.05.

Results

Sample analysis with respect to parasitaemia, age and gender

Plasmodium parasites were detected in a total of 83 samples out of 233 collected in the survey with 87.9% and 4.8% of isolates harboring *P. falciparum* and *P. malariae* monofections respectively and 7.2% being *P. falciparum*/*P. malariae* mixed infections. The asexual

P. falciparum parasitaemia among positive samples ranged from 40 to 144,640 parasites/µl of blood and mean (SEM) of 6,847 (2,789) parasites/µl [95% CI: 1,258 - 12436]. Overall mean age (SEM) of study participants was 7.23 (0.21) years [95% CI: 6.7 - 7.6]. The male to female sex ratio (M/F) was 0.83 (106/127) and a higher proportion (42.5%) of children aged 6 to 10 years were observed in the studied population (Table 2). The means of hemoglobin level in the study group stratified by age, gender and asymptomatic *P. falciparum* parasitaemia are given in table 3. Hemoglobinaemia was significantly higher in parasitized children [mean ± SEM = 10.19 ± 0.12 (95% CI: 9.95 - 10.42)] compared to non-parasitized [mean ± SEM = 10.87 ± 0.08 (95% CI: 10.70 - 11.04)] ($P < 0.0001$) but the difference was not statistically significant with parasite count ranges ($P = 0.53$).

Variables sub-groups		Asymptomatic malaria		P-value
	Total (n = 233)	Positive (n = 83)	Negative (n = 150)	
Mean age, Mean ± SEM (years)	7.23 (0.21)	7.20 (0.34)	7.24 (0.28)	0.94
Age groups, n (%)				0.25
3 - 5	85 (36.5)	28 (12.0)	57 (24.5)	
6 - 10	99 (42.5)	41 (17.6)	58 (24.9)	
11 - 14	49 (21.0)	14 (6.0)	35 (15.0)	
Gender, n (%)				0.83
Male	106 (45.5)	37 (15.9)	69 (29.6)	
Female	127 (54.5)	46 (19.7)	81 (34.8)	
Use of preventive method, n (%)				0.12
Yes	158 (67.8)	51 (21.9)	107 (45.9)	
No	75 (32.2)	32 (13.7)	43 (18.5)	

***Table 2**: Demographics, malaria prevention and clinical profile: comparison of variables based on P. falciparum asymptomatic malaria by PCR.*

Variables	Hemoglobin, Mean ± SEM, (g/dl)	95% Confidence interval	P-value
Age groups (years)			0.09
3 - 5	10.07 ± 0.18	9.69 - 10.50	
6 - 10	10.14 ± 0.16	9.80 - 10.50	
11 - 14	10.90 ± 0.45	9.90 - 11.93	
Gender			0.24
Male	10.38 ± 0.21	9.95 - 10.80	
Female	10.08 ± 0.15	9.78 - 10.38	
Parasitology			< 0.0001*
Positive	10.19 ± 0.12	9.95 - 10.42	
Negative	10.87 ± 0.08	10.70 - 11.04	
Parasite count ranges (Parasites/µL)			0.53
< 1000	10.45 ± 0.17	10.09 - 10.80	
1000 - 4999	10.19 ± 0.33	9.50 - 10.89	
≥ 5000	10.04 ± 0.19	9.60 - 10.46	
Number of clones			0.45
Monoclonal	10.01 ± 0.20	9.60 - 10.44	
Polyclonal	10.28 ± 0.15	9.98 - 10.60	

***Table 3**: Relationship between hemoglobin level, age, gender and asymptomatic P. falciparum infection.*

SEM: Standard error of mean

P. falciparum msp-2 infection clones and allelic diversity

Msp-2 block 3 gene was successfully amplified for 92% (76/83) of *P. falciparum* positive isolates. Overall, a range of one to six infection clones per isolate was observed (Figure 2). The estimated total number of different *msp-2* genotypes was 44 (23 for *FC27* and 21 for *IC/3D7*). 25% of infections were monoclonal. A total of 19 (25.0%) samples were co-infected simultaneously by *FC27* and *IC/3D7* types. There was no statistical difference between monoclonal and polyclonal infections with respect to hemoglobinaemia (See Table 3).

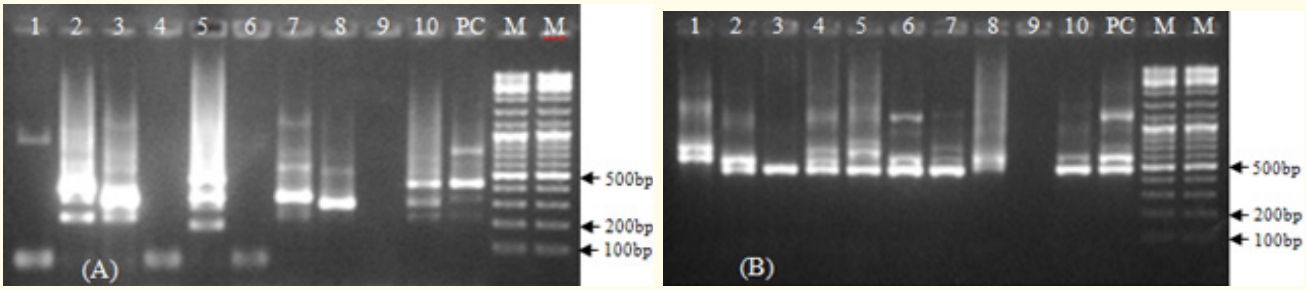

Figure 2: *Gel profile of msp-2 genotypes. (A): FC27 allelic families and (B): 3D7 allelic families. Lanes 1 - 10 represent P. falciparum isolates; PC: Positive control (NF54 strain); M: Molecular weight marker (100bp DNA ladder).*

Allele's frequency and heterozygosity

The *IC/3D7* allelic family was detected 143 times in our isolates compared to 53 for the *FC27* family. The sizes of all the *IC/3D7* and *FC27* alleles types from children were between 450 - 1299 bp and 200 - 849 bp respectively. The frequencies of these alleles based on PCR products size (bp) and their combinations are shown in figure 3. Allelic variants with fragments sizes 500 - 549 bp and 350 - 399 bp were predominant among *IC/3D7* (21.1%) and *FC27* (30.2%) alleles respectively (Figure 3). Expected heterozygosity was almost the same for *IC/3D7* locus (H_E = 0.92) and *FC27* locus (H_E = 0.93).

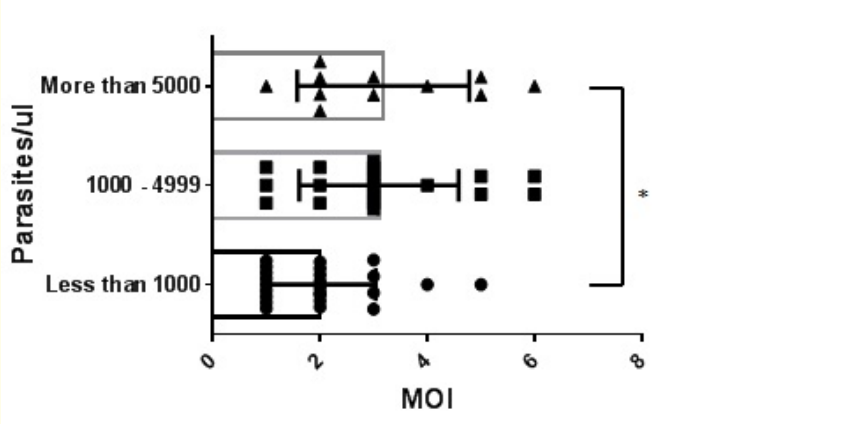

Figure 3: *Parasitaemia according to multiplicity of infection in msp-2 gene (*significance difference, P < 0.05).*

Multiplicity of infection

The overall mean (SEM) multiplicity of *msp-2* genotypes per infection were 2.45 (0.15) (95% CI: 2.15 - 2.8). MOI was seen to increase significantly with parasite count ranges (low and moderate-to-high) (Figure 4). Mean MOI was also compared according to age groups,

sex of children and use of preventive method against malaria. The results presented in table 4 indicates that MOI seems to be higher in children aged between 11 - 14 years, among male and in those using preventive method to fight malaria. However, the differences in MOI between these groups were not statistically significant (P > 0.05).

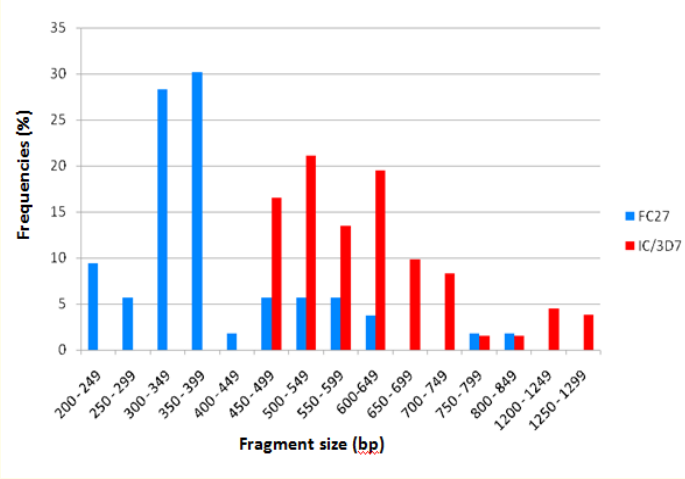

Figure 4: *Fragments size polymorphism and alleles frequency of msp-2 block 3 gene among asymptomatic malaria children.*

Discussion

The present study was undertaken to study the asymptomatic infections with respect to MOI in natural infections. MOI is influenced by various factors like age, transmission intensity and seasonal variation [29-31]. Several previous studies have shown varied results ranging from disease risk to disease protection though the presence of these differences have not been clearly stated [32-34]. Study from Kilifi reported the presence of more than one clones with increased risk of subsequent malaria in low-moderate transmission [35]. The *msp-2* genotyping is a well-established molecular marker reported similarly from other regions in epidemiological studies as evident in our study too where *msp-2* alleles were detected in 92% of the asymptomatic patients too [10,27,36,37]. The number of clones was highest in 11 - 14 years in our study indicating the tolerance to antigentically variant infections is higher in older year old children hence the presence of multiclones whereas; the lower level of immunity in young children is probably responsible for presence of lower number of multiclones in them [38]. This suggests that infections persisting in population are responsible for malaria immunity seen in our study too, where chronic infections showed reduced risk of subsequent malaria. Also, a plausible explanation for observed association between parasite clones and protection from symptomatic malaria could be that children who have been exposed to antimalarials develop immunity allowing low-grade parasitaemia to persist asymptomatically with multiclones in a semi-immune population [38,39]. We found the numbers of clones were statistically significant by increase in the level of parasitaemia but age or gender of the patient could not be correlated significantly. The presence of high parasitaemia increases the chances of detecting multiclone in a patient has also been reported previously and can be explained by the fact that there are more parasites present in the sample used for PCR amplification [40,41].

Besides, the level of parasitaemia in a host, several other factors can also affect the number of clones present in the person at the collection time [41]. The true estimate of genetically distinct clones in asymptomatic infections are highly underestimated which could be attributed to the host-parasite interaction, dynamics of circulating clones and technical limitations. Asymptomatic infections in children which could be multiclonal in nature can persist in low transmission seasons and may also have developed immunity to protect from new infections. But children with single clone infections will be more susceptible to novel infections on the onset of transmission seasons. Though the age could not be significantly related to the asymptomatic infections in our study but it has been reported previously that the

influence of age on MOI is highly dependent upon the malaria endemicity of the region [42]. The findings in our current study shows that the multiclonal infections and asymptomatic malaria are in agreement with other previous studies [43,44].

Other studies have reported age-dependent MOI in intense perennial transmission areas [45]. Our results also support these findings but the lack of correlation needs to be studied more with more number of samples. Understanding the basis of multiclonal infections in host immunity and the impact of different interventions will go a long way in development and evaluation of malaria control strategies in future. Further studies should be undertaken to unravel the mechanisms underlying the association between multiplicity of infections with asymptomatic malaria.

Conclusions

The asymptomatic *P. falciparum* field isolates from Cameroon exhibited a high degree of genetic polymorphism in *msp-2* allele typing with multiple clones. Multiplicity of infection was found to be high with increase in parasitaemia indicating a probable role in the progress of disease from asymptomatic to symptomatic. In our study the use of *msp-2* proved to be an informative marker of Cameroonian parasite population diversity as polymorphisms with diverse allele types was identified in *msp-2* among *P. falciparum* field isolates in this study.

Authors' Contributions

Designed and supervised the study: MSA, NNAR; designed and data collection of the study: DNRR, KMML, MSR; Conceived, designed and supervised the experiments: VS; Performed the experiments: DNNR, PT, AA; Analysis and interpretation of data: DNRR, VS; Wrote the first draft of the manuscript: DNRR, VS; Revised the manuscript: DNRR, NNAR, VS, KMML, PT, MSR and MSA. All authors have read and approved the final version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interests.

Acknowledgments

We are very thankful to the parents and their children for participating in the study, as well as to our field staff. We also express our gratitude to the NAM and ST centre, India for the Research Training Fellowship to Mr Dongang Nana Rodrigue Roman for research work.

Bibliography

1. Kantele A and Jokiranta TS. "Review of cases with the emerging fifth human malaria parasite, Plasmodium knowlesi". *Clinical Infectious Diseases* 52 (2011): 1356-1362.

2. Millar SB and Cox-Singh J. "Human infections with Plasmodium knowlesi-zoonotic malaria". *Clinical Microbiology and Infection* 21.7 (2015): 640-648.

3. World Health Organization. World malaria report, (2015).

4. Miller LH., *et al*. "The pathogenic basis of malaria". *Nature* 415.6872 (2002): 673-679.

5. Ekala MT., *et al*. "Allelic family-specific humoral responses to merozoite surface protein 2 (MSP2) in Gabonese residents with Plasmodium falciparum infections". *Clinical and Experimental Immunology* 129.2 (2002): 326-331.

6. Takala SL., *et al*. "Genetic diversity in the Block 2 region of themerozoite surface protein 1 (MSP-1) of Plasmodium falciparum: additional complexity and selection and convergence in fragment size polymorphism". *Infection, Genetics and Evolution* 6.5 (2006): 417-424.

7. Kiwuwa MS., *et al*. "Genetic diversity of Plasmodium falciparum infections in mild and severe malaria of children from Kampala, Uganda". *Parasitology Research* 112.4 (2013): 1691-1700.

8. Gupta P., *et al*. "Genetic Profiling of the Plasmodium falciparum Population Using Antigenic Molecular Markers". *The Scientific World Journal* (2014).

9. Bouyou-Akotet MK., *et al*. "Genetic polymorphism of merozoite surface protein-1 in Plasmodium falciparum isolates from patients with mild to severe malaria in Libreville, Gabon". *Parasite* 22 (2015): 12.

10. Mohammed H., *et al*. "Genetic diversity of Plasmodium falciparum isolates based on MSP-1 and MSP-2 genes from Kolla-Shele area, Arbaminch Zuria District, southwest Ethiopia". *Malaria Journal* 14 (2015): 73.

11. Duah NO., *et al*. "Genetic diversity of Plasmodium falciparum isolates from uncomplicated malaria cases in Ghana over a decade". *Parasites and Vectors* 9 (2016): 416.

12. Kilama W and Ntoumi F. "Malaria: a research agenda for the eradication era". *Lancet* 374.9700 (2009): 1480-1482.

13. Genton B., *et al*. "A recombinant blood-stage malaria vaccine reduces Plasmodium falciparum density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea". *Journal of Infectious Diseases* 185.6 (2002): 820-827.

14. Healer J., *et al*. "Allelic polymorphisms in apical membrane antigen-1 are responsible for evasion of antibody-mediated inhibition in Plasmodium falciparum". *Molecular Microbiology* 52.1 (2004): 159-168.

15. Ghansah A., *et al*. "Monitoring parasite diversity for malaria elimination in sub-Saharan Africa". *Science* 345.6202 (2014): 1297-1298.

16. Smythe JA., *et al*. "Identification of two integral membrane proteins of Plasmodium falciparum". *Proceedings of the National Academy of Sciences of the United States of America* 85.14 (1988): 5195-5199.

17. Campo JJ., *et al*. "Impact of the RTS,S malaria vaccine candidate on naturally acquired antibody responses to multiple asexual blood stage antigens". *PLoS One* 6.10 (2011): e25779.

18. Roy SW., *et al*. "Evolution of allelic dimorphism in malarial surface antigens". *Heredity* 100.2 (2008): 103-110.

19. Smythe JA., *et al*. "Structural diversity in the 45-kilodalton merozoite surface antigen of Plasmodium falciparum". *Molecular and Biochemical Parasitology* 39.2 (1990): 227-234.

20. Smythe JA., *et al*. "Structural diversity in the Plasmodium falciparum merozoite surface antigen 2". *Proceedings of the National Academy of Sciences of the United States of America* 88.5 (1991): 1751-1755.

21. EDS-MICS. Enquête Démographique et de Santé et à Indicateurs Multiples (2011): 213.

22. Mbenda HGN., *et al*. "Does malaria epidemiology project Cameroon as 'Africa in miniature'?" *Journal of Biosciences* 39.4 (2014): 727-738.

23. Williams JR. "The Declaration of Helsinki and public health". *Bulletin of the World Health Organization* 86.8 (2008): 650-652.

24. Cheesbrough M. "District laboratory practices in tropical countries, part 1. 2nd edition". Cambridge University Press (2005): 246-248.

25. Snounou G., *et al*. "Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections". *Molecular and Biochemical Parasitology* 58.2 (1993): 283-292.

26. Snounou G., *et al*. "Biased distribution of msp1 and msp2 allelic variants in Plasmodium falciparum populations in Thailand". *Transactions of the Royal Society of Tropical Medicine and Hygiene* 93.4 (1999): 369-374.

27. Mwingira F., *et al*. "Plasmodium falciparum msp1, msp2 and glurp allele frequency and diversity in sub-Saharan Africa". *Malaria Journal* 10 (2011): 79.

28. Kimbi HK., *et al*. "Malaria and haematologic parameters of pupils at different altitudes along the slope of Mount Cameroon: a cross-sectional study". *Malaria Journal* 12 (2013): 193.

29. Ntoumi F., *et al*. "Age-dependent carriage of multiple Plasmodium falciparum merozoite surface antigen-2 alleles in asymptomatic malaria infections". *American Journal of Tropical Medicine and Hygiene* 52.1 (1995): 81-88.

30. Mayor A., *et al*. "Plasmodium falciparum multiple infections in Mozambique, its relation to other malariological indices and to prospective risk of malaria morbidity". *Tropical Medicine and International Health* 8.1 (2003): 3-11.

31. Mara SE., *et al*. "Genetic diversity of Plasmodium falciparum among school-aged children from the Man region, western Côte d'Ivoire". *Malaria Journal* 12 (2013): 419.

32. Müller DA., *et al*. "Prospective risk of morbidity in relation to multiplicity of infection with Plasmodium falciparum in São Tomé". *Acta Tropica* 78.2 (2001): 155-162.

33. Henning L., *et al*. "A prospective study of Plasmodium falciparum multiplicity of infection and morbidity in Tanzanian children". *Transactions of the Royal Society of Tropical Medicine and Hygiene* 98.12 (2004): 687-694.

34. Males S., *et al*. "Long-term asymptomatic carriage of Plasmodium falciparum protects from malaria attacks: a prospective study among Senegalese children". *Clinical Infectious Diseases* 46.4 (2008): 516-522.

35. Färnert BA., *et al*. "Transmission-dependent tolerance to multiclonal Plasmodium falciparum infection". *Journal of Infectious Diseases* 200.7 (2009): 1166-1175.

36. Migot-Nabias F., *et al*. "Red blood cell polymorphisms in relation to Plasmodium falciparum asymptomatic parasite densities and morbidity in Senegal". *Microbes and Infection* 8.9-10 (2006): 2352-2358.

37. Vafa M., *et al*. "Multiplicity of Plasmodium falciparum infection in asymptomatic children in Senegal: relation to transmission, age and erythrocyte variants". *Malaria Journal* 7 (2008): 17.

38. Doolan DL., et al. "Acquired immunity to malaria". *Clinical Microbiology Reviews* 22.1 (2009): 13-36.

39. Moormann AM. "How might infant and paediatric immune responses influence malaria vaccine efficacy?" *Parasite Immunology* 31.9 (2009): 547-559.

40. Contamin H., *et al*. "PCR typing of field isolates of Plasmodium falciparum". *Journal of Clinical Microbiology* 33.4 (1995): 944-951.

41. Färnert A., *et al*. "Genotyping of Plasmodium falciparum infections by PCR: a comparative multicentre study". *Transactions of the Royal Society of Tropical Medicine and Hygiene* 95.2 (2001): 225-232.

42. Arnot D. "Unstable malaria in Sudan: the influence of the dry season. Clone multiplicity of Plasmodium falciparum infections in individuals exposed to variable levels of disease transmission". *Transactions of the Royal Society of Tropical Medicine and Hygiene* 92.6 (1998): 580-585.

43. Agyeman-Budu A., *et al*. "Trends in multiplicity of Plasmodium falciparum infections among asymptomatic residents in the middle belt of Ghana". *Malaria Journal* 12 (2013): 22.

44. Sondén K., *et al*. "Asymptomatic Multiclonal Plasmodium falciparum Infections Carried Through the Dry Season Predict Protection Against Subsequent Clinical Malaria". *Journal of Infectious Diseases* 212.4 (2015): 608-616.

45. Konaté L., *et al*. "Variation of Plasmodium falciparum msp1 block 2 and msp2 allele prevalence and of infection complexity in two neighbouring Senegalese villages with different transmission conditions". *Transactions of the Royal Society of Tropical Medicine and Hygiene* 93.1 (1999): 21-28.

Volume 13 Issue 1 November 2017