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Role of genetic factors and ethnicity on the multiplicity of *Plasmodium falciparum* infection in children with asymptomatic malaria in Yaoundé, Cameroon

Dongang Nana Rodrigue Roman [a,b,c], **Ngono Ngane Rosalie Anne** [a], **Vineeta Singh** [c,*], **Koanga Mogtomo Martin Luther** [a], **Ngonde Essome Marie Chantal** [b,d], **Mouelle Sone Albert** [e]

[a] *Department of Biochemistry, Faculty of Sciences, University of Douala, P.O Box 24157, Douala, Cameroon*

[b] *Institute of Medical Research and Medicinal Plants Studies (IMPM), P.O Box 13033, Yaoundé, Cameroon*

[c] *Cell Biology Laboratory and Malaria Parasite Bank, ICMR-National Institute of Malaria Research, Sector-8, Dwarka, New Delhi 110077, India*

[d] *University Hospital Centre, P.O Box 1364, Yaoundé, Cameroon*

[e] *Department of Clinical Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, P.O Box 24157, Douala, Cameroon*

[*] Corresponding author.

E-mail address: vineetas_2000@yahoo.com (V. Singh).

Abstract

In this cross-sectional study, we investigated host genetic factors and ethnic variation in circulating *Plasmodium falciparum* merozoite surface protein 2 (msp-2) clones among children with asymptomatic malaria.

Isolates from seventy two asymptomatic malaria children were used for genotyping block 3 of msp-2 gene by nested polymerase chain reaction (PCR). Sickle cell trait and glucose-6-phosphate dehydrogenase (G6PD) deficiency were analysed by restriction fragment length polymorphism of DNA products from PCR targeting

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codons 6 and 68 of the beta-globin (HBB) and G6PD genes respectively. ABO blood group was typed by agglutination method.

A total of forty two msp-2 genotypes (20 for 3D7 and 22 for FC27) were detected for an average (standard error of mean) multiplicity of infection (MOI) of 2.45 (0.16). The MOI was statistically the same among the five identified ethnic groups ($P = 0.83$). The overall prevalence of sickle cell trait and G6PD deficiency were 12.50 % and 22.22 % respectively. MOI was similar between children with Hb AA and Hb AS genotypes ($P = 0.42$). MOI was significantly high among children with a mutant G6PD genotype ($P = 0.017$). MOI was significantly higher in blood group O than group A ($P = 0.03$).

Our findings show that although ethnicity and sickle cell trait have no association with MOI, the association was observed with G6PD genotype and ABO group. The results suggest the need for extension and expansion of the current study in order to investigate the mechanisms involved.

Keywords: Infectious disease, Clinical genetics, Epidemiology, Pediatrics

1. Introduction

Malaria is a deadly parasitic disease caused by *Plasmodium* species transmitted to man by a mosquito bite of the *Anopheles* genus. Five species of *Plasmodium* are able to infect humans but *P. falciparum* has been recognized for the major cause of morbimortality throughout human history, especially in children under five years of age [1]. In countries where malaria is endemic, MOI defined as the number of different *P. falciparum* strains co-infecting a single host is frequent and can be a useful indicator of the transmission level as well as immune status [2]. In high transmission settings, majority of *P. falciparum* infections are asymptomatic or subclinical [3]. Asymptomatic parasite population detected by microscopy and/or other methods have been reported in several sites and is an important obstacle to controlling malaria [3, 4]. Previous studies conducted in Mozambique and other sub-Saharan African regions including Ghana, Kenya, Senegal, Gabon and Nigeria showed that a large proportion of individuals harbor asymptomatic infection [5, 6, 7, 8, 9, 10, 11].

In some malaria endemic areas, the clinical outcome of the infection and its progression to pathological complications depends on many factors involving the specific and dynamic combination of host and parasite properties [12, 13]. With respect to the host, it seems likely that the ability to establish an effective immune response against *P. falciparum* infection may involve genetic factors of the host. Today, it is well recognized that *P. falciparum* is a driving force of evolution has helped to shape the human genome and can select genes that contribute to resistance [14, 15].

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G6PD deficiency and sickle cell trait are counted amongst the well-characterized human genetic defects [16, 17, 18]. Sickle-cell hemoglobin (HbS) is a structural variant of normal adult hemoglobin (HbA) caused by a point mutation: substitution of valine by glutamic acid at position 6 of the β-globin subunit (βS) in the beta-globin (HBB) gene [19]. G6PD deficiency characterized by reduced G6PD enzyme activity though a genetic disorder, but can remain asymptomatic [18]. Although these mutations are distributed globally, they are more prevalent in tropical and subtropical regions [20]. Sickle cell traits and a number of other human genetic traits, including G6PD deficiency and related hemoglobinopathies, are predominantly present in populations living in malaria endemic regions and have been suggested to confer some level of protection against severe forms of the disease [21, 22, 23, 24].

In addition, since the discovery of the ABO blood group, there is growing interest in their potential role in infectious diseases. Previous studies in patients with tumors [25] and viral infections [26] found ABO blood group associations. Singh et al. [27] observed a significant association between malaria and ABO blood groups where individuals with antigen O were the most susceptible. In Zimbabwe [28], Fischer and Boone found that Group O individuals were protected against cerebral malaria. In Cameroon, numerous studies on the pathophysiology of malaria have already been done [29, 30, 31, 32, 33]; however, very few have focused on the potential relationships between red blood cell polymorphisms and malaria infection [34, 35]. In this study, to investigate the influence of host factors on the infecting parasite population, we compared the number of merozoite surface protein 2 (msp-2) parasite clones per infection among asymptomatic isolates from Cameroonian children in relation to ethnicity, sickle cell trait, G6PD deficiency and ABO blood groups.

2. Materials and methods

2.1. Study design and data collection

Briefly, a cross-sectional survey was conducted in asymptomatic children living in the Mvan neighborhood located at the periphery of the Yaoundé city, central region (Cameroon), where year-round malaria transmission occurs. This area is characterized by the presence of several tribes; but the most numerous are Ewondo, Bulu and Eton. The study took place from March to April 2016 in 236 children aged 3−14 years. The inclusion criteria included i) obtaining consent from the parent or legal representative of the child, ii) living permanently in the area, iii) axillary body temperature of $<37.5\ ^{\circ}C$, iv) absence of recent treatment received for malaria (at least in the last 7 days) and v) lack of symptoms suggestive of malaria or any other severe systemic illness. Blood samples were collected using Vacutainer® tubes (Nanjing

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Everich Medicare Co Ltd, China) and EDTA.K3 as anti-coagulant. A small aliquot of whole blood was blotted on to Whatman filter Paper number 1 (Maidstone, UK) following the manufacturer's instructions, for parasite genotyping and erythrocytes polymorphisms studies. The remaining blood was immediately used to determine malaria parasite status and density, as well as blood group. The study was approved by the Institutional Ethical Review Committee of University of Douala. Informed consent from the parent or guardian was required prior to inclusion in the study.

2.2. Determination of blood group

ABO blood groups were typed by agglutination using commercial antisera (Cypress Diagnostics, Langdorp, Belgium) as described previously [36]. Briefly, three drops of whole blood were added on a clean glass slide; then a drop of antiserum for blood groups A, B and AB was applied. The blood cells and the antigen were then homogenized for detection of eventual agglutination.

2.3. Parasite assessment

Parasitaemia was determined by microscopic examination of Giemsa-stained thick blood slides using WHO (2010) methods [37]. A blood smear was considered negative if parasites were not detected after examination of at least 200 oil-immersion fields of the thick smear. To estimate the approximate number of parasites per microliter of blood, the number of malaria parasites counted on the thick film against 200 leukocytes was multiplied by 40, based on the assumption that the average leukocyte count was 8,000/μl of blood. Molecular identification of parasites species and msp-2 block 3 genotyping detecting 3D7 and FC27 allelic types were carried out by nested polymerase chain reaction (PCR) as previously described [38, 39].

2.4. Multiplicity of msp-2 infection

Multiplicity of infection was defined as the number of different *P. falciparum* strains co-infecting a single host for the examined gene. Mean multiplicity of infection (MOI) was estimated by dividing the total number of distinct msp-2 genotypes detected by the number of positive samples [40].

2.5. Molecular identification of G6PD variants and sickle cell trait

After DNA extraction (QIAamp DNA Mini Kit, Qiagen, Germany) of the dried blood spot samples, sickle cell trait and G6PD deficiency (due to G202A mutation) were determined by PCR-Restriction Fragment Length Polymorphism (RFLP). The 110 base pairs (bp) containing codon 6 of the HBB gene and the 109 bp containing codon 68 of the G6PD gene were amplified independently using Eppendorf

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Mastercycler Gradient (Eppendorf, Germany) according to protocols previously described [41, 42, 43, 44] with some modifications. Briefly, 4 μL of DNA extract were added to a final volume of 25 μL in a reaction mixture containing 1X Taq buffer with 15 mM $MgCl_2$; 10 mM of dNTPs mix (2.5 mM each), 0.4 μM of each primer and 1.6 U of Taq polymerase enzyme (Table 1). The PCR reaction conditions included an initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 57 °C (for sickle cell trait) or 63 °C (for G6PD) for 30 sec, extension at 72 °C for 30 sec and a final extension at 72 °C for 5 min. The PCR products were separated on 3 % agarose gel pre-stained with ethidium bromide and DNA bands were visualized under a UV transilluminator by using the Alpha Innotech system (Alpha Innotech-Genetic Technologies, Inc, USA).

2.6. RFLP analysis of PCR- amplified fragments

Seven microliters of the amplified fragment were treated with 0.3 μL of restriction endonuclease enzymes (Thermo Scientific, Waltham, USA) *Bsu36 I* (10 U/μL) in the case of the point mutation causing the sickle cell trait or *Hin1 II* (5U/μL) in case of G6PD deficiency; and digested at 37 °C for 16 hours in 10X buffer tango in a total volume of 20 μL. Subsequently, the digestion products pre-stained with ethidium bromide were separated on a 3% agarose gel and visualized under ultraviolet light and the sizes of the PCR products were estimated using a 50 bp DNA ladder marker (Thermo scientific, Waltham, USA). The *Bsu36 I* enzyme has a recognition site at codon 6 in the normal HBB gene cleaving it into two fragments (56 bp and 54 bp), while the fragment amplified from DNA of sickle cell mutation remained uncleaved (110 bp). For G6PD analysis, the sizes of the restriction fragment were 109 bp for the normal allele; while the mutant allele cleaved and gave two bands (63 bp and 46 bp) [Table 1].

Table 1. PCR/RFLP conditions used to identify the sickle cell and G6PD genetic variants of individuals in the present study.

Mutations types	Codons	Primer oligonucleotide sequence (5′−3′)	Amplicon size (bp)	RFLP pattern (fragment size in bp)		
				RE	Wild-type	Mutant
(Val → Met)	68	GTG GCT GTT CCG GGA TGG CCT TCT G CTT GAA GAA GGG CTC ACT CTG TTT G	109	*Hin1 II*	109	63, 46
(Glu → Val)	6	ACA CAA CTG TGT TCA CTA GC CAA CTT CAT CCA CGT TCA CC	110	*Bsu361 I*	54, 56	110

RFLP: Restriction Fragments Length Polymorphisms; RE: Restriction Enzyme; bp: base pairs.
Val: Valine; Met: Methionine; Glu: Glutamic acid.

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2.7. Data management and analysis

The Fisher's exact test was used to compare the distribution of msp-2 allelic family with respect to different host genetic variants in asymptomatic *P. falciparum* children. Mann Whitney and Kruskal-Wallis tests were performed to compare the number of distinct parasite alleles per infected isolate in the subset of patients harboring parasites. All statistical analyses were performed using GraphPad Prism demo (version 6.05, GraphPad Software Inc) softwares. *P* values of <0.05 were considered to indicate statistical significance.

3. Results

3.1. Characteristics of the study population

A total of eighty three children with *P. falciparum* asymptomatic malaria met the inclusion criteria and were enrolled in the study. 7 participants were excluded because of the unsuccessfully genotyping of msp-2 gene and 4 due to the lack of ethnicity information. Finally, seventy two children from 3 to 14 years with asymptomatic malaria were admitted to participate in the study. The distribution of different ethnic groups was 41/72, 15/72, 5/72, 6/72 and 5/72 for the Ewondo, Bulu, Eton, Bassa and Haoussa respectively (Table 2). The mean age $\pm$ standard error of mean (SEM) of the children was 6.9 $\pm$ 0.3 years (95 % CI: 6.2−7.6) and 52.8 % (38/72) were female.

3.2. Multiplicity of infection and ethnicity

A total of forty two different alleles were detected in msp-2 family typing. These alleles comprised 20 alleles of 3D7 and 22 of FC27 allelic families. The 3D7 allelic family was present in all ethnic groups and predominated among the Ewondo (58.6%), Bassa (83.3%), Haoussa (80.0%) and Bulu (66.7%). Polyclonal infections with both allelic families (3D7 + FC27) were observed in all populations (Table 2). The overall average (SEM) number of clones per infection in the population was 2.45 (0.16). Among the Ewondo, Bassa, Haoussa, Bulu and Eton, the mean multiplicity of infection was 2.4, 2.2, 2.0, 2.7 and 3.0 respectively and no significant difference was observed between these values using the Kruskal-Wallis test ($P = 0.83$) (Table 2).

3.3. Relation between multiplicity of msp-2 genotypes and erythrocyte variants

Gel profile illustrating the PCR amplicons as well as the RFLP fragments that we obtained is given in Fig. 1. The proportion of individuals having sickle cell trait and G6PD deficiency in the study population was 9/72 (12.50 %) and 16/72

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Table 2. Genotyping of *P. falciparum* msp-2 polymorphic region block 3 according to the ethnicity.

Ethnicity	Msp-2 allelic family types	No. of isolates	Allele frequency (%)	Allele size range (bp)	MOI
Ewondo	3D7	24	58.6	450−1300	2.4
	FC27	6	14.6	200−750	
	3D7 + FC27	11	26.8		
	Total	**41**	**100.0**		
Eton	3D7	1	20.0	600−700	3.0
	FC27	1	20.0	340	
	3D7 + FC27	3	60.0		
	Total	**5**	**100.0**		
Bassa	3D7	5	83.3	480−1300	2.2
	FC27	0	0.0	-	
	3D7 + FC27	1	16.7		
	Total	**6**	**100.0**		
Haoussa	3D7	4	80.0	500−1300	2.0
	FC27	0	0.0	-	
	3D7 + FC27	1	20.0		
	Total	**5**	**100.0**		
Bulu	3D7	10	66.7	480−1200	2.7
	FC27	2	13.3	300−360	
	3D7 + FC27	3	20.0		
	Total	**15**	**100.0**		

MOI: Multiplicity of infection.

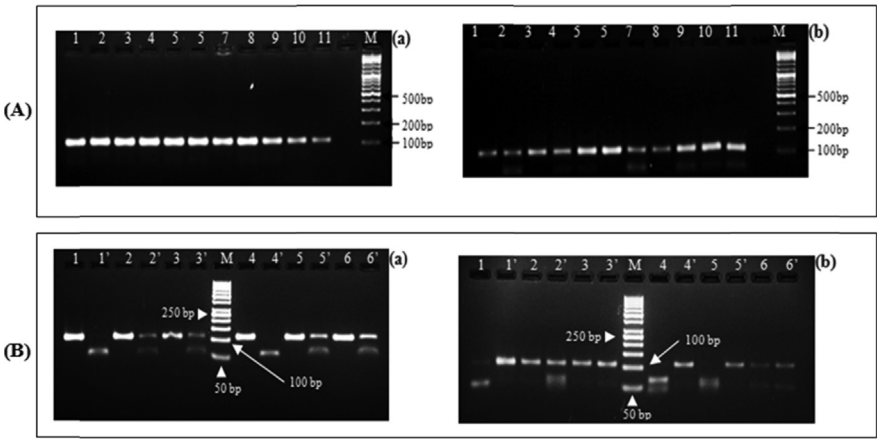

Fig. 1. Gel picture showing PCR amplicons and RFLP fragments. (A): represents PCR products, lines 1 − 11 are samples for sickle cell (a) and G6PD studies (b); line M represents 100 bp ladder molecular size marker. (B): presents restrictions fragments after digestion with enzyme. Lines 1, 2, 3, 4, 5 and 6 shows bands before digestion and lines 1', 2', 3' 4' 5' and 6' after digestion for the same isolates. Line M represents 50 bp ladder molecular size marker. G6PD: Glucose-6-phosphate dehydrogenase.

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(22.22 %) respectively. In order to determine the effect of erythrocyte polymorphism on multiplicity of *Plasmodium* infection, we compared the mean values of the number of the parasite strains co-infecting the children carrying normal genotypes with those carrying mutants (Fig. 2). The results indicate that participants with G6PD deficiency (mean ± SEM = 3.25 ± 0.39) had a significantly higher mean multiplicity of infection compared to normal individuals (mean ± SEM = 2.24 ± 0.16) [P = 0.017] (Fig. 2). Similarly, the analysis of the relationship between the ABO blood group system revealed that the multiplicity of the infection was on average significantly higher in group O (mean ± SEM = 2.66 ± 0.20) compared to A (mean ± SEM = 2.10 ± 0.34) (P = 0.03); but statistically equal to group B (mean ± SEM = 2.5 ± 0.40) [P > 0.05] (Fig. 2). The distribution of the clones of the msp-2 gene (FC27 and 3D7) according to the erythrocyte variants is given by Fig. 3. Compared to the children with Hb AS genotype, children with normal genotype were significantly more infected with FC27 allelic family (P = 0.01).

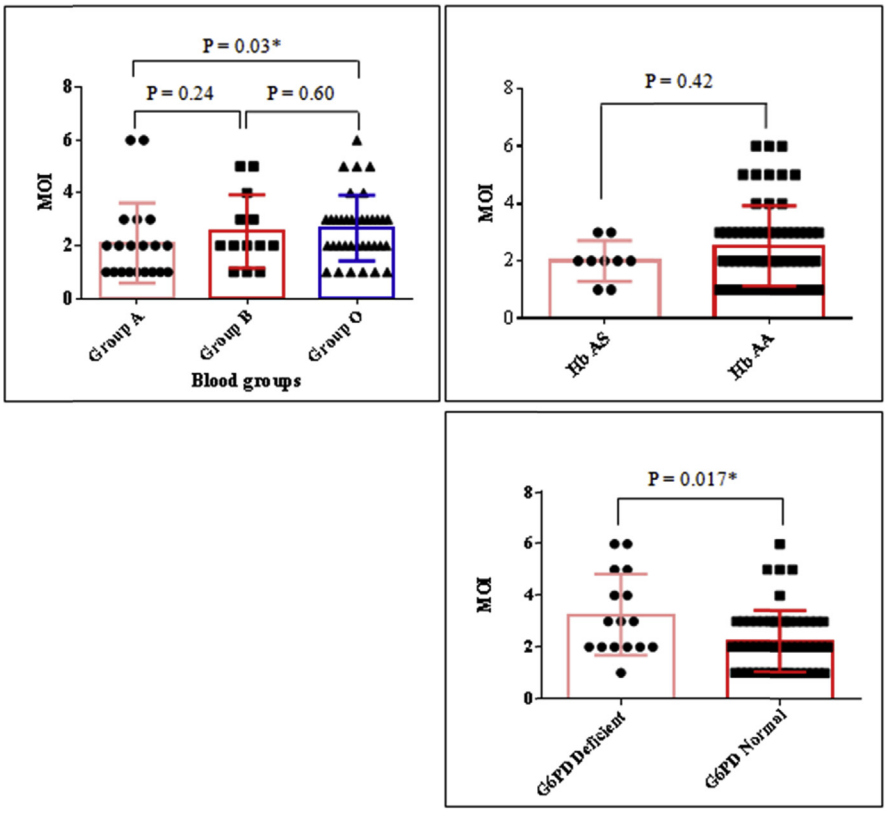

Fig. 2. Multiplicity of infection (MOI) according to erythrocyte polymorphism in asymptomatic malaria children (*: significant difference at 0.05 level). Hb AS: Children with sickle cell trait, Hb AA: Children with normal haemoglobin; G6PD Deficient: children with the genotype resulting in a deficiency in the enzymatic activity of glucose-6-phosphate dehydrogenase (G6PD), G6PD Normal: children with the genotype conferring a normal enzymatic activity of G6PD; MOI: Multiplicity of infection.

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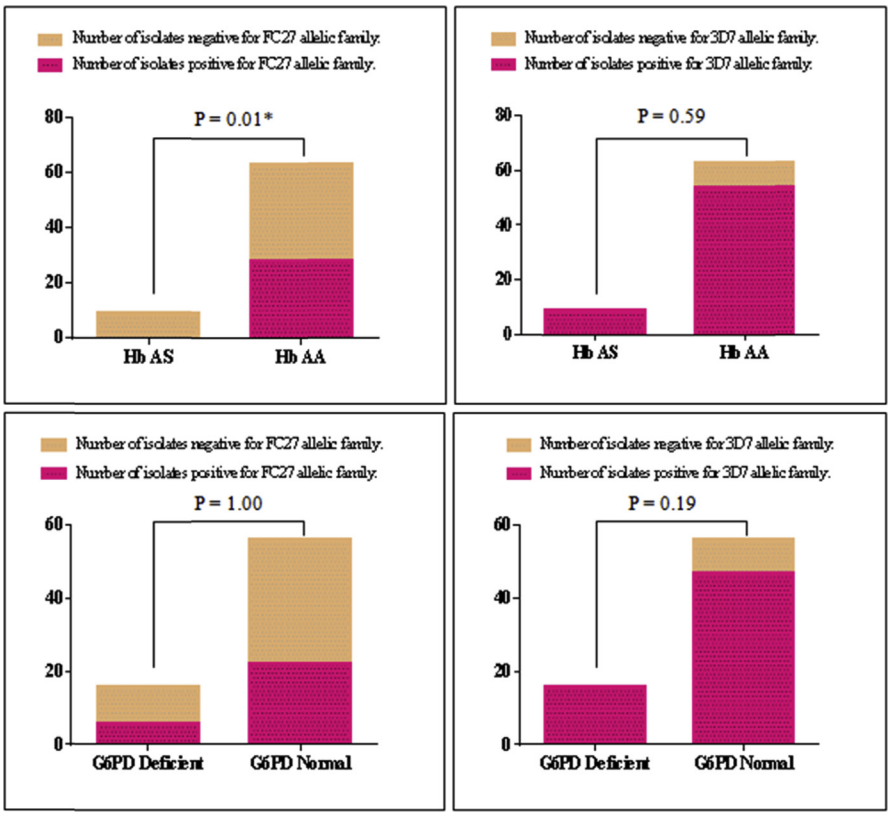

Fig. 3. Allele frequencies and erythrocyte polymorphism (*: statistical significant with Fisher's exact test) Hb AS: Children with sickle cell trait, Hb AA: Children with normal haemoglobin; G6PD Deficient: children with the genotype resulting in a deficiency in the enzymatic activity of Glucose-6-phosphate dehydrogenase (G6PD), G6PD Normal: children with the genotype conferring a normal enzymatic activity of G6PD; MOI: Multiplicity of infection.

4. Discussion

This study was undertaken to assess the influence of the number of msp-2 parasite clones per infection in *P. falciparum* asymptomatic children in relation to ethnicity, sickle cell trait, G6PD deficiency and ABO blood groups. Seventy two asymptomatic malaria children were evaluated for erythrocytic variants along with MOI. In Cameroon very few studies have focused on the relationship between malaria and erythrocytic variants [34, 35]. This is the one of the recent study to provide data on the erythrocytic variants and multiplicity of *P. falciparum* isolates in asymptomatic phenotype in Yaoundé, Cameroon.

No significant association was observed between the MOI and ethnicity. Although this study does not discuss the factors that make it possible to understand this result, the most likely explanation could lie in the intrinsic characteristics of the immune responses of the population studied. Moreover, the candidate alleles of the genes determining the resistance to *P. falciparum* infection, such as the IL4-524T gene, are found in a part of the Cameroonian population [45]. The non significant variation

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in the average number of clones observed in our comparative analysis would then be consistent with the hypothesis that ethnic groups living in this area would probably control the parasitic infection in the same way, certainly by means of immunomodulatory factors. Our observation is contrary to that obtained by Paganotti et *al.* in Burkina Faso, which found the number of clones per infection, was lower in the Fulani ethnic group compared to Mossi; with a greater difference in children under one year old and those over five years old [46].

Influence of MOI with erythrocytic variants was examined in the current study. MOI was significantly higher in group O compared to group A but not in group B; suggesting a protective effect of O antigen against clinical forms of malaria. Unlike the blood group, no effect of Hb AS on the multiplicity of infection was noted in this study. This corresponds to the reports of Senegal [47] but contrary to what was shown in Gabon [48]. This disagreement could be explained by differences in age ranges of the study population. However, the mean values we obtained revealed interesting trends suggesting that the multiplicity of infection tends to be lower in individuals of Hb AS genotype. Futhermore, the alleles of the FC27 family were significantly more abundant in the Hb AA children than in the carriers of the sickle cell trait. Some studies have shown the influence of hemoglobin status on the distribution of allelic families of the msp-2 gene [47]. Such an effect was not observed by Mockenhaupt et al. and Kiwanuka et al. [49, 50]. Therefore, further studies are needed to clarify this issue. MOI was significantly high in children with G6PD deficiency compared to those with normal gene. This observation is not in agreement with that of other reports [51]. A plausible explanation is the geographical variation of the genetic diversity of *P. falciparum* i.e. that the clones identified in our study area are probably different from those observed by these authors. Thus, there is probably a mechanism by which the parasite clones identified in this study would resist the G6PD deficient cell environment.

5. Conclusions

The number of clones per infection is not influenced by ethnicity in our study area**.** Inherited blood disorders including sickle cell trait and G6PD deficiency are present and have some associations with MOI in asymptomatic malaria. However, further longitudinal studies with large sample size are needed to better understand the mechanisms involved.

Declarations

Author contribution statement

Dongang Nana Rodrigue Roman: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Vineeta Singh: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ngono Ngane Rosalie Anne, Koanga Mogtomo Martin Luther, Ngonde Essome Marie Chantal: Contributed reagents, materials, analysis tools or data.

Mouelle Sone Albert: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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