Genetic diversity of *Plasmodium falciparum* and genetic profile after artemisinin-based combination therapy (ACTs) deployment in Cameroon for the management of uncomplicated malaria in Children

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Abstract

Background *Plasmodium falciparum* is the number one cause of malaria morbidity and mortality. Several methods of intervention have been deployed in Cameroon with an attempt to reduce malaria transmission. But evaluation methods mostly based on microscopy and immunology have proven to be cumbersome and expensive. This study aimed at analyzing the genetic diversity of *P.falciparum* and the impact of ACTs deployment on MOI Method 350 clinical isolates were collected between 2012 and 2013 and, three *P. falciparum* loci namely, *msp-1*(block2), *msp-2* (block3), and *glurp*, (region II) characterized by nested PCR and DNA sequencing. Results From this study, a total of 16 different *pfmsp1* were identified, including K1, MAD20 and RO33 allelic families. The K1 and MAD20 were the predominant polymorphic allelic types at the *msp-1* gene, whereas alleles belonging to 3D7/IC were more frequent at the *msp-2* gene. A peculiarity of this study is that RO33 revealed a monomorphic pattern among the *msp-1* allelic type. *Msp-1* and *msp-2* revealed considerably greater parasite diversity than *glurp*. A total of 27 different *msp-2* genotypes were recorded of which 15 belonged to the 3D7-type and 12 to the FC27 allelic families. Alignment of peptides encoded by *pfmsp1* and *Pfmsp2* reveals that K1 polymorphism had the highest similarity in the *P.fmsp1* and *Pfmsp2* clade followed by MAD20 with 93% to 100% homology. Indicating that *P. falciparum* isolates from Cameroon present high identity with allelic sequences from other areas in Africa, suggesting that vaccine developed with k1 and MAD20 of *Pfmsp1* allelic variant could be protective for Africa children. The MOI ranged from 2.51 for *msp1* to 3.82 for *msp2*. The overall heterozygosity ranged from 0.55 for *msp-1* to 0.96 for *msp-2* consistent with the genetic pattern observed in hyperendemic areas. Conclusion The present study reveals that isolates from South West Region of Cameroon are mainly polyclonal with high MOI and highly diverse in respect to both *msp-1* and *msp-2* despite ACTs deployment aiming at reducing malaria transmission. This study lays emphasis on the use of MOI and genotyping of both *msp-1* and *msp-2* in the evaluation of malaria control intervention in malaria endemics countries.

Background

In spite of enhanced control efforts, malaria continues to be a major public health problem in sub-Saharan Africa and Southeast Asia, and *Plasmodium falciparum* infection is prevalent in most of the endemic countries. The World Health Organization (WHO) estimated that, 219 million malaria cases occurred worldwide of which 78% was in Africa. About 407,000 fatal cases were registered, 92% in Africa and 61% of the global death in children under 5 years of age *P. falciparum* being the major cause of all deaths [1,2]. Malaria in Cameroon is caused by three human malaria species: *P. falciparum*, *P. ovale* and *P. malariae*. The prevalence of *P. falciparum* is about 99% while *P. malariae* and *P. ovale* share the remaining 1%. The most deadly malaria is caused by *P. falciparum*. Malaria transmission in Cameroon depends on the population of *Anopheles arabiensis*, a member of the *An. gambiae* Giles complex, a principal malaria vector in Cameroon [3,4]. Malaria is a leading public health problem in Cameroon and is reported as the first cause of morbidity and mortality [5,6], accounting for 16% outpatient visits, 20% hospital admissions and 27% inpatient deaths [6-8]. Despite the current efforts to control malaria in

Cameroon, the situation has not improved, mainly due to the increasing problems of vector resistance to insecticides [10] and resistance to almost all antimalarial drugs including ACTs [11-14]. Merozoite surface protein (MSP-1) and merozoite surface protein-2 (MSP-2) are 2 proteins causing immune responses in humans [15,16] and are important candidates for development of blood stage malaria vaccines [17-19]. The *msp-1* gene is located on chromosome 9 and contains 17 blocks of sequences, of which 7 are variable, flanked by conserved regions [20]. The block 2 *msp-1* is particularly polymorphic and 3 distinct allelic families have been described as *MAD20*, *K1* and *Ro33* [21-25]. The *msp-2* gene is located on chromosome 2 composed of five blocks of which the most polymorphic is the central block 3 [26]. The polymorphic central domain of the gene encoding MSP-2 belongs to 2 distinct families; 3D7 and Fc27 [27-29]. Allelic forms of these antigen genes have been reported from different parts of the world [18,23-32]. Differences in allelic types as well as the number of repeat sequences in *msp-1* and *msp-2* can be detected by PCR, followed by dot-blot hybridization [33]. Size polymorphism is also used not only for the *msps* but also for other markers including *Glurp* and *Csp* in which variations are also detected by sequencing. Genetic diversity of *P. falciparum* populations determines the intensity of malaria transmission [34-37], thus providing baseline data for any antimalarial drug efficacy trial and the possibility of implementing control strategies based on modern intervention or vaccines. Merozoite surface proteins 1 and 2 genotyping is widely used in malaria molecular epidemiology studies to assess the allelic diversity and multiplicity of infection as a proxy of transmission level in molecular monitoring of interventions [32,38]. The *Pfmsp1*, *Pfmsp2* and *glurp* are commonly selected because they are located on different chromosomes, and this reduces the likelihood of linkage disequilibrium [39,40]. Genotyping of these genes has been effectively used to trace individual clones over time in cohort studies and to measure duration of infection [41,42]. Different clones of *P. falciparum* can be determined using regions of highly polymorphic genes coding for MSP-1 and MSP-2, and GLURP. These markers are equally essential in distinguishing recrudescence from reinfection of the parasite in an anti-malarial treatment trial [29,43-46], since the discriminating power of these markers is dependent on the extent of allelic diversity and on the frequency of each allele within a population [29,47]. Genetic diversity of *P. falciparum* has been used to implement specific strategies for control and to evaluate the impact of interventions on changes in malaria epidemiology [10,111]. However, in Cameroon, there are no or limited data on MOI and *P. falciparum* genetic diversity respectively, especially when *msp-1*, *msp-2* and *glurp* need to be considered together as molecular markers in genotyping studies. Thus, the aim of this study was to characterize the highly polymorphic genetic markers of *P. falciparum* field isolates, including the merozoite surface protein 1 and 2 (*msp-1*), the merozoite surface protein 2 (*msp-2*) and glutamate rich protein (*glurp*) and to determine the allele distribution and factors influencing the MOI and heterozygosity as indicator of malaria parasite transmission dynamics

Methods

1. Study site

This study was performed in the Cameroon Development Corporation (CDC) which is an agro-industrial company based in the South West region of Cameroon specialised in the production of rubber, palm oil etc. Cameroon is distinguished by an overall climate which varies with terrain, from tropical rain forest with mean annual temperature of 24.5°C in the south coastal regions to 26.5°C in the semiarid Northern regions. Concerning the Elevation extremes, the lowest point is the Atlantic Ocean at 0 meter and the highest point is Fako (peak of Mount Cameroon) at 4,095 meters above the sea level. The CDC is a parastatal company hosting rural communities in the South West region of the country which is about 30 km south of Buea town and 45 km from Douala (figure 1). The CDC company has headquarters in Limbe town headed by a Director, with a population of about 45,000 workers. The population is heterogeneous consisting primarily of peasant farmers with over 60 % coming from the North West and western region of Cameroon. Limbe has 8 primary and two secondary schools. The community has good access roads, good pipe-borne water and electricity supplies. There is a good health system consisting of two reference hospitals as primary health centers in Limbe town and twenty three satellite clinics located in different towns of the south west region of Cameroon. Apart from the CDC Primary Health Centre and hospitals, there are also private clinics, mission and government health centers and hospitals. The South West region of Cameroon has a Cameroonian-type equatorial climate that favors the proliferation of mosquitoes, the vector of malaria. The climate is characterized by fairly constant temperatures and two seasons: a short dry season (November–February) and a long rainy season (March–November) with abundant precipitation (2,000–10,000 mm) [48]. The mean annual rainfall is 2,625 mm, relative humidity is constantly high (75%–80%), and the temperature varies from 18°C in August to 27°C in March [3]. In general, malaria transmission is intense and perennial in the South West region, with peak periods corresponding to the rainy season [48]. Three anophelines vectors including *Anopheles gambiae*, *A. funestus* and *A. nili* have been identified in malaria transmission in South West Region. *A. gambiae* the most dominant in terms of aggressiveness and activity [3,4,22] accounts for up to 72.7% of transmission, Infection rates of 87 infective bites/person/year [4] and overall entomologic inoculation rate (EIR) estimated recently at 3.93 infective bites/person/night [22]. In addition, houses are grouped into camps which are structured buildings provided by the company to accommodate the workers and their families.

2. Sampling and Parasite collection

P. falciparum isolates were collected from microscopically diagnosed *P. falciparum* positive patients aged six months to 6 years, in clinics and hospitals of CDC Cameroon. Consent to participate in this study was obtained from parents or guardians and blood sample was taken from each patient using finger prick for thick and thin blood films. 2-3 drops of blood were collected on 3 MM Whatman filter paper. A total of 315 blood samples were collected from 23 clinics and 2 hospitals of the CDC Cameroon belonging to 2 different health districts.

3. Microscopy

Blood films were stained with diluted Giemsa stain and then examined microscopically for the presence of malaria parasites; 200 fields under 1000× magnification were examined from the thick film before the slide was considered negative. For positive slides, parasitaemia (parasite density) was determined by counting only the asexual stages against 200 white blood cells (WBC) and then multiplied by 25, assuming the average of total WBC count of individuals equal to 8000 cells/μl of blood. The level of parasitaemia was graded as low (< 1000 parasites/μl of blood), moderate (1000 - 9999 parasites/μl of blood) and severe ($\geq$ 10,000 parasites/μl of blood).

4. Molecular identification and genotyping

Genomic DNA was extracted from blood spots collected on filter papers. Briefly, a disc of the filter paper was punched out from the blood spot using a paper puncher and placed in 1.5 ml centrifuge tubes using clean forceps. Genomic DNA was extracted using Qiagen blood and tissue kit (QIAGEN, Germany) according to the manufacturer's instruction.

Nested PCR amplification and allele detection

DNA was eluted using 50 μl AE (10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0) elution buffer (QIAGEN, DNeasy® Blood & Tissue Kit, Cat. no. 69506, Germany) and kept at -20°C until used for PCR. Genomic DNA was amplified by Nested PCR using allelic specific Primers (Table 1) and conditions for PCR amplification were followed as previously described [15,39,49] for family specific allele analysis of *msp-1* (block 2), *msp-2* (block 3) and Glurp (region II). In the primary PCR, a 25 μl PCR mixture was used containing 1 μl of DNA template, 0.2 μM of each primer, 1x TBE buffer, 250 μM of dNTPs, nuclease free water, 1 U of Taq polymerase enzyme, 2 mM MgCl₂, 10 mM KCl, and 10mM Tris-HCl, at pH 8.3. All reagents were from TIAGEN (Biotechnology, Inc., Beijing). Cycling conditions for the primary PCR were as follows; starting with three single steps of denaturation at 94°C for 5 minutes, annealing at 58°C for 2 minutes and extension at 72°C for 2 minutes. This was followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute and extension at 72°C for 1 minute, then a single annealing step at 58°C for 2 minutes and final extension at 72°C for 10 minutes. *P. falciparum* genotypes were further analyzed by amplification of the two highly polymorphic regions of *msp-1* (Block 2) and *msp-2* (Block 3) using nested-PCR as previously described [49] with slight modifications for the cycling conditions of the secondary PCR. Briefly, oligonucleotide primers sets (Suppl. 1, Table1), were used for detecting the different families (K1, MAD20 and RO33 in MSP-1; FC27 and IC in MSP-2). Three microliters of primary PCR products were used as the DNA templates in the secondary PCR which had similar concentrations to the primary PCR. The cycling conditions for the secondary PCR were as follows: starting with a single step of denaturation at 95°C for 10 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. PCR reaction mixtures were incubated in a thermal cycler.

The secondary PCR products were separated by electrophoresis at 100 volts on 1.5 % molecular grade agarose gel (Caisson, Utah, USA), stained with ethidium bromide, submerged in 0.5 X TBE (Tris-borate EDTA) buffer and visualized by UV transilluminator (BioDoc-It UVP, Cambridge, UK) at 302 nm on gel documentation system. The number and size of DNA fragments were estimated based on their mobility related to a 100bp DNA ladder (Vivantis, Selangor Darul Ehsan, Malaysia). DNA fragment sizes were binned into different classes of 20 and 50 bp ranges with each bin assigned as an allele. Alleles in paired samples were considered a match for *msh2* and *msh1* if within 20 base pairs and for *glurp* if within 50 base pairs [27,30]

5. Multiplicity of infection (MOI) and heterozygosity (He)

Multiclonal infections were defined as those having more than one allele in at least one locus out of the loci genotyped. The MOI was determined by calculating the number of different alleles at any one locus detected in the sample[19]; single infections were those with only one allele per locus at all of the genotyped loci. Isolates with more than one genotype were considered as polyclonal infection while the presence of a single allele was considered as monoclonal infection. The mean multiplicity of infection (MOI) was determined as the quotient of the total number of *P. falciparum* genotypes detected in MSP-1 or MSP-2 by the number of samples positive for either *msh-1* or *msh-2* [50].

As a measure for genetic diversity, the expected heterozygosity (HE) which represents the probability of being infected by two parasites with different alleles at a given locus and ranging between 0 and 1 [29] was calculated by using the following formula:

$HE = [n/(n-1)] [(1-\sum p_i^2)]$, where n is the number of isolates sampled and p_i is the allele frequency at a given locus.

6. Statistical analysis

Data was analyzed using the SPSS for windows software version 17. For descriptive analysis, proportion was used to present the distribution of different allelic families while the mean was used to present the multiplicity of infection (MOI). Independent chi square test was used to compare the mean MOI according to gender, and parasitaemia. A p-value ≤ 0.05 was considered indicative of a statistically significant difference.

7. *msh-1* and *msh-2* sequence analysis

Purified PCR products of isolates representing different alleles of *msh-1* and *msh-2* were sequenced in both directions with the primers of the secondary PCR using the ABI PRISM® BigDye™ terminator Ready Reaction Cycle Sequencing Kit (Biometra Thermocycler, England) according to the manufacturer's

instruction. The sequences were then analyzed using the DNASTAR software package (DNASTAR, Madison, WI). The sequences were used to correct the estimated molecular weight and to confirm the nature and size of the amplified product. To understand the identity of Cameroonian isolates with respect to isolates of other regions, sequence data available in public domains were downloaded for allelic families of *msp-1* and *msp-2* and aligned using ClustalW method (EMBL-EBI, Hixton, and Cambridge, UK), then analysed by MEGA version 5.1 (<http://mega.software.informer.com/5.1b/>). We sought to identify sequence differences using BLASTN sequence homology searches. Each allelic family *msp-1* (including K1, MAD20 and R033) and *msp-2* (including FC27 and 3D7/IC) were analyzed separately in order to estimate the average number of nucleotide substitutions for each *msp-2* allele, and to examine the mode of evolution of FC27 type repeats. We assembled and aligned individual repeat units with DNASTAR and BLAST searched in GenBank to analyze the distribution of the pairwise proportion of nucleotide differences among individual repeat units using isolates derived from GeneBank, namely the isolate from Gabon accession number AY372506 for FC27, the isolate HM568631 from India for 3D7/IC allelic family. As for *msp-1* alleles, the isolate from Brazil (accession:JX416338) for the alignment of K1-type allele, while the isolates EU032224 from central Sub Sahara Africa and AY138508 from Iran were used as reference isolate for the sequence analysis of R033 and MAD20, respectively.

Results

1. Study profile

Out of 315 DNA samples at baseline, 137 samples for *msp-1* and 307 samples for *msp-2* were successfully amplified by PCR, among which 107 for *msp-1* and 243 for *msp-2* were successfully sequenced.

2. Genetic diversity and haplotype frequency

The number of genotypes observed at each marker is shown in Table 2. Sixteen (16) different *msp-1* genotypes were observed, representing K1 (9 genotypes), MAD20 (6 genotypes) and R033 (1 genotype) allelic families. The *msp-1* fragment sizes ranged from 153 bp - 335 bp, while the *msp-2* fragments ranged from 140 to 568 bp both for the FC27 and IC allelic families (Table 2).

The majority (87.5%) of these genotypes especially for the k1 (100%) and MAD20 (97.5 %) belonging to the *msp1* allelic family occurred at a frequency below 10% (Table 2). However, one genotype the MAD20 family (167-187bp) occurred above 10%. The R033 family was found to be monomorphic with an amplified fragment size of 155 bp and occurred at a frequency of 78.1% (107/137) (Table 3) of the overall *msp1* genotypes.

The *msp-2* diversity and genotype frequencies are shown in Figure 2a and 2b (Suppl. 2; figure 2a, 2b) while Figure 2c shows the overall frequencies of *msp-2* genotypes. A total of 27 different *msp-2* genotypes (size range from 140 to 568 bp) were recorded of which 15 belonged to the 3D7-type and 12 to the FC27 allelic families (Table 2). The majority of these genotypes occurred at a frequency below 10%

(figure 2c). However, 1 genotype from the 3D7/IC allelic family (420 bp-440 bp), 2 from the FC27 family (176 bp-201 bp and 201 bp-226 bp) occurred above 10% (figure 2c). The 3D7/IC family was found to be highly polymorphic ($p=0.01$) as compared to FC27 (Suppl. 2; figure 2a and 2b).

The *glurp* diversity and genotype frequencies are shown in Figure 4. A total of 5 different *glurp* genotypes (size range from 597 to 817 bp) were recorded with the majority of the allelic families occurring at above 10 %. However, only two alleles occurred below 10% of which, 4.67% and 9.34% belonged to the 550-600 bp and 652-702 bp allelic group.

In *msp-1*, more than half of the *msp-1* positive samples harbored all the three types of alleles of the *msp-1* gene. Thus the combination of RO33, MAD20 and K1 allelic families was identified with an overall frequency of 57.50% (Figure 4a). The RO33 allelic family was predominant as it was identified in 78.10% (107/137) of the samples. One third (33%) of the blood samples positive for *msp-1* were identified as monoclonal infections while two-third (66 %) exhibited a polyclonal pattern of K1, MAD20 and RO33 combination in a set of two or three alleles (Figure 4a). Among the polyclonal infections, K1/RO33, K1/MAD20 and RO33/MAD20 constituted 6.66%, 1.6% and 0.83% of the *msp-1* positive isolates respectively. The distribution of the identified *msp-2* allelic families is illustrated in Figure 4b. Overall, 11.42 % of *msp-2* positive isolates were identified as monoclonal infections either for 3D7/IC or FC27 allelic families against 88.56% *msp-2* positive isolates (figure 4b) displaying a polyclonal pattern of infections. The frequency of the 3D7/IC and FC27 haplotypes combinations was found to be higher than the frequency of samples with only 3D7 or FC27 allelic families (figure 4b).

3. Multiplicity of infection and heterozygosity

P. falciparum isolates in this study had higher rates of multiple genotypes infection with an overall mean multiplicity of infection of 3.16. The mean MOI for *msp-2* loci was the highest (3.82), as compared with *msp-1* (2.51) Table 3). In the children, there was no difference in mean MOI between male and female ($P=0.1$) (Table 4). However, the influence of parasite density on genetic diversity showed that children with parasite density ranging from 2000 to 5000 had the lowest MOI (Table 5). Meanwhile those with parasite ranging from 20000 and above exhibited the highest MOI difference ($P=0.001$), as compared with various range of parasite densities.

The mean MOI was found to be higher in male patients whose parasitaemia ranged from moderate to high level, than that of male patients with low parasitaemia. The expected heterozygosity (HE) was calculated which used to estimate the fraction of all parasites that would be heterozygous for any of the two loci. Table 3 shows generally high HE values in each allelic family ranging from 0.5 to 0.65 for *msp-1* and from 0.94 to 0.96 for *msp-2*.

The influence of parasite density on genetic diversity showed that children with parasite density ranging from 2000 to 5000 had the lowest MOI (table 5) meanwhile those with parasite ranging from 20000 and above exhibited the highest MOI as compared with various ranges of parasite densities.

4. Sequence analysis of genetic polymorphism of *m*sp-1 block 2 and *m*sp-2 block3

The *m*sp-1 block 2 and *m*sp-2 block 3 DNA fragments from PCR products with either similar or different molecular sizes in agarose gels were sequenced with the aim of further estimating the genetic diversity of the parasite population. Partial gene sequences were obtained from each end of the PCR product using the same primers for the nested PCR reactions, then DNA sequences assembled and BLAST searched in GenBank to identify identical or similar sequences. Analysis of *m*sp-1 and *m*sp-2 sequence data revealed above 80% identity of study isolates among themselves in general and above 83% with isolates from other countries with a few exceptions (Suppl. 2; Table 6). Especially, the R033 allelic sequences of Cameroonian isolates were all very identical and had shown above 99% similarity (Suppl. 2; Table 6) with sequences reported for isolates of Kenya [51] and Indonesia [52]. In K1 family, 93 to 99% similarity was observed with K1 allelic sequences reported for isolates of Tanzania [53], Indonesia (accession no. U72950), Vietnam and Brazil [54]. Likewise, 83 to 100% identity was observed in MAD20 family with isolates of Iran [55], Sudan [56], Vietnam [54]. Allelic families of *m*sp-2, FC27 and 3D7/IC showed 97 to 100% identity with isolates of Gambia [57], Brazil (accession no. DQ115969) Tanzania (accession no. AY378316), and Gabon (accession no. AY372518). No significant identity was found with either the Nigerian or the Brazilian isolates with respect to the 3D7/IC allelic family of this study. However 89 to 97 % identity were recorded with isolates from Gambia and Thailand [57] with the highest identity recorded with the isolates from India (97%) [58].

Discussion

The aim of this study was to perform a molecular characterisation of highly polymorphic markers in *P. falciparum* clinical isolates. In this first study conducted after the introduction of artemisinin-combination therapies, it was observed that *P. falciparum* field isolates in Cameroon exhibited a high degree of genetic polymorphism in *P. falciparum* *m*sp-1 and *m*sp-2 markers. These genes encoding for individual functional proteins expressed on the surface of the merozoite appear to play an essential role in the invasion of the red blood cell [59,60] and have captivated a lot of interest as potential vaccine candidates and as drug targets for inhibiting blood-stage replication [61-63]. Our findings revealed a high proportion of multiclonal isolates and MOI and a total of 16 different *Pfmsp*-1 and 27 different *Pfmsp*-2 gene types . This figure is not exhaustive since it is obvious that with nested PCR genotyping some of the sub-populations present in mixed infections would not be fully typed by all amplification steps [42,63]. Moreover, more amplified product was found for the *m*sp2 gene than *m*sp1 amplified products, this could be explained by non-synonymous substitutions introduced in template DNA that could jeopardize the proper annealing of the primer at its binding site in *m*sp1 gene or could be explained by the fact that natural selection is more efficient when acting on *m*sp-1 than *m*sp-2 [59,64] suggesting that *m*sp-1 as compared with *m*sp2 proteins are under strong functional constraints in a complex interaction with the host leading to an increased in host's immunological response [65]. The two markers including *m*sp-1 (16 genotypes) and *m*sp-2 (27 genotypes) revealed considerably greater parasite diversity than *glurp* (5 genotypes). Thus, the observed genetic polymorphism in the these two *P. falciparum* major merozoite surface proteins *Pfmsp*1 and *pfmsp*2 could be explained by balancing selection occurring as a result of

different mechanisms of interaction with the host [66]. This distribution of families of *msh-1* and *msh-2* and their allelic variations were similar to that reported from other countries with meso- to high endemicity of malaria [67, 68]. Genotyping procedure recommended in antimalarial drug trials stipulates consecutive analysis of the three markers starting with *msh2* or *glurp*, and then *msh1* [111]. Based on allelic profile of each gene obtained in this study, parasitological outcome assessment could be more accurate when both markers *msh-1* and *msh-2* are included in the genotyping of recurrent parasitemias in antimalarial drug trials, consistent with other studies [42]. Our findings equally raise concern over the use of *glurp* genotyping in antimalarial drug trials since this marker revealed limited allelic families as compare to *msh-1* and *msh-2*.

1. Genetic diversity for *msh-1* and *msh-2* Allelic families

The present study reported higher numbers of alleles (43 alleles for both MSP-1 and MSP-2) than previously reported to be circulating in the study area and in the central region of Cameroon [22,27]. A peculiarity of in this study is that RO33 was found to be monomorphic and the most predominant allele type of *msh1* compared to the polymorphic K1 and MAD20 allelic families in agreement with previous studies where RO33 was found to be the most predominant allelic family for *msh1* locus [39,54,67-70]. However, our finding does not corroborate other studies in which RO33 was identified as the least predominant allelic variant type while MAD20 was the most predominant allelic family of the *msh1* locus [22, 34]. This discrepancy could be attributed to the difference in the degree of transmission intensity. Another peculiarity of our study is that, the RO33 family of *msh-1* did not show any polymorphism, with only 1 variant (155 bp) detected. This result differs from that of Gabon and West Uganda, where the Ro33 family was polymorphic with three and four allelic variants, respectively [15, 71], but was close to that in Senegal [72] and Brazil [73], showing the monomorphic nature of RO33 family of *msh-1*. The allelic variant K1 was the second highly distributed after RO33. This does not corroborate previous finding in the same region of Cameroon [22]. but is consistent with most findings in areas of holoendemic, mesoendemic, and hyperendemic malaria, in which the allelic variant K1 was predominant[39].The predominance of Ro33 and K1 allelic family could be attributed to the balancing selection acting on these two allelic variants[74]. The MAD20 allelic variant was the least predominant among the *Pfmsp1* allelic family in agreement with other studies conducted Africa including the Gambia, Nigeria, and Gabon [54,70]. This result is does not corroborate other studies reporting the predominance of MAD20 allelic variant over K-1 and RO33 allelic variants [23, 39, 74-76. This low distribution of MAD20 allelic variant could be partly explained by a purifying selection acting on MAD20 allelic type as compared with RO33 and K1 allelic type. This could equally be attributed to a single nucleotide substitution in DNA template that hinders proper annealing with primers designed to amplify the MAD20 allelic type [77].. An association between the distribution of k1 allelic families with severe malaria has been investigated [67,78] while theRO33 allelic family has been frequently reported in asymptomatic malaria cases[21,34]. The predominance of Ro33 seems to be beneficial for the host since the presence of this allele type is related to reduced risk of clinical malaria[67,78].A significant correlation has been established between the genetic diversity of the *msh-1* gene of *P. falciparum* and parasite density. Likewise an association has

been observed between MSP-1 allele diversity and age group on one hand, and between *m*sp-1 allele diversity and gender among asymptomatic patients on the other hand[79].

In *m*sp-2 the allelic families IC/3D7 and FC27 were almost of equal frequencies consistent with other findings [55], but in contrast with previous reports showing a significant predominance of FC27 over the 3D7/IC allelic family [34]. The frequencies of individual *m*sp2 genotypes were low with 88.9% occurring at a frequency $\leq 10\%$. However 2 (16.7%) genotypes, belonging to the FC27 and 1 (6.7 %) belonging to the 3D7/IC allelic family were found at frequencies of above 10%. High genetic diversity and low allelic frequencies have been reported previously from other sites including Gabon [15], Uganda [44], Senegal [81], Burkina Faso [82] and Honduras [36].

Diversity, expressed as expected heterozygosity (*He*), ranged from 0.55 for *m*sp-1 to 0.96 for *m*sp-2 suggesting that the parasite population in Cameroon, exhibits intermediate to higher heterozygosity reflecting intermediate to high transmission pattern [30] consistent with the findings in Uganda, Congo and Zimbabwe showing a high heterozygosity ranges between 0.78 and 0.8 for *m*sp-2 [34,83]. The correlation between the genetic variation of *P. falciparum* and malaria endemicity has been described [34-36, 71]. In areas with declining endemicity, it is reported that the number and diversity of alleles (heterozygosity) decrease with decreasing *P. falciparum* transmission [32,83]. The present study reveals that the *m*sp-2 gene is highly polymorphic as compared with *m*sp 1 gene. This observation is different in low transmission settings where high diversity has been recorded for *m*sp-1 as compared with the *m*sp-2 gene [23,32]. Hence, the high allelic diversity together with the low frequency of individual circulating alleles observed in the present study increase the discriminatory power of *m*sp-1 and *m*sp-2 to differentiate between recrudescence and re-infection. Thus, this study reinforces the importance for the genotyping of *P. falciparum* based on *m*sp-1 and *m*sp-2 in antimalarial drug efficacy trials, to distinguish between re-infection as recrudescence and emphasize on the importance of implementing *m*sp-1 and *m*sp-2 genotyping in effective malaria management and malaria control strategies in Cameroon and in endemic areas.

2. Polyclonal infection expressed as the MOI values

In this study most patients with *P. falciparum* infections were infected with multiple genetically distinct parasite variants with high level of polyclonal infections observed among *m*sp-2 (88.6%) and *m*sp-1 (33.8%) positive isolates. Such a pattern of parasite structure is typical among *P. falciparum* populations in areas of high transmission, such as in sub-Saharan Africa where more than 10 variants can be routinely detected in an individual [32,62-64] and selection among these variants in the host is likely to play an important role in parasite diversity. In contrast, in areas of low transmission, such as in Asia or Latin America, patients may have infections with as few as a single variant[37,84]. No data on MOI was available in Cameroon before the introduction of ACT renders it difficult to draw a conclusive statement on the impact of ACTs introduction on MOI. Nevertheless, in this study, the polyclonal infection expressed as the MOI values were heterogeneous across the different loci, and the mean MOI was highest for *m*sp-2

than *msp-1* in accordance with previous studies in neighbouring countries with high intensity of malaria transmission including Congo and Gabon [85]. Higher MOI in Cameroon can be the results of multiple infectious mosquito bites or transmission of genetically diverse sporozoite inoculum from a single mosquito bite. Genetically distinct malaria parasites in natural populations have an extremely high rate of genetic recombination during the sexual stages resulting in multiple strains being transmitted simultaneously [86]. Effective recombination of parasites and mutation occurring in several rounds of DNA replication cycles will likely continue to maintain this genetic diversity. During genetic recombination, novel combinations of alleles can be generated offering beneficial features to the parasite, as driven by positive selection enabling spread alleles through the population. Since the evolutionary selection of malaria occurs both within individual hosts and within populations, determining the number of strain in an infection might be an important indicator of transmission intensity [87]. Effective malaria control measures (ACTs deployment, distribution of ITNs) have successfully reduced malaria transmission in many hyperendemic regions of sub-Saharan Africa. After an intervention, the malaria parasite population structure and transmission rate in these regions is expected to become similar to the low transmission rates of the regions of Southeast Asia and South America. The declining malaria transmission, as a result of scaling up interventions, has been shown to affect the genetic diversity pattern and population structure of *P. falciparum* [38,112]. Therefore the high MOI observed in this study is a reflect of high intensity of malaria transmission in Cameroon, despite several control strategies deployed at the health facilities and in the community such as distribution of free ACTs treatment and mosquito bed nets. This is in agreement with previous findings that observed an increase of MOI with an increase in malaria endemicity [35,36] and a low MOI for *msp-1* and *msp-2* correlated with a low intensity of malaria transmission [37].

Therefore regular molecular epidemiological surveys need to be performed in order to monitor the genetic diversity of *P. falciparum* populations in different regions of Cameroon and the world, and then correlate parasite genotypes to the disease phenotypes [79,88-90]. Previous studies reported a reduced risk of clinical malaria associated with polyclonal infections [91] and high rate of severe malaria in individual harbouring mono-infections and very common genotypes [92, 93]. Our findings revealed an increase in the mean MOI according to parasite density but not according to the gender of patients which is consistent with previous studies showing significantly high MOI in patients with moderate to high transmission [55, 94] . In the present study, most of the positive samples were from children aged 0-5 years, and this limited range of age constraint examining the correlation between the MOI and age. However, previous studies showed a pattern of greater MOI in older individuals than younger individuals reflecting more previous exposure to infection [95] while conflicting findings, indicated decreased MOI with age [96,97]. Thus, determining the MOI in endemic area including Cameroon is very important since it can be used to predict clinical outcome and target population with higher attention.

3. Genotyping by gel electrophoresis and direct sequencing

In this study, the polymorphic surface antigens *msp2*, *glurp*, and *msp1* genes for 304 isolates were successfully amplified by Nested PCR and genotyped using agarose gel electrophoresis of which 127

msp-1 and 297 *msp-2* gene fragments with single band were selected for further characterisation by direct sequencing. In this study, the lengths of the repeat units, whose number varies between the different allelic variants, was taken into account to set bin width of 20 bp for *msp-1* and 50 bp and *msp2* then *glurp* [30]. However, variations in bin width [44,98] and the different fragment sizing methods need to be standardized to facilitate the comparison of data for a particular marker between studies. Although, genotyping method using gel electrophoresis may face some confounding factors including the variability in the electrophoretic migration of a given DNA fragment, this genotyping method could be very important where alternative method are not available and is widely used for *P. falciparum* genotyping [88,89] beside capillary electrophoresis [40,69,99,100] recognise to have higher resolution power than gel electrophoresis in the ability to distinguish between allelic variants of amplified fragments. However, PCR artefacts are a challenge for this method and MOI determination is often underestimated not only with gel electrophoresis but also with capillary electrophoresis analysis especially when genotyping strategy do not considered separate nPCRs for each allelic family [42]. In contrast to previous studies, a separate nPCR followed by direct sequencing was performed in the present study enabling accurate determination of the fragments sizes for *msp1* and *msp2* allelic families as well as sequence motifs and nucleotide differences. The development of single-nucleotide polymorphism-based genotyping techniques and next-generation sequencing, might provide highly diverse haplotype markers with sufficient resolution to detect minority population in a mixed infection[101]. Thus, Next generation sequencing technologies and genome-wide characterization could be an alternative strategy to accurately analyse polyclonal infections [103] although complete haplotype characterization of multiclonal infections remains a challenge due to PCR artefacts and sequencing errors [103,104]. Therefore, in order to accurately study the competition and selection between variants in mixed malaria infection, new tools, more sensitive to detect minority populations and quantitative for relative parasite population sizes are required [105]. Another strategy could be the combination of sequencing method and efficient computational tool for an effective characterisation of allelic variants. A good number of software packages are being developed to analyse genome-wide SNP data of field isolates for the estimation of the presence of multiple genotypes, especially minor allele in multiclonal infections [106,107]

4. Sequence analysis of genetic polymorphism of *msp-1* and *msp-2*

This study showed highly diverse nature of *P. falciparum* isolates of Cameroon in respect to length and sequence motifs. Sequencing and gene alignment confirmed the identity *Pfmsp1* and *Pfmsp2* polymorphisms. Thus, when performing the gene alignment, we found high similarity between the peptides in *Pfmsp1* and *pfmsp2* of Cameroon and in the other regions in Africa. However, from all peptides analyzed, the region of the alignment corresponding K1 polymorphism had the highest similarity among all the species in the *Pfmsp* clade included in this study ranging from 93% to 99% homology with previously described polymorphism in isolate from Kenya and Tanzania. The MAD20 peptide sequence polymorphism was the second most conserved with 83% to 100% homology between *P. falciparum* isolates of Cameroon and other region of Africa and other region of the world. Thus the development of a vaccine based on K1 and MAD20 allelic variant could likely be effective in providing immune protection against malaria in those regions in Africa, although it is not yet known to what extent the high allelic

diversity within the *K1-like* and *MAD20-like* allelic types is of immunological significance [66]. However, previous analysis indicated more serological variation among the allelic sequences of the K1-like compared to the MAD20-like type [108,109], and more effort has been made to incorporate the repeat sequence variation of the K1-like alleles in recombinant antigens towards design of a multivalent vaccine [64,77,110] and more than 500 different *msp1* block 2 allelic sequences has been described, providing a reference for molecular epidemiological studies and potentially for design of a multi-allelic vaccine [66]. Sequencing and immunologic characterization of other allelic variants such as MAD20 for *Pfmsp1*, alongside with 3D7/IC for *pfmsp2* should be conducted to obtain more useful information.

Conclusions

In this first study conducted five years after the introduction of artemisinin-based combination therapies, we investigated the genetic diversity of *P. falciparum* isolates from children aged 6 months to 6 years. The present study shows that field isolates of South West Region of Cameroon were found to be mainly polyclonal with high MOI and highly diverse in respect to both *msp-1* (block 2) and *msp-2* (central repeat region, block 3). These markers appear to be highly polymorphic with low allelic frequencies as compared to *glurp*. This observation reinforced the value of *msp-1* and *msp-2* markers of *P. falciparum* for PCR correction of treatment outcomes in classifying recurrent post-treatment *P. falciparum* episodes as recrudescence or new-infections in drug clinical trials. This study lays emphasis on the use of both *msp-1* and *msp-2* genes in monitoring the trend of malaria epidemiology, and the use of MOI as an important indicator in the evaluation of malaria control interventions. The High MOI observed in this study is an indication that malaria transmission remains high in Cameroon despite a large distribution of ACTs and calls for intensifying control intervention. Besides, our findings reveal that population structure of *P. falciparum* isolates is identical in Cameroon as revealed by presence of common allelic composition and the high level of identity among allelic sequences from Cameroonian isolates and that of other areas in Africa and in the world and that *P. falciparum* population is a mixture of different strains. Therefore, vaccine developed with k1 and MAD20 of *Pfmsp1* allelic variant could be protective for Africa children but this finding will require further genetic and immunological characterisation. It is anticipated that, clonal selection could not be the uniform across the country considering the varied climate range observed in Cameroon moving from the northern region (with sahel desert) to the south (with equatorial dense forest) corresponding to different malaria epidemiology. Therefore, it will be relevant to carry out studies on the genetic diversity of *P. falciparum* isolates from other regions of Cameroon with varied malaria epidemiology as well as longitudinal studies involving other malaria transmission related markers such as CSP to further understand the clonal fluctuations associated with transmission intensity.

Abbreviations

ACTs:	artemisinin-based combination therapy
CDC:	Cameroon Development Corporation

EDTA:	Ethylen diamine tetraacetic acid
He:	Heterozygosity
MOI:	Multiplicity of infection
Pfmsp1:	Plasmodium falciparum merozoite surface protein 1
Pfmsp2:	<i>Plasmodium falciparum</i> merozoite surface protein 2
Glurp:	glutamate rich protein
CSP:	Circumsporozoite protein
PCR:	polymerase chain reaction
TBE:	Tris-borate EDTA

Declarations

Ethics approval and consent to participate

Parent or guardian of participants provided written informed consent before enrollment in this study in accordance with the principles of the World Health Assembly of 1975 on Ethics in Human experimentation and the Helsinki Declaration. All malaria patients received antimalaria drug free of charge. This study was approved by the Ethics Committees of the Ministry of Public Health Cameroon and by the provincial CDC authorities and retrospectively registered with controlled-trials.com in the website:

<https://clinicaltrials.gov/ct2/show/NCT02974348> under the registration number NCT02974348

Consent for publication

Not applicable

Availability of data and material

All data generated and/or analysed during this study are included in this published article [and its supplementary information files] and are available from the corresponding author on reasonable request.

Competing interests

All authors declared no conflict of interest.

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Authors' contributions

MTN and ZZN designed and organized the study. ZZN, JHC and SMR supervised the study. MTN, PFG, XZ and JHC carried out the field and laboratory work and preliminary data analysis. MTN drafted and wrote the manuscript. PFG analysed data and wrote the manuscript. All authors read and approved the final manuscript.

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Tables

Table1. Sequence of primers of *msh1* and *msh2* genes of *P. falciparum* from Cameroon

<i>Msp-1</i> (Block2)	Primary PCR	CHM1-OF: 5' CTAGAAGCTTTAGAAGATGCAGTATTG-3' CHM1-OR: 5' CTAAATAGTATTCTAATTCAAGTGGATCA-3'
	Secondary PCR	CHM1-KF: 5' AAATGAAGAAGAAATTACTACAAAAG GTGC-3' CHM1-KR: 5' GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3' CHM1-MF: 5' AAATGAAGGAACAAGTGAACAGCTGTTAC-3' CHM1-MR: 5' ATCTGAAGGATTTGTACGTCTTGAATTACC-3' CHM1-RF: 5' TAAAGGATGGAGCAAATACTCAAGTTGTTG-3' CHM1-RR: 5' CATTTGAAGGATTTGCAGCACCTGGAGATC-3'
<i>Msp-2</i> (block3)	Primary PCR	M2-OF: 5' ATGAAGGTAATTAACACATTGTCTATTATA-3' M2-OR: 5' CTTTGTACCATCGGTACATTCTT'3'
	Secondary PCR	M2-FCF: 5' ATATTAAGAGTGTAGGTGCARATGCTCCA-3' M2-FCR: 5' TTTTATTTGGTGCATTGCCAGAACTTGAAC-3' M2-ICF: 5' AGAAGTATGGCAGAAAGTAAKCCTYCTACT-3' M2-ICR: 5' GATTGTAATTCGGGGGATTCAAGTTTGTTCG-3'
<i>Glurp</i> (RegionII)	Primary PCR	CHG-OF: 5' -TGAATTTGAAGATGTTCACTGAAC-3' CHG-OR: 5' -GTG GAATTGCTTTTCTTCAACACTAA-3'
	Secondary PCR	CHG-NF: 5'-TGAATTTGA AGA TGT TCA CAC TGA AC-3' CHG-OR: 5'-GTG GAATTGCTT TTTCTTCAACAC TAA-3'

Table 2. Base pair range and number of detected genotypes of the respective *msp-1* and *msp-2* gene families in *P. falciparum* isolates from 315 malaria patients in Cameroon

Marker	<i>msp-1</i>			<i>msp-2</i>	
	K1	MAD20	R033	FC27	3D7/IC
Number of different genotypes per allele	9	6	1	12	15
Allele range (bp)	153-335bp	175-205bp	155bp	140-387bp	200-568bp
Total per locus		16			27
Genotypes frequency for allele type occurring at a frequency <10%	9 (100%) (0%)	5 (97.5 %)	0	10 (83.3%) (95.3 %)	14
Total per allele		3 (87.5%)		24 (88.9%)	
Genotypes frequency for allele type occurring at a frequency >10%	0 (0%) 1(100%)	1(16.7%)		2 (16.7%) 1 (6.7%)	
Total per allele		2 (12.5 %)		3 (11.1 %)	

Table 3. Heterozygosity, and multiplicity of infection (MOI) per *msp1* and *msp2* allelic families in *P. falciparum* isolates from 315 malaria in Cameroon.

	Frequency	No. fragments	He	MOI
K1 (%)	80 (58.39%)	197	0.65	2.46
MAD20 (%)	40 (29.19%)	40	0.50	1.00
R033 (%)	107 (78.10%)	107	0.50	1.00
Total/ <i>msp1</i>	137	344	0.55	2.51
3D7(IC)	295 (96.1%)	621	0.99	2.10
FC 27	298 (97.1%)	552	0.94	1.88
Total/ <i>msp2</i>	307	1183	0.96	3.82

Table 4. Distribution of MOI by gender among children with falciparum malaria

	PCR+	No. 3D7/IC fragments	No. FC27 fragment	No. FC27 + 3D7 fragment	MOI
Female	157	43	21	175	1.52
Male	150	76	22	202	2
Total	307	119	43	377	

Table 5: Distribution of msp2 allele by parasite density among children with falciparum malaria

	PCR+	No. 3D7/IC fragments	No. FC27 fragment	No. FC27 + 3D7/IC fragment	Total fragments	MOI
2000-5000	123	54	39	95	188	1.53
5001-10000	36	6	4	50	60	1.67
10001-15000	43	31	24	63	118	2.7
15001-20000	36	24	27	35	86	2.8
20001-Above	69	64	51	115	230	3.3
	307	203	162	358	723	2.6

Figures

Figure 1

Map of major health facilities of the Cameroon Development Corporation (CDC), South West Region of Cameroon and Geographic map of Cameroon, Source;
<https://www.worldatlas.com/webimage/countrys/africa/cm.htm>

Figure 2

2a. The frequencies for 3D7/IC allelic family of msp-2 genotypes (Suppl. 2; figure 2a, 2b,2c) 2b. The frequencies for FC27 allelic family of msp-2 genotypes (Suppl. 2; figure 2a, 2b, 2c) 2c. Relative allele frequency in the sequenced fragments and adjusted to the overall population of msp2 (Suppl. 2; figure 2a, 2b, 2c)

Figure 3

Allele frequency of *P. falciparum* glurp

Figure 4

4a. Distribution of msp-1 haplotypes. 4b. Distribution of haplotypes for msp2 allelic family

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