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Polymorphism in two merozoite surface proteins of *Plasmodium falciparum* isolates from Gabon

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Abstract

Background: *Plasmodium falciparum* antigenic diversity and polymorphism confuses the issue of antimalarial vaccine development. Merozoite surface protein (MSP)-1 and -2 are two highly polymorphic vaccine candidates. Characterisation of their precise polymorphism in endemic regions may facilitate the design of an effective vaccine.

Methods: Isolates obtained in 52 Gabonese children presenting with uncomplicated malaria were genotyped by nested-PCR of *msp-1* block 2, and *msp-2* block 3, to analyze both parasite population polymorphism and clone fluctuations.

Results: Twenty-five and 19 different alleles were respectively obtained for *msp-1* and *msp-2* loci, the RO33 family of *msp-1* being poorly polymorphic. Four cases of non-random distribution of alleles were reported of the FC27, and/or 3D7 families of *msp-2*. All but two isolates were composed of more than one genotype, and the multiplicity of infection (MOI) was 4.0. Neither parasite density nor age was related to MOI. Clone fluctuations were studied for ten subjects who were sampled again at reappearance of parasites in blood. Disappearance and reappearance of alleles were observed following treatment, suggesting difficulties in assessing polymorphism and in distinguishing reinfection from recrudescence.

Conclusion: *P. falciparum* polymorphism is extensive in Southeast Gabon, and most of infections are composed of multiple clones. The fluctuation of clones contributes to parasite diversity.

Background

One main limitation to the development of a vaccine against *Plasmodium falciparum* is the antigenic diversity related to *P. falciparum* polymorphism. Merozoite surface protein (MSP)-1 and MSP-2 are two proteins causing immune response in humans [1–4] and are prime vaccine candidates. Perhaps as part of an immune evasion mechanism, *msp-1*₁₉ and *msp-2* DNA sequences include variable blocks generating antigenically diverse forms [4,5].

The polymorphism at these two loci is mainly assessed by the number of repeats [6–8], which can be used to distinguish by size the different alleles after PCR amplification.

The phenomenon of clone fluctuation, which consists of kinetic changes in the relative load of each parasite subpopulation present in blood [9,10], is important to consider when studying *P. falciparum* diversity. Strain-specific immunity is largely responsible for protective immunity

to malaria parasites [11,12]. In malaria endemic areas, infections due to multiple parasite clones are frequent [11,13–16], and clone fluctuation may be a logical strategy to slow down the appearance of strain-specific responses. However, mechanisms other than immune evasion may be involved in peripheral blood genotypes' fluctuations, including sequestration in the deep vasculature, strain competition, and clearance effect related to either strain-specific immune response or treatment.

Allelic diversity and clone fluctuation have rarely been assessed in the same study, although their concomitant existence highly complicates the analysis of *P. falciparum* polymorphism. We studied both mechanisms in *P. falciparum*-infected children from Gabon and discuss the implications of our findings.

Subjects and Methods

Study area and blood collection

Bakoumba, a village from southeast Gabon in the Haut-Ogooué province, belongs to a meso- to hyper-endemic area for *P. falciparum* malaria [17]. The average EIR for February and March 2000, the time of the study, was 0.83 infective bite per man per night, due to *Anopheles gambiae*, *Anopheles funestus*, and *Anopheles moucheti* (Elissa *et al.*, personal communication). Samples were collected during a treatment efficacy study of sulfadoxine-pyrimethamine combination and amodiaquine carried out between January and June 2000 [18]. This study involved 252 subjects aged between six months and 10 years. Patients were followed weekly during four weeks after treatment (Day 0), as well as any other day if the child was unwell. At enrolment, and during the follow-up, finger-prick blood was sampled for thick smears and collection on filter paper. Patients involved in the present study were the 52 children in which treatment failed, and for which multiple parasitised blood samples were available. Ethical clearance was given by the Ethics Committee of the International Centre for Medical Research of Franceville, and informed consent was obtained from parents or guardians.

DNA preparation and PCR amplification

Blood collected on filter-paper was dried and conserved at room temperature until extraction. DNA was prepared by chelex extraction, as described [19], and stored at -20° C. The oligonucleotides primers were designed from published sequences, as listed in the Nucleotide Blast Database, to amplify block 2 of *msp-1* [20], and block 3 of *msp-2* [5]. The two genes were amplified by nested PCR, each amplification with conserved, or family-specific primer pair, being done separately, as described [18]. PCR products were electrophoresed on 1.5% agarose gels, and DNA visualized by ultraviolet transillumination after ethidium bromide staining. Fragments obtained were compared by

size. For comparison, purified genomic DNA from W2, HB3 and 3D7 laboratory strains were amplified and electrophoresed similarly. All migrations were repeated at least twice.

Data analysis

The multiplicity of infection (MOI), or number of genotypes per infection, was calculated as the highest number of genotypes at any of the two MSP loci. Relations between the MOI and age or parasite density were studied using the Spearman correlation test. The prevalence of each family was calculated as the percentage of D0 samples containing allele(s) of this family. The distribution of the specific families was estimated at each of the two loci as the percentage of fragments assigned to one family within the overall number of fragments. The occurrence of specific associations of paired alleles was analysed by the Fisher's exact test.

Results

Samples and genotyping strategy

Fifty-two samples collected at enrolment were used for describing parasite allelic diversity. Additional samples were collected from the same subjects, at first reappearance of parasites in blood, 7 to 21 days following treatment. In 10 subjects, a third sample was obtained 5 to 14 days later. Finally, a fourth sample was available from one subject. All genotyped samples were positive for *msp-1* and *msp-2* PCR.

P. falciparum allelic diversity

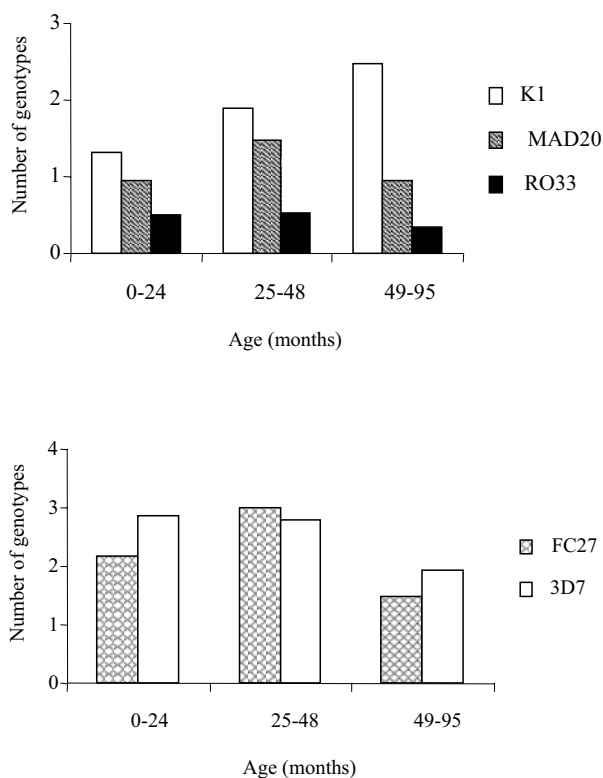
Among the 52 D0 samples, at the *msp-1* locus, 14 alleles of the K1 family ranged from 135 to 330 bp; 8 MAD20 alleles ranged from 190 to 280 bp; and 3 of the RO33 family were of 160, 230, and 250 bp (table 2). For the *msp-2* locus, 11 FC27 alleles ranged from 300 to 600 bp; and 8 3D7 alleles ranged from 360 to 590 bp. Forty-seven (90.4%) D0 samples harboured K1 parasites, 33 (63.5%) MAD20 parasites, and 19 (36.5%) RO33 parasites. For *msp-2*, 34 (65.4%) isolates contained FC27 parasites, and 43 (82.7%) 3D7 parasites. At each locus, family distribution was 52.5%, 32.5%, and 15.0% for K1, MAD20, and RO33 of *msp-1*; and 49.7% and 50.3% for FC27 and 3D7 of *msp-2*. Four specific associations of two alleles were over-represented. In the FC27 family, the 500 and 560 bp ($P = 0.004$), and the 530 and 600 bp alleles ($P < 0.0001$) were positively associated. In the 3D7 family, the 500 bp allele was associated positively to the 560 bp allele ($P = 0.01$) and negatively to the 530 bp allele ($P = 0.01$).

Multiplicity of infection

Before treatment, MOI (\pm SE) was 4.0 (\pm 0.26). All but two samples contained at least two distinct parasite lines. The two single clonal infections were composed by parasites belonging to the K1 family of *msp-1*, and to the FC27 and

Table 1: Sequences of oligonucleotide primers used to amplify merozoite surface protein (*msp*)-1 and *msp*-2 polymorphic regions of *Plasmodium falciparum* isolates from Gabon.

Gene	Primer	Sequence	Notes
<i>msp-1</i>	msp1-C1	5'-AAGCTTTAGAAGATGCAGTATTGAC	Conserved
	msp1-C2	5'-ATTCATTAATTTCTTCATATCCATC	Conserved
	K1a	5'-GAAATTACTACAAAAGGTGCAAGTG	K1 family-specific
	K1b	5'-AGATGAAGTATTTGAACGAGGTAAGTG	K1 family-specific
	MAD20a	5'-AAATGAAGGAACAAGTGGAACAGCTGTTAC	MAD20 family-specific
	MAD20b	5'-ATCTGAAGGATTTGTACGTCTTGAATTACC	MAD20 family-specific
	RO33a	5'-TAAAGGATGGAGCAATACTCAAGTTGTTG	RO33 family-specific
	RO33b	5'-CATCTGAAGGATTTGCAGCACCTGGAGATC	RO33 family-specific
<i>msp-2</i>	msp2-C1	5'-ATGAAGGTAATTAACATTGTCTATTATA	Conserved
	msp2-C2	5'-CTTTGTTACCATCGGTACATTCTT	Conserved
	FC27a	5'-GCAAAATGAAGGTTCTAATACTAATAG	FC27 family-specific
	FC27b	5'-GCTTTGGGTCCTTCTTCAGTTGATTC	FC27 family-specific
	3D7a	5'-AGAAGTATGGCAGAAAGTAAGCCTCTACT	3D7 family-specific
	3D7b	5'-GATTGTAATTCGGGGGATTTCAGTTGTTCC	3D7 family-specific

**Figure 1**
Mean number of genotypes in merozoite surface protein (*msp*)-1 and -2 polymorphic families of *Plasmodium falciparum* collected in the blood of 52 children, 0 to 95 months old, presenting with a malaria attack in Gabon, by age.

3D7 family of *msp-2*, respectively. MOI was not correlated to age (Figure 1, Spearman correlation, $P > 0.1$), although the number of genotypes of the K1 family increased with age (Spearman correlation, $P = 0.04$).

Clonal fluctuations

Following treatment, parasites reappeared in the blood of 10 children in which 2 or 3 additional blood samples were obtained. Three types of fluctuating figures of *msp-1* and *msp-2* molecular patterns were obtained (Figure 2). In type a, one or several initial allele(s) transiently vanished and recurred thereafter. In type b, new alleles appeared at intermediate and/or final points, although some initial alleles may persist. In type c, alleles observed at intermediate and final points were all present at D0. Variations in parasite densities were not related to appearance or disappearance of alleles (Spearman test for MOI and parasite density, $P > 0.1$). The disappearance of alleles, as in type c, could be attributed to treatment efficacy, and cannot be considered as parasite fluctuation. Recurrent parasites with identical genotype as initial parasites are likely drug-resistant parasites. Among the 5 families of *msp-1* and *msp-2* genes, a or b fluctuating types were observed in 9 of the 10 children. The figure of the child n° 170 was of type c for all 5 families.

Discussion

This work investigated the polymorphism of *P. falciparum* subpopulations in Gabon, by nested-PCR typing. This method does not allow one to distinguish alleles of identical size, but differing in sequences [20]. Competition between alleles during PCR amplification has previously been described [21]. Identical field samples were genotyped in different laboratories [22], and the main difference lied in the number of alleles detected. Our parasite

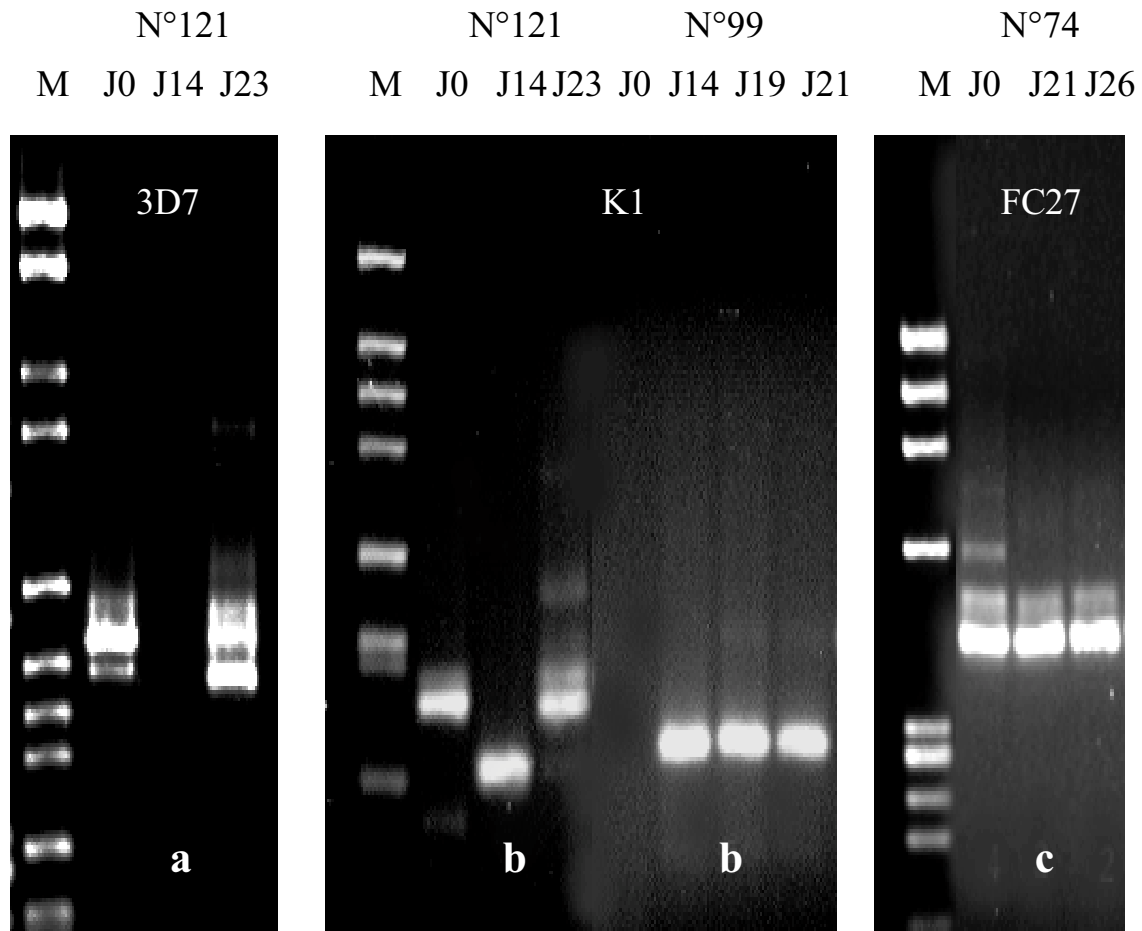


Figure 2

Examples of clonal fluctuations in successive blood samples between Day 0 and Day 28. The different clonal fluctuation figures were classified by type a, b, or c. Type a: one or several of the initial allele(s) transiently vanished, and were present again at the final point. Type b: new alleles appeared at intermediate and/or final points, although some of the initial alleles may be still present. Type c: alleles observed at intermediate and final points were all present at D0.

isolates, as most field samples, contained several clones with polymorphic *msp-1* and *msp-2* sequences at the variable blocks that may differ considerably in size, nucleotide sequence, and repeat copy number [7,23]. In such conditions, nested-PCR remains a suitable method to study *msp* polymorphism. Nevertheless, the results should be considered with the method limitations in mind, as variations in sensitivity were seen throughout experiments for weak bands, as well as in the estimation of band's length. When the same samples were electro-

phoresed several times, faint bands were inconsistently visible, demonstrating variations in technique sensitivity, due to unmanageable changes in temperature or moisture content. Estimation of band's length was liable to disparities between and within gels. Given these limitations, it is important to replicate all PCR products migrations.

This study showed high allelic diversity, as 25 and 19 different alleles were respectively present at the *msp-1* and *msp-2* loci. However, the RO33 family of *msp-1* was poorly

Table 2: Merozoite surface protein (*m*sp)-1 and *m*sp-2 polymorphism in isolates from 52 Gabonese children presenting with a *Plasmodium falciparum* malaria attack, 2000.

	<i>m</i> sp-1		<i>m</i> sp-2			Total
	K1	MAD20	R033	FC27	3D7	
Number of distinct bands	14	8	3	11	8	44
Number (%) of samples containing alleles of the corresponding family	47 (90.4)	33 (63.5)	19 (36.5)	34 (65.4)	43 (82.7)	
% of fragments assigned to the corresponding family	52.5	32.5	15.0	49.7	50.3	
MOI* (± SE)	1.6 (± 0.1)	1.0 (± 0.1)	0.5 (± 0.1)	1.8 (± 0.2)	1.8 (± 0.2)	4.0 (± 0.3)

* Multiplicity of infection

polymorphic, with only 3 different alleles detected. At the *m*sp-2 locus, FC27 and 3D7 alleles were nearly equally distributed, whereas alleles belonging to the K1 family were more frequent at the *m*sp-1 locus. This situation differs from that of Dienga [13], a village from the same area of Gabon (both villages being 45 km apart), where the R033 family was the most frequent at the *m*sp-1 locus in isolates from asymptomatic subjects. However, a single R033 allele was detected, confirming the reduced polymorphism of this family. In a more recent study in Dienga [24], R033 and FC27 alleles were mainly found during asymptomatic infections, whereas K1, MAD20, and 3D7 were predominant in isolates from clinical infections. Such a differential distribution according to clinical status was not observed in Cameroonian [24], Senegalese [14], nor Kenyan [25] isolates. In Senegal [14] and Thailand [26], the R033 family was poorly polymorphic, as opposed to the other MSP-1 and MSP-2 families. The *m*sp-2 locus is usually highly polymorphic, and FC27 and 3D7 families are both highly represented [14,26,27].

Multiple bands were observed in all but two samples, and the MOI was 4.0, probably as a consequence of the high transmission level [14,27]. The very high MOI may also be explained by the clinical and immunological status of the patients, although conflicting results are present in the literature. Some studies indicated that MOI decreases with age in asymptomatic subjects after 15 years [28,29]. In our study, age was not related to the number of genotypes, in agreement with several studies achieved with symptomatic or asymptomatic subjects [14–16].

Ten subjects were studied at multiple points during four weeks. In nine, alleles fluctuated with time. Either alleles transiently disappeared, or additional alleles appeared (and eventually disappeared) during the follow up. We cannot rule out that clonal disappearance was the result of treatment activity, and its reappearance due to reinfection by a new anopheles bite. However, parasite population showed a very rich polymorphism of *m*sp-1 and *m*sp-2 loci composed of at least 22 different alleles, with the most

common allele not exceeding 28% of all alleles of a given family. The concomitant reinfection by several clones similar to those present in the initial infection would be very unlikely. Alleles fluctuations were more likely related to variations of the alleles ratios in peripheral blood, as reported over a several months period in the absence of malaria transmission [30]. In holoendemic areas, Daubersies *et al.* [9] concluded that a rapid turnover of parasite populations inoculated by mosquitoes has occurred, whereas Farnert *et al.* [10] suggested fluctuations of inherently synchronous subpopulations. Several mechanisms may be involved including sequestration in the deep vasculature, strain competition, strain-specific immune response, and/or parasite immune evasion. In our study, treatment efficacy is also responsible for the disappearance of sensitive clones, and drug-resistance is liable for recrudescence parasites. The appearance of new allele(s) could be attributed to reinfection by new clone(s), or to detection of genotype(s) present earlier at undetectable concentration. Clonal fluctuation causes huge difficulties in determining the precise composition of a parasite population. PCR distinction between recrudescence and newly inoculated parasites is critical in treatment efficacy studies. Only recrudescence clones can be warranted, while reinfections cannot be ascertained.

Conclusions

Although the method had showed limitations, this study illustrated high polymorphism in Southeast Gabon with respectively 25 and 19 different alleles at *m*sp-1 and *m*sp-2 loci. Infections were highly complex, malaria attacks being characterized by a mean of four genotypes per infection. Clonal fluctuation increases infection complexity and makes polymorphism analysis difficult. These results illustrate two forms of *P. falciparum* complexity in a high transmission area: sequence polymorphism and clonal fluctuation.

Authors' Contributions

Field work was done at Bakoumba Hospital, Gabon. Laboratory work was conducted at CIRMF, Gabon. Agnès Au-

bouy carried out the laboratory work. The three authors participated in the design of the study, the analysis and the interpretation of the results, as well as in preparing the manuscript.

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