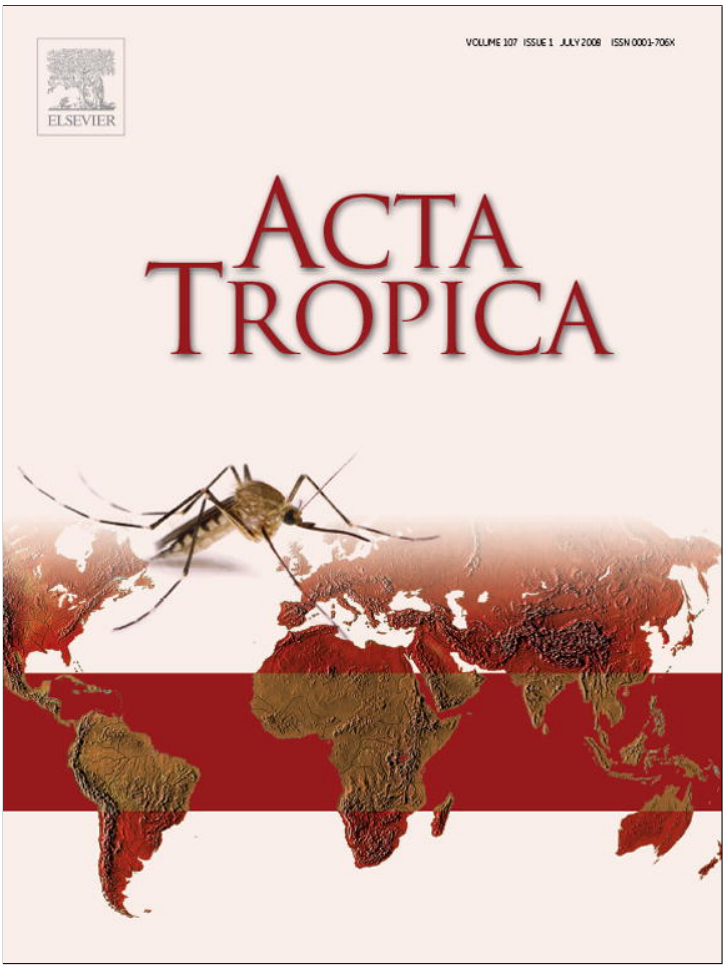

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Genetic diversity and genotype multiplicity of *Plasmodium falciparum* infections in symptomatic individuals living in Bangui (CAR)

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ABSTRACT

This study provides the first estimate of the genetic diversity and genotype multiplicity of *Plasmodium falciparum* infections in symptomatic individuals living in Bangui (Central African Republic, CAR). Three hundred thirty six clinical isolates were used for analysis of parasite population polymorphism and genotyped by nested-PCR of *msp*-1 block 2, and *msp*-2 block 3. We found a very high level of polymorphism, with, respectively, 17 and 25 different alleles at the *msp-1* and *msp-2* loci and a high percentage of multiclonal infections (42.7% with *msp-1* and 76.7% with *msp-2*), with a mean of 1.7 genotype with *msp-1* and 2.8 with *msp-2*.

We observed that (i) multiclonal infections and allelic polymorphism of *msp-2* were significantly more frequent in Southern districts than in Northern districts of Bangui suggesting that the epidemiological features of *P. falciparum* may vary within Bangui and (ii) showed that immunocompromised HIV-positive patients tend to have a lower average number of *msp-2* allele per isolate than immunocompetent patients.

1. Introduction

One of the major constraints faced in developing an effective vaccine against asexual stages of *Plasmodium falciparum* is antigenic diversity within parasite populations. *P. falciparum* is a highly polymorphic parasite with considerable heterogeneity of molecules. Some surface antigens present highly polymorphic regions grouped into allelic families such as Merozoite Surface Proteins 1 and 2 (*msp-1, msp-2*).

Multiplicity of infection (MOI), defined as the minimum number of parasite genotypes carried by an infected individual, has been shown that the most infected individuals carry more than one parasite genotype in both low endemicity areas such as Sudan (Babiker, 1998) or Thailand (Brockman et al., 1999) and in regions with higher transmission rates such as Gambia, Tanzania, Senegal and Gabon (Babiker et al., 1997; Ntoumi et al., 1995; Ranford-Cartwright et al., 1997; Smith et al., 1999a). Most of these molecular epidemiological studies have indicated that the multiplicity of infections is a useful indicator of the dynamics of parasite infections and of immune status: MOI depends on both the age and clinical status of the patient (Smith et al., 1999b). Therefore, it has been speculated that interventions to control the disease such as long-term chemoprophylaxis, impregnated bed nets (Frasser-Hurt et al., 1999) or immunisation against malaria (Haywood et al., 1999) might interfere with the multiplicity of infection in individuals by affecting parasite density loads and the spectrum of the disease.

In order to examine genetic diversity and genotype multiplicity of *P. falciparum* populations, field isolates were collected in symptomatic individuals in Bangui, capital of the Central African Republic (CAR) during the peak period for malaria. PCR amplification of the polymorphic regions of the merozoite surface protein 1 (*msp-1*) and merozoite surface protein 2 (*msp-2*) marker genes, encoding two molecules expressed during the asexual blood stages of infection and widely used in molecular epidemiological field studies (Babiker et al., 1997; Konate et al., 1999) was performed. Results of these data were used to describe how multiplicity of infection varies in accordance to age, parasite density and the district of Bangui inhabited. We also investigated influence of HIV infection on genetic diversity and genotype multiplicity of *P. falciparum* by comparing multiplicity of infection between HIV-seronegative and HIV-seropositive patients and between CD4 count levels in HIV-seropositive patients.

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2. Materials and methods

2.1. Study area

This study was conducted in Bangui, capital of the Central African Republic (CAR) between March and July 2004. Bangui is located beside the Oubangui River in the heart of Central Africa, to the immediate north-east of the Democratic Republic of Congo (geographic co-ordinates: 7°00′N, 21°00′E). The climate is tropical and rainfall levels are highest from April to November. Mean daily temperature varies from 19 to 32 °C. Malaria transmission occurs throughout the year, with peaks at the beginning and end of the rainy season. Malaria is hyperendemic in this region and *P. falciparum* is the predominant malaria species.

In 2002, a sentinel serosurveillance study conducted by the Institut Pasteur de Bangui in the CAR showed that the prevalence of HIV-seropositivity reached 15% in Bangui (Matsika-Claquin et al., 2004).

2.2. Sample collection

Patient isolates were obtained from symptomatic individuals from Bangui with uncomplicated *falciparum* malaria (presence of *P. falciparum* on blood films and absence of symptoms of severe malaria), before treatment. These patients attended several health centres in the northern (Boy Rabe, Gobongo) and the southern (La Kouanga and Ouango) districts of Bangui. Most of the patients included at Boy Rabe or La Kouanga health centres were already known as HIV seropositive status. These both areas apart from the influence of the Oubangui River in the south do not differ by social level, human interventions or HIV seroprevalence. No recent data concerning the vector species or entomological inoculation rate (EIR) were available.

Venous blood samples were collected in a tube coated with EDTA (Vacutainer tubes®, Becton Dickinson®, Rutherford, NJ, USA) and were sent to the malaria unit laboratory at the Pasteur Institute of Bangui within 4 h in controlled cool box at 4 °C. Blood samples were immediately centrifuged and the erythrocyte pellet was frozen at −20 °C until its use for DNA extraction. For each patient, demographic characteristics such as age, sex, district of residence in Bangui (as living for more than 6 months) and HIV status (when it was already known) were noted. We also offered HIV testing to each patient, with counselling before and after the test but a non-acceptance to test for HIV was not exclusion criteria.

Patients were treated with the amodiaquine plus sulfadoxine-pyrimethamine combination or with quinine, as recommended by the National Malaria Control Programme in the CAR (Menard et al., 2005).

2.3. Microscopic examination

Giemsa-stained thin and thick blood smears were examined to check for mono-infection with *P. falciparum* and to determine parasite density. The slides were read by experienced technicians at the health centre examining at least 100 oil-immersion fields before a slide was considered as negative. For those who were found to be infected with malaria parasites, the parasite density was calculated at the Pasteur Institute of Bangui by counting the number of asexual parasites per 200 white blood cells (WBC), and adjusting with the exact WBC count performed by the haematological analyser.

2.4. HIV serology and CD4 count

HIV status was determined with plasma samples by using an algorithm derived from strategy II of the WHO and evaluated at Institut Pasteur de Bangui in 2003 (Menard et al., 2003a). Briefly, the first test used to screen samples was an enzyme-linked immunosorbent assay with Vidas HIV DUO© kit (BioMérieux© France, Marçy l'Etoile) and the second test used to confirm positive results obtained with the first test was the Determine Abbott© rapid immunochromatographic test (antigen source: combined recombinant and synthetic peptides, storage temperature: 2–30 °C, Abbott Laboratories©, Tokyo, Japan).

A Coulter AcT Diff 2 analysor which was standardized against a 4C plus blood control was used for whole-blood analysis of haematological parameters. Lymphocyte subsets were analysed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems©, San Jose, CA) with two monoclonal antibodies (MAbs) (aCD4-aCD8) as it was described previously (Menard et al., 2003b).

2.5. DNA extraction and PCR

The DNA template for nested PCR was prepared from the whole blood sample. Parasite DNA was extracted from 100 μl of thawed erythrocyte pellets by heating with 0.1 M NaOH at 100 °C for 3 min. The mixture was then centrifuged and the supernatant treated with 250 μl lysis solution (0.1 M Tris–HCl, Triton 100-X, 1 M NaCl, 10× SDS, 0.5 M EDTA) and 20 mg/ml proteinase K at 37 °C for 1 h, followed by two phenol/chloroform (1:1) extractions (Ntoumi et al., 1995). The DNA was then precipitated with ethanol pelleted by centrifugation, resuspended in 100 μl of distilled water, and stored at −20 °C.

PCR genotyping was performed as described by Snounou et al. (1999), using repetitive regions found in two polymorphic genetic makers, namely *msp-1* (block 2) and *msp-2* (block 3). Allelic variants of *msp-1* (MAD20, K1 and RO33) and *msp-2* (3D7 and FC27) were detected by allelic family specific nested PCR. PCR conditions and the primer sequences have been described in detail by Snounou et al. (1999). All PCR amplifications (Gene Amp PCR system 9700®, Applied Biosystems®, Courtaboeuf, France) contained a positive control (purified genomic DNA from W2, HB3 and 3D7 reference strains from the Malaria Research Reference Reagent Resource Center, MR4/ATCC, Manassas, Virginia for *msp-1* and *msp-2* allelic families) and a negative control (containing no target DNA).

2.6. Allele distribution, multiplicity of infection

The prevalence of each family was calculated as the percentage of samples containing at least one allele from that family. The distribution of the specific families was estimated at each of the two loci as the percentage of fragments assigned to a given family. Multiclonal infections were defined as infections with isolates of more than one genotype. The multiplicity of infection (MOI) or number of genotypes per infection was estimated as the highest number of alleles detected at either of the two loci.

2.7. Statistical analysis

Data were analysed using EpiInfo 2000 software® and MedCalc v.8.0.0.1 software®. With these softwares all necessary descriptive statistics as well as parametric (*t*-test, one-way analysis of variance) and non-parametric methods (Spearman's correlation coefficient, Kruskall–Wallis test) were calculated. The total number of multiple infections and the number of infections belonging to the allelic families (MAD20, K1, RO33; 3D7 and FC27) were analysed with respect to age, parasite density, district of Bangui inhabited, HIV serological status and CD4 count level. Spearman's correlation coefficients were calculated to assess associations between multiplicity of infection and parasite densities or CD4 count level. For the multivariate analysis, variables with a *P*-value <0.25 were initially

Table 1
Demographic, clinical and parasitological characteristics of symptomatic individuals from Bangui (CAR) with uncomplicated falciparum malaria

Characteristics	*n*	Values
Patients		
No. of males/females	307	140/167
Mean age (CI95%), years	304	15.2 (13.4–16.9)
Temperature (°C) (mean)	243	38.1 (±1.0)
No. of inhabited in Northern/Southern districts in Bangui	312	177/135
No. of HIV serology performed, positive/negative	163	42/121
Mean parasite density (CI95%), no. of parasites/μl	308	16,816 (12,416–21,216)

introduced into the model and removed following a backwards-stepwise selection procedure to leave only those with a *P*-value <0.05 in the final model.

2.8. Ethical approval

As the CAR has no national ethics committee, this study was approved by the expert committee for anti-malarial drug policy and the Ministry of Health in CAR. Written informed consent was obtained from all patients.

3. Results

3.1. Patient isolates

Three hundreds thirty six patient isolates from symptomatic individuals with uncomplicated *falciparum* malaria in Bangui were collected between March and July 2004 and were used to describe the allelic diversity of the parasites involved. The demographic, clinical and parasitological characteristics of these patients are given in Table 1. No significant difference was found between HIV serological status and district of Bangui inhabited: 26.4% (19/72) of HIV+ in the Southern districts compared to 25.3% (23/91) of HIV+ in the Northern districts of Bangui ($P = 0.98$). It should be noted that HIV seroprevalence found in our study was over-estimate because of the patients included at Boy Rabe or La Kouanga health centres were already known as HIV seropositive status.

3.2. P. falciparum allelic distribution

Alleles of *msp-1* and *msp-2* loci were classified according to the size of their PCR-amplified fragments. Both genetic markers and the corresponding allelic families were very diverse. We obtained amplicons for 281 (83.6%) genotyped samples in *msp-1* PCR and for 332 (98.8%) genotyped samples for *msp-2* PCR. MAD20-type was detected in 212 *P. falciparum* isolates (75%), with 9 alleles varying in size between 160 and 295 bp, K1-type in 42 (14.9%), with 5 alleles varying in size between 250 and 380 bp and RO33-type in 109 (38.8%) with 3 different alleles (75% of 160 bp, 16% of 230 bp and 9% of 250 bp). For *msp-2* locus, 243 samples (73.2%) of allelic family FC27 and 286 samples (86.1%) of 3D7 were positive. We identified 14 FC27 alleles (205–500 bp) and 11 3D7 alleles (245–555 bp).

At each locus, the family distribution was 10.5% for K1, 61.0% for MAD20, 28.5% for RO33 of *msp-1*, and 45.8% and 54.2% for FC27 and 3D7, respectively, of *msp-2*.

3.3. Multiplicity of infection and prevalence of multiclonal infections

Among the 258 samples with positive PCR products for *msp-1* and *msp-2*, a maximum of eight genotypes in three patient isolates was detected using *msp-1* and *msp-2*. The distribution of the genotype number per isolate was as follows: 13.6% of the isolates were monoclonal (35/258), 25.2% (65/258) contained at least two genotypes, 24.8% (64/258) carried three genotypes, 18.6% (48/258) four genotypes, 12.0% (31/258) five genotypes, 3.1% (8/258) six genotypes, 1.6% (4/258) seven genotypes and 1.2% (3/258) contained at least eight genotypes. Details by loci and allelic family are shown in Table 2.

Analysis of *msp-1* and *msp-2* alone detected 42.7% and 76.7% of the multiclonal infections, respectively, whereas the combination of the two markers revealed 86.4% of multiclonal infections. The estimated mean number of genotype was 1.7 (CI95%: 1.6–1.8) with *msp-1* and 2.8 (CI95%: 2.7–3.1) with *msp-2*.

3.4. Univariate analysis

3.4.1. Influence of age

The influence of age on genetic diversity and genotype multiplicity of *P. falciparum* infections showed that children in the age group of 3–5 years had the highest average number of different genotypes in their samples: 1.96 with *msp-1* and 3.12 with *msp-2*. The average number of *msp-1* and *msp-2* bands per isolate was slightly lower in the older age groups (Table 3), but this difference was not significant ($P = 0.78$ for *msp-1* and $P = 0.15$ for *msp-2*). Furthermore, the frequencies of multiclonal infections were not significantly different between age groups (range from 80.9% for age class 16–25 years to 100% for age class 6–10 years, $P = 0.24$). There was also no significant influence of age on distribution of allelic families of *msp-1* or *msp-2*.

3.4.2. Influence of parasite density

The association between multiplicity of the target genes and stratified parasite density groups was calculated by Spearman correlation. Here, *msp-2* ($n = 304$, Spearman's $r = 0.185$, CI95%: 0.07–0.29, $P = 0.001$) showed a significant positive correlation.

Table 2
Distribution of the number of distinct alleles per sample by loci and allelic family of symptomatic individuals from Bangui (CAR) with uncomplicated falciparum malaria

Number of distinct alleles detected	Number of samples (%)						
	msp-1				msp-2		
	K1	RO33	MAD20	Total	3D7	FC27	Total
1	36 (11.5)	85 (27.2)	142 (45.4)	150 (57.3)	120 (38.3)	127 (40.6)	72 (23.3)
2	5 (1.6)	18 (5.8)	51 (16.3)	65 (24.8)	98 (31.3)	47 (15.0)	73 (23.6)
3			7 (2.2)	35 (13.4)	39 (12.5)	43 (13.7)	67 (21.7)
4			1 (0.3)	7 (2.7)	9 (2.9)	12 (3.8)	52 (16.8)
5				4 (1.5)	2 (0.6)	1 (0.3)	27 (8.7)
6				1 (0.4)			10 (3.2)
7							5 (1.6)
8							3 (1.0)

Table 3
Parasite density (μl) and means number of *msp-1* and *msp-2* alleles by age groups, district of Bangui inhabited and HIV status in symptomatic individuals from Bangui (CAR) with uncomplicated falciparum malaria

Variable	Mean number of					
	n	msp-1 allele	*P*[a]	*n*	msp-2 allele	*P*[a]
Stratified age groups (years)						
0–2	69	1.75	0.78	77	2.87	0.15
3–5	49	1.96		54	3.12	
6–10	25	1.64		29	3.03	
11–15	13	1.77		16	3.00	
16–25	47	1.49		56	2.71	
>25	52	1.70		68	2.42	
Stratified parasite density groups (parasites/μl)						
0–500	16	1.62	0.41	22	2.31	0.03
501–5000	114	1.59		137	2.77	
5001–20,000	80	1.68		91	2.85	
>20,000	49	1.87		54	3.37	
District of Bangui inhabited						
Northern	154	1.58	0.10	174	2.61	0.0015
Southern	108	1.81		134	3.18	
HIV status						
HIV-seropositive	39	1.57	0.95	41	2.31	0.11
HIV-seronegative	103	1.56		118	2.74	
Stratified CD4 count groups (cell/μl)						
0–99	4	1.00	0.08	4	1.25	0.0075
100–200	6	1.33		8	1.87	
>200	21	1.80		22	2.72	

[a] For Mann–Whitney *U*-test.

The association was also significant for *msp-2* by using the Kruskall–Wallis test (Table 3). In *msp-2*, mean of parasite density of 235 samples with multiclonal infections was 18,482 parasites/μl and differed significantly ($P = 0.0017$) from the mean among the 69 monoclonal infections (12,004 parasites/μl).

A significant correlation was found between parasite density and age by using the Spearman correlation: $n = 299$, Spearman's $r = -0.394$, CI95%: −0.48 to −0.29, $P < 0.0001$. On the other hand, no significant correlations were found between parasite density and district of Bangui inhabited ($P = 0.94$), HIV serological status ($P = 0.20$) or CD4 count for HIV+ ($P = 0.72$).

3.4.3. Influence of district of Bangui inhabited

The average number of *msp-2* bands per isolate was significantly higher in Southern districts than in Northern districts of Bangui ($P = 0.0015$), as it is shown in Table 3. Moreover, *msp-2* showed that multiclonal infections were significantly more frequent in Southern districts (86.6%) than in Northern districts of Bangui (69.5%) ($P = 0.0002$, OR: 2.8, CI95%: 1.5–5.1).

No significant relation was found between *msp-1* allelic family distribution and district in Bangui inhabited. For *msp-2* allelic family distribution, FC27 and 3D7 were significantly more frequent in Southern districts than in Northern districts of Bangui: FC27 (80.7% versus 68.4%, $P = 0.007$, OR: 1.9, CI95%: 1.1–3.3) and 3D7 (90.4% versus 81.9%, $P = 0.017$, OR: 2.1, CI95%: 1.1–4.1).

No significant correlations were found between district of Bangui inhabited and age groups ($P = 0.62$), HIV serological status ($P = 0.85$) or CD4 count for HIV+ ($P = 0.32$).

3.4.4. Influence of HIV serological status

The average number of *msp-1* or *msp-2* bands per isolate was not significantly influenced with the HIV serological status (Table 3). However, *msp-2* showed that multiclonal infections were significantly more frequent ($P = 0.02$) in HIV-seronegative patients ($n = 28$, 76.3%) than in HIV-seropositive patients ($n = 17$, 58.5%).

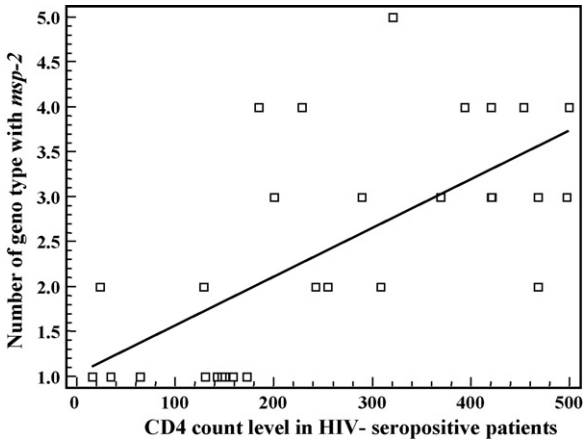

Fig. 1. Correlation between number of genotype with msp-2 and CD4 count level in HIV-seropositive patients ($n = 34$, Spearman's $r = 0.379$, CI95%: 0.047–0.636, $P = 0.03$).

3.4.5. Influence of CD4 count in HIV-seropositive patients

The average number of *msp-2* bands per isolate was significantly higher for patients within the higher CD4 count ($P = 0.0075$), as it is shown in Table 3. The association between multiplicity of infections and CD4 count level in HIV-seropositive patients was calculated by Spearman correlation and showed a significant positive correlation with *msp-2* ($n = 34$, Spearman's $r = 0.379$, CI95%: 0.047–0.636, $P = 0.03$) (Fig. 1). In *msp-2*, mean of CD4 count level of 22 samples with multiclonal infections was 384 μl^{-1} and differed significantly ($P = 0.03$) from the mean among the 12 monoclonal infections (216 μl^{-1}).

3.5. Multivariate analysis

A multivariate analysis was performed on the average number of *msp-2* bands per isolate to control confounding factors. The analysis was split according the HIV serological status. Within the HIV-seronegative patients group, age group, parasite density and district of Bangui inhabited were entered into the model. The average number of *msp-2* bands per isolate was always significantly higher in Southern districts than in Northern districts of Bangui ($P = 0.011$), but the association disappeared for parasite density ($P = 0.19$). Within the HIV-seropositive patients group, parasite density, district of Bangui inhabited and CD4 count were entered into the model. The average number of *msp-2* bands per isolate was significantly higher in Southern districts than in Northern districts of Bangui ($P = 0.009$) and higher in patients with higher CD4 count level ($P = 0.03$).

4. Discussion

We investigated, for the first time, the genetic diversity and genotype multiplicity of *P. falciparum* infections in symptomatic individuals living in Bangui. As it was noticed by several authors (Viriyakosol et al., 1995), increasing our knowledge of the genetic diversity of *P. falciparum* may improve our understanding of the pathological mechanisms of malaria, the processes of acquired immunity, the spread and genetic background of drug resistance and transmission conditions.

We observed a very high level of allelic diversity in this study. The number of different *msp-1* and *msp-2* alleles observed among the 281 samples genotyped for *msp-1* (83.6%) and the 332 samples genotyped for *msp-2* (98.8%) was large: 17 different alleles from the allelic families tested were detected for *msp-1*, and 25 alleles were detected for *msp-2*. However, as not all of the sam-

ples giving positive microscopy results gave amplicons in nested PCR-based genotyping experiments (55 of 336 samples with negative PCR results for *msp-1* and 4 of 313 samples with negative PCR results for *msp-2*), we must have underestimated the degree of genetic diversity. Sequence polymorphism at the primer sites, DNA degradation during storage or the presence of PCR inhibitors may account for these findings.

At the *msp-1* locus, MAD20 was the most polymorphic allelic family, with nine different alleles, and was the allelic family most frequently detected in isolates. At the *msp-2* locus, FC27 was the most polymorphic allelic family, with 14 different alleles. Consistent with previous reports (Aubouy et al., 2003; Basco et al., 2004; Hoffmann et al., 2001; Snounou et al., 1999), we found that the *msp-2* locus was more polymorphic than the *msp-1* locus and that the FC27 and 3D7 families were strongly represented and almost identically distributed.

Multiple bands, reflecting multiclonal infections, were observed in 42.7% and 76.7% of samples tested with *msp-1* and *msp-2*. The estimated mean number of genotype was 1.7 with *msp-1* and 2.8 with *msp-2* were comparable with data previously published from holoendemic areas in Africa (Aubouy et al., 2003; Ekala et al., 2002; Hoffmann et al., 2001; Jordan et al., 2001; Magesa et al., 2001; Missinou et al., 2001; Ntoumi et al., 1995, 2000; Ranford-Cartwright et al., 1997; Smith et al., 1999b; Zwetyenga et al., 1998), probably reflecting the high malaria transmission levels.

Among the isolates that were investigated in this study, multiplicity of infection was highest in the age group 3–5 years with allelic families of *msp-1* (average 1.96 genotypes) *msp-2* (average 3.14 genotypes). Multiplicity of infection appeared to decrease with age and thus with the acquisition of protective immunity, but unlike in other studies (Konate et al., 1999), this trend was statistically not significant.

In our study, *msp-2* clearly appeared to be more polymorphic than *msp-1.* This genetic marker was the best to estimate the multiplicity of *P. falciparum* infection in symptomatic patients in showing significant relations with the parasite density, the district of Bangui inhabited or the CD4 count level in HIV-seropositive patients. Except for the parasite density, these significant relations were confirmed by using multivariate analysis, allowing to control the confounding factors.

Although the northern and southern districts of Bangui were separated by only a few kilometres, it was very surprising to find a significant difference between the percentage of multiclonal infections or the average number of *msp-2* alleles per isolate and the district of Bangui. However, this finding, already described in Dakar by Trape et al. (1992) highlights that the epidemiological features of *P. falciparum* may vary within a given city. Moreover, since several reports (Paul et al., 1998) have shown a positive association between the EIR and the mean number of clones in the human host, our data led us to suppose that the EIR is higher in the south districts than in the north districts in Bangui, probably explained by the influence of the Oubangui River in the south, whose marshy banks are permanent larval lodgings. Obviously, this result must be confirmed with further studies such as entomological studies or *P. falciparum* population structure with neutral microsatellite markers studies.

One other interesting finding of this study was to find by using multivariate analysis in HIV-seropositive patients group that the multiplicity of infection of *msp-2* was significantly correlated with the CD4 count level ($P = 0.03$). Indeed, we observed that immunocompromised patients with their lower than normal CD4+ T lymphocyte counts tended to have a lower multiplicity of infection than immunocompetent patients. These data seem to confirm the role of cellular immunity in controlling malaria infection. Our hypothesis should be that immunocompromised patients by depleting their CD4+ T lymphocytes, were losing their protective immunity by alteration and restriction of their repertoire of CD4+ cells, leading the lost of the ability to control opportunistic infections and other infectious diseases such as malaria infections. Therefore, immunocompromised patients will tend to become symptomatic more rapidly after fewer infective bites and thus fewer parasite genotypes than immunocompetent patients. However, it is obvious because of the limitations of this study that this hypothesis must be confirmed and further studies must be carried out.

Conflict of interest

None.

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