

Original article

Genetic diversity and population structure of *Plasmodium falciparum* isolates from Dakar, Senegal, investigated from microsatellite and antigen determinant loci

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Abstract

We investigated the genetic diversity and the population structure of 32 *Plasmodium falciparum* blood sample isolates (25 from Dakar city and suburbs and seven from other localities in Senegal) with two different types of molecular markers, 19 microsatellite and four antigenic determinant loci. Under the same technical procedure, microsatellite loci showed a mean number of alleles greater than that of antigenic loci. Both markers revealed that 15.6% of blood samples were multi-infected. Mean expected heterozygosity calculated from microsatellites and antigens was similar, 0.74 and 0.70, respectively. Significant linkage disequilibrium was observed from microsatellite loci and antigenic determinant loci. This suggests a non-panmictic structure for this sample that could be explained by two non-exclusive hypotheses: (i) a particular mating system (i.e. clonality), and/or (ii) a population structure in *P. falciparum* (i.e. Wahlund effect). Urban samples could have been drawn from a heterogeneous set of foci with different level of parasitic transmission. Moreover, no relationship was found between multilocus genotypes and different parameters (i.e. age, sex and blood group of parasitized patients; number of trophozoites per microliter of blood). The results are discussed taking into account recently published studies on malaria population biology. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

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1. Introduction

Malaria is the most prevalent parasitic disease worldwide. Among the four species which are causative agents in humans, *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, the first accounts for the majority of infections and is the most lethal. According to the World Health Organization (WHO) [1], 300–500 million clinical cases are diagnosed each year and more than 90% of all cases are located

in sub-Saharan Africa. Mortality due to malaria is estimated to be over one million persons per year. If young African children from rural areas are the principal victims, pregnant women, non-immune travelers, refugees and displaced persons also belong to high-risk groups [1]. In Africa, 550 million persons live in malaria-infested areas, and in West Africa, previous studies [2] defined different epidemiological patterns. These patterns are related to climatic, phytogeographic and transmission (entomological inoculation rates (EIR) and periodicity of transmission) characteristics and are as follows: (1) area of stable malaria (throughout the year as in the equatorial area) with a high EIR (as much as 1000 infected bites/man/year), (2) area with a long seasonal outbreak (6–8 months as in tropical areas) with 100–400 infected bites/man/year, (3) area with intermediary short

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seasonal outbreaks (<6 months as in the Sahelian area) with 2–20 infected bites/man/year, and (4) area of unstable malaria with very short and uncertain outbreaks (as in deserts or mountains). But in each of these main patterns, variability of transmission rates can be observed, due to different ecological parameters and human activities (i.e. dams, agricultural structures). Concerning urban malaria, transmission rates are weaker than in rural areas, because of a lower density of *Anopheles* vectors [3]. So, malaria can be contracted all over the year with the same intensity or with a seasonal outbreak, in relation with the climatic position of the city. Variations from one part to another part of the same city (i.e. city center and suburbs) are observed [3–5]. A great percentage of severe malaria is diagnosed because inhabitants have developed little immunity according to the low malaria transmission, which occurred in urban zones. The WHO [1] refers to “weekend malaria” in Africa which happens when inhabitants return to their rural settings, and this phenomenon seems to be becoming an increasing problem.

As reported by Babiker and Walliker [6], great genetic diversity of *P. falciparum* is common in countries with a wide range of transmission intensities; indeed, a correlation has been observed between the extent of genetic polymorphism and the level of transmission intensity. Previous studies [6,7] also relate that multi-infection is relatively frequent, and a positive relationship has been reported in three African countries between EIR and the number of *Plasmodium* genotypes in patients. Knowing that *P. falciparum* is a hermaphrodite parasite where both selfing and outcrossing can occur, these population dynamic parameters of malaria are crucial for the genetic structures and the epidemiological characteristics of this disease (i.e. spread of resistance, local adaptation to mosquitoes or humans and morbidity). The debate on this topic is controversial and remains very sharp, but it is evident that the knowledge of population biology of malaria at different geographic scales is essential to understand the boundaries and the functioning of malaria foci.

A clonal structure of *P. falciparum* populations was first proposed [8], but other authors [6] argued that in highly endemic areas outcrossing occurs frequently enough to lead to panmictic structures, and to generate a great diversity of molecular genotypes. For example, Day et al. [9] wrote that if self-fertilization can be relatively frequent, then the relatively infrequent outcrossing could be sufficient to produce the apparent panmixia observed in different populations, and the authors proposed to investigate population structure in smaller areas. In recent papers [10,11], the authors raised the problem of malaria population biology in the presence of the different population dynamics encountered in malaria foci. Thus, the problem is still under debate, but we recommend reading Pollak [12] and Awadalla and Ritland [13] for an understanding of the respective roles of partial inbreeding and contrasting mating systems in population genetics.

More and more studies were conducted to analyze the genetic diversity, the dynamics and the population structure of *P. falciparum* using different molecular tools, which constitute indirect markers since it is impossible to study the population biology of this parasite by the usual ecological markers. But the majority of the molecular markers used in the different analyses belong to genes determining antigens [14–18], and multilocus enzyme electrophoresis [18]. Currently, few works refer to microsatellite loci [10,19,20]. Microsatellite loci present a high variability and are considered to be neutral Mendelian markers; they thus constitute appropriate tools for population genetics investigations when compared with the usual malaria markers such as merozoite surface proteins (MSP1 and MSP2) or circumsporozoite surface proteins (CSPs). Indeed, these genes can be under selection [21] and present less genetic variability than microsatellite loci [10].

Senegal has high rates of malaria transmission in rural areas, but a relatively low EIR occurs in Dakar [3,22]. Moreover, mixing of populations and mobility of persons inside the town (i.e. center and suburbs) and movements between all parts of the country are frequent. So, the parasite population could be sub-structured and more heterogeneous than in rural areas. Thus, the goal of this study is to investigate the genetic structure presented by infected blood samples of *P. falciparum* collected mainly in Dakar city and its suburbs. Moreover, in order to compare information carried by putative neutral and non-neutral markers, we analyzed 19 microsatellite loci and four antigenic determinants: two merozoite surface proteins (MSP1, MSP2), glutamate rich protein (GLURP) and CSP.

2. Materials and methods

2.1. Parasite samples and DNA preparations

Blood samples were collected in Senegal from 32 patients infected with *P. falciparum*; most of them (25) were from Dakar. The seven other samples came from different localities in Senegal, and served as outgroup for comparison. The samples were collected from September to December 1996. Patients did not receive any anti-malarial drug treatment before the blood sampling. Origin, age, sex, blood group and number of trophozoites per microliter of blood of the different patients are given in Table 1.

Two-hundred microliters of red blood cell pellets were suspended in 200 μ l of sterile H₂O, supplemented by 100 mM NaCl, 10 mM EDTA and 45 μ l of 10% SDS and 8 μ l of Rnase at 500 μ g/ml. The mixture was incubated for 2 h at 37 °C. Then 10 μ l of proteinase K at 10 mg/ml was added and the mixture was incubated for 2 h at 55 °C. Then DNA was isolated using phenol–chloroform extraction and dissolved in 20 μ l of sterile water.

Table 1
Characteristics of the *P. falciparum* (Pf) samples collected from patients in Senegal

Sample code	Locality	Sampling date	Age	Sex	Blood group	Parasitemia**
Pf 1	Tambacounda	09/13/1996	36	M	A	112,000
Pf 2	Dakar	09/24/1996	14	M	A	97,360
Pf 3	Dakar	09/25/1996	7	M	O	60,560
Pf 4	Dakar	09/26/1996	16	M	O	18,400
Pf 5	Dakar	10/01/1996	17	F	B	111,760
Pf 6	Dakar	10/03/1996	15	F	A	23,840
Pf 7	Dakar	10/07/1996	20	M	A	7,040
Pf 8	Dakar	10/09/1996	20	M	A	21,440
Pf 9	Dakar	10/14/1996	23	M	A	27,200
Pf 10	Casamance	10/14/1996	10	M	O	12,240
Pf 11	Dakar	10/15/1996	20	M	A	138,000
Pf 12	Dakar	10/16/1996	24	M	O	23,200
Pf 13	Dakar	10/16/1996	34	M	O	3,440
Pf 14	Dakar	10/21/1996	15	F	O	75,360
Pf 15	Dakar	10/21/1996	13	M	O	36,400
Pf 16	Fatick	10/22/1996	45	M	O	63,200
Pf 17	Dakar	10/23/1996	18	F	A	44,640
Pf 18	Fatick	10/28/1996	8	F	A	76,320
Pf 19	Dakar	10/28/1996	11	F	O	37,600
Pf 20	Dakar	10/29/1996	30	F	A	156,960
Pf 21	Linguère	11/05/1996	25	M	A	5,600
Pf 22	Dakar	11/05/1996	18	F	O	37,040
Pf 23	Dakar	11/05/1996	10	F	AB	49,920
Pf 24	Kaolak	11/06/1996	19	M	O	9,600
Pf 25	Casamance	11/06/1996	20	M	B	4,400
Pf 26	Dakar	11/07/1996	27	M	B	92,160
Pf 27	Dakar	11/11/1996	26	M	A	7,040
Pf 28	Dakar/Pikine *	11/14/1996	17	F	O	56,320
Pf 29	Dakar	12/03/1996	10	F	A	33,600
Pf 30	Dakar	12/09/1996	28	M	B	1,600
Pf 31	Dakar	12/09/1996	19	M	B	60,000
Pf 32	Dakar	12/10/1996	26	M	O	110,400

* Dakar suburb.

** Parasitemia: number of trophozoites/ μ l of blood.

2.2. Microsatellite loci

The 19 selected loci (Table 2) have all been described [19]; one locus (GenBank accession number AL034558) was described by Churcher et al., but is unpublished. PCR conditions were as follows: the amplification was performed in a reaction mixture of 20 μ l containing around 20 ng of genomic DNA, 1X reaction buffer, 2.5 mM MgCl₂, 80 μ M of each deoxynucleoside triphosphate, 6 pmol of each primer and 1.3 U of *Taq* polymerase (Promega). The thermal profile involved one cycle with denaturation at 94 °C for 2 min, followed by 30 cycles of 20 s at 94 °C, 10 s at 45 °C, 10 s at 40 °C and 30 s at 60 °C. After PCR amplification, 8 μ l aliquots of the reaction mixture were electrophoresed on 8% Long Ranger TM acrylamide gel with Tris–borate buffer 1X. The DNA bands were visualized by silver staining [23].

2.3. *MSP1*, *MSP2*, *GLURP* and *CSP*

For *MSP1*, *MSP2* and *GLURP*, we used the primers and the PCR conditions already described [24]. We did not

differentiate the three allelic families of *MSP1* block 2 [25] and the two allelic families of *MSP2* block 2 [26]. We analyzed only the size of *MSP1* and *MSP2* amplified products in order to be in the same conditions of characterization as for microsatellite loci. For *CSP*, we amplified the central region using CS3 and CS4 primers already proposed [27]. The amplification was performed in a PTC 100 thermocycler (MJ-Researcher). Twenty microliters of each amplification were loaded on 1.6% agarose gels, separated by electrophoresis in 1X TBE, stained with ethidium bromide, and examined by illumination with UV.

2.4. Statistical analysis

Genetic polymorphism was measured by the number of alleles per locus (A) and Nei's unbiased expected heterozygosity [28] using F-STAT, v1.2 [29] adapted to haploid data (unbiased expected heterozygosity corrected by $(2n - 1)/(2n - 2)$, where n is the sample size according to GENETIX v. 4.01 [30]).

The genotypic linkage disequilibrium was tested by the Fisher exact test performed by the GENEPOP package

Table 2
Molecular marker characteristics

Locus name (present study)	Marker name [19]	dbSTS or GenBank access	Chromosome location	Number of alleles per locus
P1	TA35	G38826	4	10
P2	TA111	G38830	5	3
P3	PJ2	G37826	7	11
P4	TA80	G38857	10	4
P5	TA119	G38863	11	8
P6	TA31	G38864	11	7
P7	TA125	G38868	11	10
P8	TA22	G38886	14	9
P11	MDR1	G42769	5	5
P14	ARA6	G37833	8	4
P15	ARA3	G37855	12	5
P17	ARP2	G37793	13	7
P24	PfPK2	G37852	12	8
P26	TA109	G38842	6	8
P27	TA81	G38836	5	7
P28	TA42	G38832	5	5
P32	C1M25	G37999	1	5
P33	C1M8	G38013	1	12
P35	PfMAL3P2	AL034558 *	3	11
MSP1	–	–	9	5
MSP2	–	–	2	7
GLURP	–	–	10	8
CSP	–	–	3	4

* From Churcher et al. (1999), unpublished data.

version 3.2d [31]. The null hypothesis is that genotypes at one locus are independent of genotypes at the other locus. A randomization procedure (4,000,000 iterations) using the Markov chain methods [31] enabled estimation of the exact probability of random association between all possible pairs of loci. Using an exact binomial test [32] we tested if the rate of associations at the 5% level of significance differed from those expected under the null hypothesis (i.e. 0.05). For comparison with other studies on the population genetic structure of *P. falciparum*, linkage disequilibrium was also estimated using the LIAN 3.0 software developed for multilocus haploid data [33]. LIAN tests the null hypothesis of linkage equilibrium for multilocus data. Linkage equilibrium is characterized by statistical independence of alleles at all loci. LIAN tests for this independent assortment by first computing the number of loci at which each pair of the population differs. The main output file states a “standardized index of association” (I_{AS}), a measure of haplotype-wide linkage. Associations between all pairs of loci were also estimated by the method described in Garnier-Géré and Dillmann [34] (computed by GENETIX v. 4.01 [30]). Comparison of the values test obtained between microsatellite loci and antigenic markers was then undertaken by a Wilcoxon rank-sum test [32].

Cavalli-Sforza and Edwards chord distance [35] was used to build a tree reflecting the genetic relationships which link the different *P. falciparum* isolates (see Takezaki and Nei [36] for justifying the use of this distance). Genetic distances were computed by GENETIX v. 4.01 [30] and the tree was generated using the neighbor-joining method [37].

3. Results

3.1. Genetic diversity

Five blood samples (2, 12, 17, 18 and 26) were found multi-infected from both microsatellite and antigenic patterns (Table 3); they were excluded from further analysis. Nine more samples presented multi-infected patterns for only one or two loci. We scored for them the predominant band or no value when the bands were of equal intensity, as suggested by previous studies [10]. Two samples (14 and 15) presented the same allelic patterns for all loci; these samples were collected on the same day from one brother and his sister living in the same house, sleeping in the same bed, and presenting clinical symptoms for 3 and 4 d, respectively, strongly suggesting an interrupted feeding by a unique infecting anopheline. The number of alleles observed per locus is shown in Tables 2 and 4: it ranged from 3 (P2 locus) to 12 (P33 locus) for microsatellite loci, and from 4 (CSP locus) to 8 (GLURP locus) for antigenic markers. The unbiased expected heterozygosity was similar for both markers (Table 4).

3.2. Linkage disequilibrium

Despite a slight difference between the two statistical procedures (i.e. Genepop vs LIAN for antigen loci—Table 5), linkage disequilibrium analysis evidenced a strongly significant correlation between loci (Table 5). As the population structure could contribute to that result (i.e. Wahlund

Table 3
Number of microsatellite and antigen loci presenting a multi-infected pattern in five samples

Sample code	Microsatellite loci	Antigen loci
Pf 2	14	2
Pf 12	12	3
Pf 17	5	2
Pf 18	10	1
Pf 26	13	2

effect), the analysis was repeated on isolates from a single location (Dakar) with similar results. The analysis was also done separately on microsatellite and on antigenic data; if linkage disequilibrium still exists between microsatellite markers, no correlation was found between antigenic markers (Table 5). But when we compared microsatellite loci vs antigenic markers there is no difference in the mean correlation between pairs of loci from microsatellites (mean $r = 0.20 \pm 0.05$) and from antigenic markers (mean = 0.17 ± 0.02) (Wilcoxon rank-sum test, $P = 0.27$). The most parsimonious explanation of this phenomenon could be attributed to the low level of variability observed for the antigenic determinants which lead to powerless statistical tests for linkage disequilibrium. Linkage disequilibrium cannot be attributed to physical links between markers, because except for two associated pairs of microsatellite loci (i.e. P2 and P27, P5 and P6), all other associated loci are located on distinct chromosomes.

3.3. Genetic relationships between samples

Fig. 1 shows the great heterogeneity observed in *Plasmodium* genotypes which splits in several groups. In particular, we note that samples outside of the Dakar area are randomly distributed among these different groups. Moreover, Fig. 1 shows no evident association between the

different characters (i.e. sex, age, blood group and parasitemia) and genotypes.

4. Discussion

The level of microsatellite variability observed (Table 4) revealed the presence of a high genetic diversity in this sample, but the values obtained are slightly inferior to those generally observed in African areas with a stable and long season of malaria transmission. Indeed, the mean value found for Congo isolates by Durand (unpublished data) is 7.75 ± 2.62 , and mean values published by previous studies [10] from Uganda, Congo and Zimbabwe ranged from 10.00 to 10.67. However, our values are higher than those found in South America (from 2.17 to 2.50), and in Thailand (4.92 ± 0.57) and they are similar to the values observed in localities of Papua New Guinea (from 6.00 to 6.58) [10]; these areas present a low or intermediate level of malaria transmission. Our observation agrees with the results presented by others [38] who found extensive genetic diversity in isolates collected in Dakar with antigenic markers. However, the genetic variability observed for the antigenic markers is smaller than the values usually found in the literature [39,40]. It is especially true for MSP1 and MSP2, but the variability observed here is only based on allelic size and not on variations in sequences which were generally taken into account in previous studies. Nevertheless, with the same kind of analysis (i.e. allelic size) the mean number of alleles per locus is significantly smaller for antigenic markers than for microsatellites (Table 4). This result could reflect (i) differential mutation rates between microsatellite and antigenic loci, and/or (ii) in accordance with the theory on selected genes, that antigen determinants are under selection and not microsatellites.

Table 4
Unbiased expected heterozygosities (He) corrected for haploid data (27), and number of alleles observed (A)^a

Statistical characteristic	He antigens	He microsatellites	A antigens	A microsatellites
Mean	0.702	0.745	5.440	6.439
LCL	0.701	0.745	5.433	6.435
UCL	0.703	0.745	5.446	6.443

^a Lower confidence limit (LCL) and upper confidence limit (UCL) are estimated after 10,000 bootstrap simulations over loci.

Table 5
Genetic disequilibrium analysis^a

Sample origin	Sample size	Microsatellite loci		Antigen loci		Microsatellite and antigen loci	
		Genepop	I_{AS} LIAN	Genepop	I_{AS} LIAN	Genepop	I_{AS} LIAN
Dakar	21	24***	0.047+++	1 ^{NS}	0.083 ⁺	33***	0.050+++
Senegal	27	27***	0.036+++	0 ^{NS}	0.040 ^{NS}	31***	0.031+++

^a Number of significant pairs found for Dakar and Senegal (all) samples from 19 microsatellite loci (171 possible pairs), four antigen loci (six possible pairs) and with both markers together (253 possible pairs) using Genepop and standardized index of association I_{AS} using LIAN.

*** Test significant at $P < 0.001$, and ^{NS} non-significant test after an exact binomial test.

+++ Test significant at $P < 0.001$, ⁺ test significant at $P < 0.05$ and ^{NS} non-significant test after a simulation test (10,000 iterations).

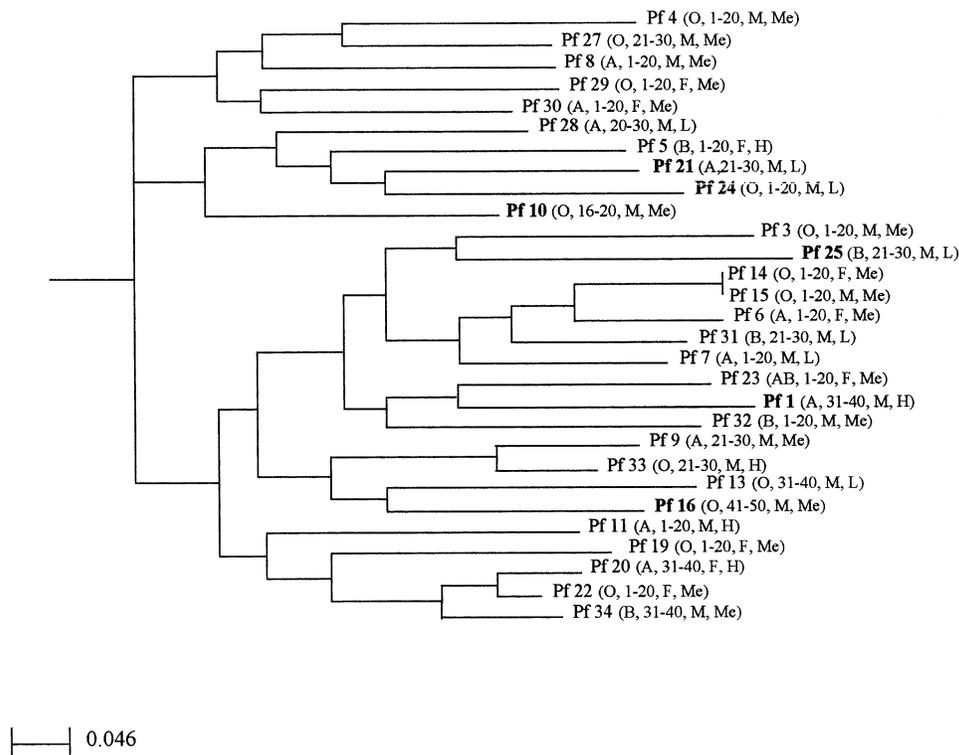


Fig. 1. Neighbor-joining tree reflecting the genetic relationships between the *P. falciparum* isolates collected from Senegal. Samples in bold characters were collected outside Dakar. Blood groups (A, B, AB, O), age classes (1–20 years, 21–30 years, 31–40 years, 41–50 years), sex (F, M) are given in parentheses. For the parasitemia level (i.e. number of trophozoites per μl of blood), we refer to L (low, $<10^4/\mu\text{l}$), Me (medium, $<10^5/\mu\text{l}$) and H (high, $>10^6/\mu\text{l}$).

The levels of unbiased expected heterozygosities found in this study with both microsatellites and antigens are comparable with other studies based on microsatellite loci: (i) Congo (0.79 ± 0.11) (Durand, unpublished data); (ii) Uganda, Congo and Zimbabwe isolates (from 0.76 to 0.8) [10]. But the heterozygosity observed in this Senegal sample is higher than those found also with microsatellite loci in South America (from 0.3 to 0.4), in Thailand (0.51 ± 0.08) and Papua New Guinea (from 0.62 to 0.65) [10]. If we observe an inferior mean number of alleles per locus compared to other areas, heterozygosity levels of this sample present the same order of magnitude as observations made in areas of high malaria transmission in which outcrossing occurs frequently [6].

Notwithstanding this great genetic diversity and frequent multi-infections, significant linkage disequilibrium clearly appeared in the present urban samples of malaria parasites. Linkage disequilibrium was also observed with microsatellite loci by others [10] in areas of low level of genetic diversity such as in some countries of South America (Bolivia, Colombia, Brazil), in areas with intermediate genetic diversity such as in Thailand or in one locality of Papua New Guinea, and in Zimbabwe, which presents a high genetic diversity. In other African countries with a high genetic diversity (Uganda and Congo), the same authors [10] did not observe linkage disequilibrium. Other authors [11] showed, from different sets of multilocus markers, strong linkage disequilibrium which could reflect a clonal

mode of propagation in Venezuela localities with low EIR. Two non-exclusive hypotheses could explain the situation observed in our Senegal sample: (i) a global non-panmictic structure of the Dakar malaria population due to a high predominance of selfing; (ii) a structuration in sub-populations of several malaria foci in Dakar (i.e. Wahlund effect). A similar situation is noticed by Durand et al. (unpublished data) who observed significant linkage disequilibrium in a sample set from Congo where malaria is stable with a high EIR and a high genetic diversity. So, these observations might underline the existence of different structured foci displaying their own population dynamics in countries with inclusive high malaria transmission.

In a study conducted in two villages in Senegal 5 km from one another displaying different transmission conditions, a study [15] showed the existence of a higher genetic diversity of malaria in the village with stable or perennial transmission (EIR: 89–350 infected bites/man/year) when compared to the village with seasonal transmission (EIR: 7–63 infected bites/man/year). In Pikine (suburb of Dakar), the transmission rate is comparable to the transmission rate observed in proximal rural areas, but the authors underlined that this site displayed many gardening areas with perennial water pools [41]. Previous studies [3] in Dakar city investigated the vector density between an area bordering a permanent marsh and an area located 600 m away. The density of *Anopheles arabiensis* decreased from the marshland to the farthest area. Children presented parasite rates

three times higher in the vicinity of the marshland, which was a main larval breeding site, than at some hundred meters away. Thus, the urban transmission level of malaria depends not only on the epidemiological pattern of the geographic area where the town is located (i.e. stable, unstable or intermediary malaria), but also on the human structures located inside the town [5]. Consequently, urban malaria displays a great heterogeneity of population structures which could explain the present results. The survey of urban malaria, which seems to constitute the “tomorrow-malaria” [1,5], must be considered through a strict characterization of the different sites of transmission, together with a maximum of inquiries about the real origin and clinical manifestations of patients. It is thus now necessary to consider the respective population structures of vectors and parasites in order to define who transmits what, where and when in malaria foci, because each site could be very delimited with its own type of transmission. However, it is obvious that these kinds of studies are currently under-represented in the literature on malaria.

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