

Genetic polymorphism of Merozoite Surface Protein 1 (msp1) and 2 (msp2) genes and multiplicity of *Plasmodium falciparum* infection across various endemic areas in Senegal

Tolla Ndiaye¹, Mouhamad Sy¹, Amy Gaye¹, Daouda Ndiaye^{1,2}

1. Laboratory of Parasitology/Mycology HALD, Cheikh Anta Diop University of Dakar, PO Box 5005, Dakar, Senegal.
2. Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA, USA.

Emails:

ndiayetola@gmail.com; symouhamad92@gmail.com; amygaye08@live.fr; dndiaye23@gmail.com

Abstract

Introduction: Despite a significant decline in Senegal, malaria remains a burden in various parts of the country. Assessment of multiplicity of *Plasmodium falciparum* infection and genetic diversity of parasites population could help in monitoring of malaria control.

Objective: To assess genetic diversity and multiplicity of infection in *P. falciparum* isolates from three areas in Senegal with different malaria transmissions.

Methods: 136 blood samples were collected from patients with uncomplicated *P. falciparum* malaria in Pikine, Kedougou and Thies. Polymorphic loci of msp1 and 2 (Merozoite surface protein-1 and 2) genes were amplified by nested PCR.

Results: For msp1 gene, K1 allelic family was predominant with frequency of 71%. Concerning msp2 gene, IC3D7 allelic family was the most represented with frequency of 83%. Multiclonal isolates found were 36% and 31% for msp1 and msp2 genes respectively. The MOI found in all areas was 2.56 and was statistically different between areas ($P=0.024$). Low to intermediate genetic diversity were found with heterozygosity range ($H_e=0,394-0,637$) and low genetic differentiation ($F_{st} \text{ msp1} = 0.011$; $F_{st} \text{ msp2} = 0.017$) were observed between *P. falciparum* population within the country.

Conclusion: Low to moderate genetic diversity of *P. falciparum* strains and MOI disparities were found in Senegal.

Keywords: Senegal, MOI, Genetic diversity, msp1, msp2.

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Introduction

Malaria control interventions showed a significant progress worldwide¹. However, malaria remains a burden in sub-Saharan Africa despite scaling up malaria interventions¹. Senegalese national malaria control program (NMCP) intensified malaria control strategies for more

than ten years including large distribution of long-lasting insecticide-treated bed nets (LLINs), use of histidine rich protein 2-based rapid diagnostic tests (HRP2-RDT) and artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated *Plasmodium (P) falciparum* malaria². Nevertheless in 2015, 492253 cases and 526 deaths due to *P. falciparum* malaria had been registered in the country³.

In Senegal, the overall population is exposed to malaria with a gradient of transmission increasing from North to South due to malaria epidemiology that occurs as a result of intensified control efforts in these areas. Indeed, malaria is hyperendemic in the South (annual incidence

Corresponding author:

Tolla Ndiaye,
Laboratory of Parasitology/Mycology HALD,
Cheikh Anta Diop University of Dakar,
P.O.Box 5005, Dakar, Senegal.
Email: ndiayetola@gmail.com

is greater than 100/1000 inhabitants) and hypoendemic in the North (annual incidence rates are now less than 5/1000 inhabitants)³. Malaria transmission pattern affects the multiplicity of infection (MOI) and genetic diversity of parasite populations, the latter's increase when transmission is high and decrease when transmission is low^{4,5}. Thus, assessment of genetic diversity and multiplicity of infection (MOI) provide insight on malaria transmission and genetic differentiation measurements such as heterozygosity (He) and fixation index (Fst) which are important data in monitoring of malaria control and elimination strategies^{4,6,7,8,9,10}.

The polymorphic loci of merozoite surface proteins (msp1 and msp2) are now well established to assess the genetic diversity of *P. falciparum* population and multiplicity of infection (MOI) which is an indicator of malaria transmission intensity in endemic areas^{9,11,12,8,13,14,15}. Additionally, many studies demonstrated that msp1 and msp2 genes are enough robust polymorphism markers to characterize the parasite population structure^{12,16} and are the gold standards to evaluate the MOI¹⁷. Msp1 gene has three allelic families K1, MAD20 and RO3318 and msp2 gene two allelic families, IC3D7 and FC27¹⁹.

The characterization of genetic diversity of *P. falciparum*

through different areas with various endemicities within a country has been extensively looked into in different parts of the world^{8,9,13,16,20,21}. However, a few studies about this topic have been performed in Senegal by Ahouidi et al²² and Konaté et al²³. Hence, it is important to have current data on genetic diversity on parasite populations through several areas in the country to better inform malaria control programs. In addition, the monitoring of malaria transmission intensity is a clear priority to malaria elimination. As MOI is an important metric of malaria transmission^{24,25,26,27,28}, it is a robust measure of changing malaria transmission and remains one of the most accurate molecular approaches to evaluate malaria parameters²⁹ in areas moving towards elimination like Senegal. It is important to have insight of malaria transmission intensity using the MOI within the country to know which area need more interventions in order to achieve the malaria elimination target in Senegal. This study aimed to investigate the genetic diversity and the multiplicity of *P. falciparum* infection of circulating parasites strains from three areas in Senegal having different endemicities by genotyping the highly polymorphic loci of msp1 and msp2 genes. Some genetic measurements such as He and Fst were also analyzed.

Materials and methods

Figure 1. Map

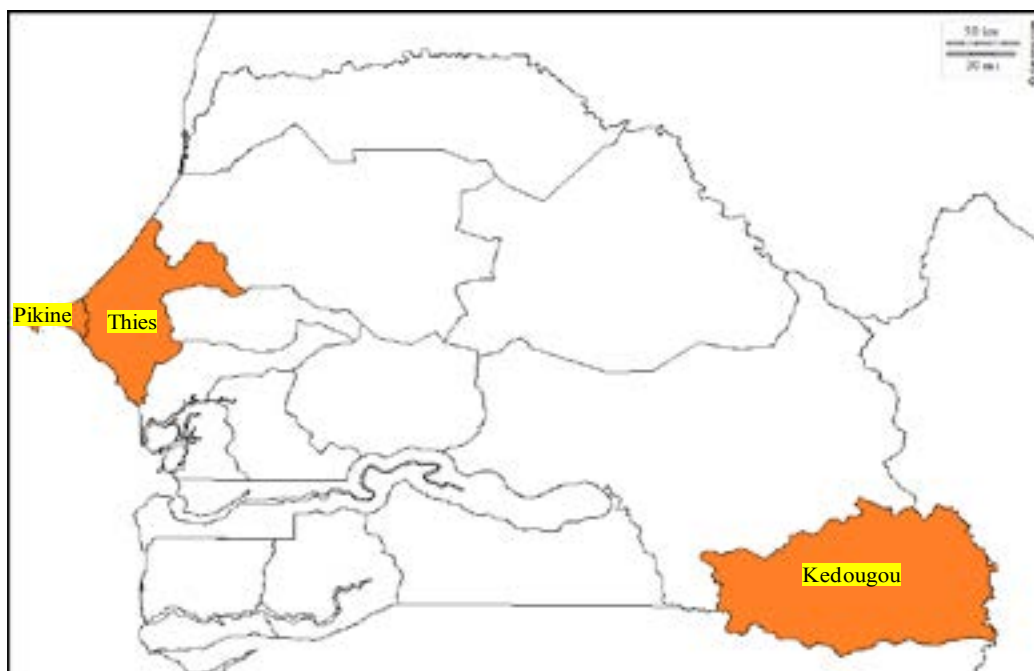


Fig. 1: Map of the three malaria study areas (Kedougou, Pikine and Thies) in Senegal. This map was generated using online website (<http://www.d-maps.com>)

Study sites

This study was carried out in three areas of Senegal with different endemicity of malaria: Pikine, Thies and Kedougou. Pikine is a sub-urban area which is 15 km the capital center (Dakar) with a heterogeneous endemicity (related to recurrent floods) with an entomological inoculation rate (EIR) ranging from 0 to 20 infectious bites per years and a malaria incidence greater than 15 malaria cases per 1000 habitants. Thies, in the west of the country at 70 km from Dakar, is a hypo-endemic area with a low EIR varying from 0 to 20 infectious bites per years and an average of 5 to 15 malaria cases per 1,000 habitants. Kedougou, located in the South-East, at 685 Km from Dakar, is hyper-endemic with an incidence higher than 15 malaria cases per 1000 habitants. In Kedougou, EIR is high ranging from 20 to 100 infectious bites/person/year².

Sample collection

This study was approved by Ethics Committee of the Ministry of Health of Senegal. Febrile patients visiting the NMCP sentinel site in these three areas during malaria transmission season were enrolled in this study after giving informed consent or guardians consent for children depending on the age of the patient. Blood samples were collected on filter-paper from patients who met the following criteria: Living in a 15-km radius of health facilities, having fever (axillary temperature ≥ 37.5 C) or history of fever in the previous 48 h, age ranging from 6 months to 75 years and uncomplicated *P. falciparum* malaria with parasite density ≥ 1000 asexual forms per microliter. Patients who presented signs or symptoms of severe malaria as defined by World Health Organization (WHO) 30 and pregnant women were not included.

DNA extraction and PCR genotyping

Parasite DNA, from filter paper was extracted using QIAamp DNA Mini kit (Qiagen[®], QIAGEN, USA) according to the manufacturer's instructions.

The polymorphic loci of *msp1* block 2 (K1, MAD20 and RO33 allelic families) and *msp2* central region (IC3D7 and FC27 allelic families) were amplified by nested PCR as described previously^{31,32}. The primer sequences are as shown in Table 1. All PCR reactions were performed out in a total volume of 20 μ l containing 6 μ l GoTaq Green Master Mix, 0.5 μ M of each primer, and 11 μ l reagent

grade water. In the first round reaction (nest 1), 1 μ l of genomic DNA was added as a template. In the second nested reaction (nest 2), 1 μ l of the nest 1 PCR product was used as DNA template. Cycling conditions for primary PCR were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 2 min and extension at 72°C for 2 min; a final extension was done at 72°C for 3 min. The cycling conditions for secondary PCR were, initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 2 min, extension at 72 °C for 2 min, with a final extension cycle of 72°C for 3 min. Positives (3D7 and Dd2) and negative (reagent grade water) controls were systematically incorporated in each PCR run. The nested PCR product were revealed by electrophoresis on 2% agarose gels stained with ethidium bromide and visualized under UV trans-illumination (VersaDoc[®], BIORAD, Hercules, USA). The size of PCR fragments were estimated using 100 bp molecular weight.

Statistical analysis

The online Biostatgv was used for statistical analysis. For all tests, the significance level was $p < 0.05$. Isolates presenting more than one allele were considered as multiclinal isolates. The chi-square test (X^2) was used to compare the frequencies of multiclinal isolates between localities. The multiplicity of infection (MOI) was calculated by dividing the total number of alleles detected for *msp1* and *msp2* genes by the total number of samples¹¹. Student's t test was used to compare MOI between localities. We used the expected heterozygosity (H_e) and genetic differentiation (F_{ST}) to assess population structure of parasites. Heterozygosity was calculated using the following formula $H_e = n/(n-1) (1-\sum P_i^2)$, where n = sample size, P_i = allele frequency as described by Nei et al.³³ and F_{st} was calculated as described previously^{22,34}.

Results

The study population characteristics

A total of 13 *P. falciparum* infected patients were enrolled from three Senegalese localities. Among these patients, 64.7% were male and 35.3% were female with a sex ratio of 1.83. Patients' age ranged from 4 to 75 years with a mean age of patients was 22.3. The parasite density ranged from 1000 to 404000 asexual forms per microliter.

Allelic distribution of *msp1* and *msp2* genes

Overall, the three allelic families (K1, MAD20 and RO33) of *msp1* gene and two (3D7 and FC27) of *msp2* gene were observed in this study.

A total of forty-two alleles types were detected for the two genes in all localities: twenty-two for *msp1* (Fig 2) and twenty for *msp2* (Fig 3).

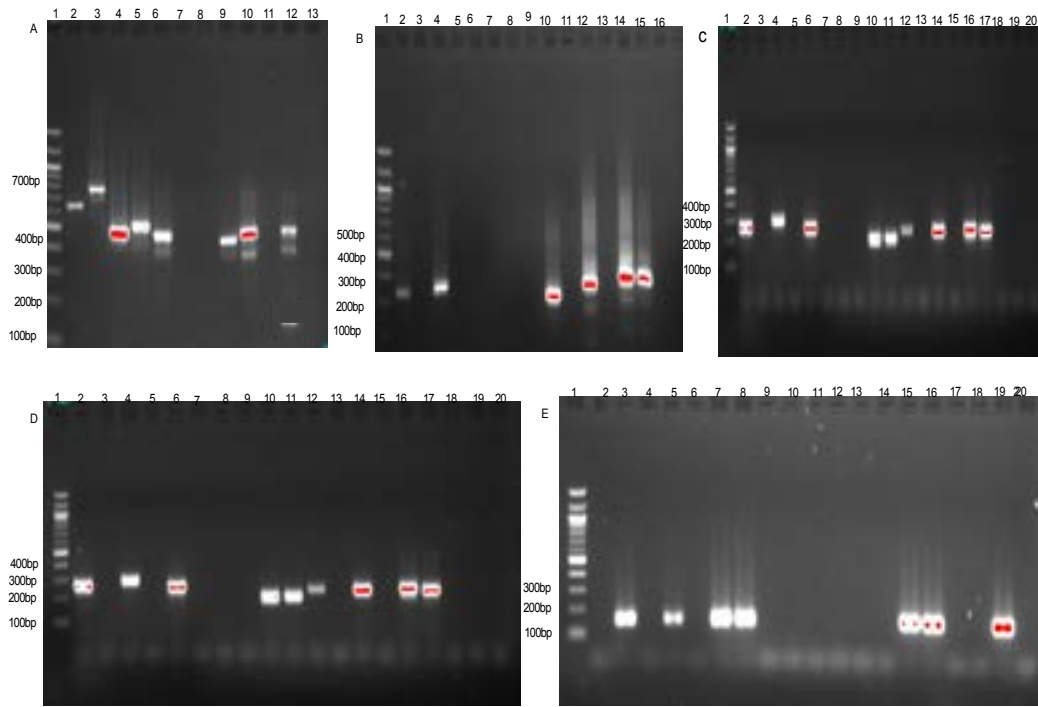


Fig.2: Banding pattern of *msp1* and *msp2* alleles of *P. falciparum*.

A = IC3D7; Lane 1, 100 bp DNA ladder; Lanes, 2–9, selected samples; Lane 10–12, Positives controls (10, IC3D7; 11, Dd2; 12, HB3); Lane 13, negative control.

B = FC27; Lane 1, 100 bp DNA ladder; Lane 10–12, selected samples; Lane, 13–15, Positives controls (13, IC3D7; 14, Dd2; 15, HB3); Lane 16, negative control.

C = K1, D = MAD20, E = RO33; Lane 1, 100 bp DNA ladder; Lanes, 2–16, selected samples; Lane 17–19, Positives controls (17, IC3D7; 18, Dd2; 19, HB3); Lane 20, negative control.

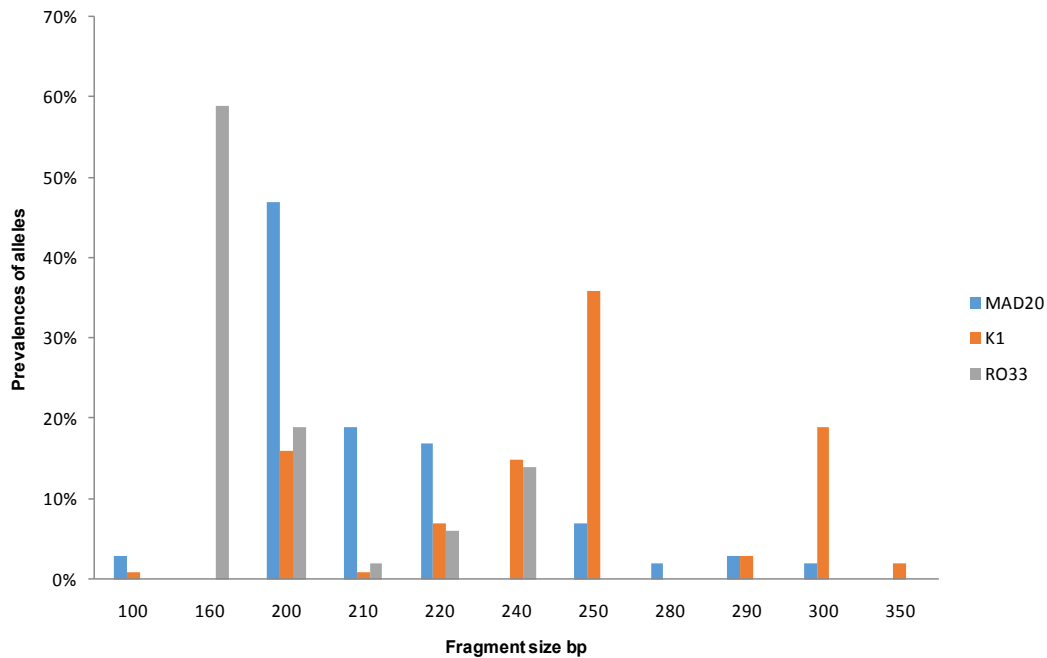


Fig.3: Prevalence of MAD20, K1, RO33 alleles distribution according to their length or number of base pairs.

In *msp1* gene, nine K1 alleles types (range from 100 bp to 350 bp), eight MAD20 alleles types (100 bp-300 bp) and five RO33 alleles types (160 bp-250 bp) were observed while for *msp2* gene, eleven IC3D7 (300 bp-800 bp) and

nine FC27 (200 bp -600 bp) alleles types were identified. Distribution of the different allelic families of *msp1* and *msp2* genes and their combinations in different localities are shown in table 2.

Table 1: Sequences of the primers used to amplify the *msp 1* and *msp 2* genes of *P. falciparum* isolates

Amplification/gene	Primers	Primer sequence
Primary PCR		
msp1	M1-OR	5'-CTA GAA GCT TTA GAA GAT GCA GTA TTG-3'
	M1-OF	5'-CTT AAA TAG TAT TCT AAT TCA AGT GGA TCA-3'
msp2	M2R	5'-ATGAAG GTA ATT AAAACATTG TCT ATT ATA-3'
	M2F	5'-CTT TGT TAC CAT CCG TAC ATT CTT-3'
Secondary PCR		
K1	M1K1R	5'-AAA TGA AGAAGA AAT TAC TAC AAAAGG TGC-3'
	M1K1F	5'-GCTTGC ATC AGC TGG AGG GCT TGC ACCAGA-3'
MAD20	M1MAD20R	5'-AAA TGA AGG AACAAG TGG AACAGC TGT TAC-3'
	M1MAD20F	5'-ATC TGA AGG ATT TGT ACG TCT TGA ATT ACC-3'
RO33	M1RO33R	5'-TAAAGG ATG GAG CAAATA CTC AAG TTG TTG-3'
	M1RO33F	5'-CAT CTG AAG GAT TTG CAG CAC CTG GAG ATC-3'
IC3D7	M2ICR	5'-AATACT AAG AGT GTA GGT GCA TATGCT CCA-3'
	M2ICF	5'-TTT TAT TTG GTG CAT TGC CAG AAC TTG AAC-3'
FC27	M2FCR	5'-AGAAGT ATG GCA GAA AGT AAC CCT TCT ACT-3'
	M2FCF	5'-GAT TGT AAT TCG GGG GAT TCA GTT TGT TCG-3'

Table 2: Distribution of allelic families of *msp1* and *msp2* genes of *P.falciparum*

Gene /Allelic families	Localities			
	Pikine (n=45)	Kédougou (n=50)	Thiès (n=41)	Total (136)
	N(%)			
Msp1				
K1	22(49)	14(28)	25(61)	61(44)
MAD20	6(13)	10(20)	2(5)	18(13)
RO33	1(2)	3(6)	4(9)	8(6)
K1+MAD20	4(9)	9(18)	2(5)	15(11)
K1+ RO33	4(9)	4(8)	7(17)	15(11)
MAD20+RO33	8(18)	4(8)	1(3)	13(10)
K1+MAD20+RO33	0	6(12)	0	6(5)
Total K1	30(66.66)	33(66)	34(82.9)	97(71)
Total MAD20	18(40)	29(59)	5(12.1)	52(38)
Total RO33	13(28.88)	17(35)	12(29.2)	42(31)
Multiclinal isolates	16(36)	23(46)	10(25)	49(36)
Msp2				
IC3D7	20(44)	26(52)	25(60)	71(52)
FC27	11(24)	7(14)	5(13)	23(17)
IC3D7 + FC27	14(32)	17(34)	11(27)	42(31)
Total IC3D7	34(76)	43(86)	36(88)	113(83)
Total FC27	25(56)	24(48)	16(40)	65(48)
Multiclinal isolates	14(32)	17(34)	11(27)	42(31)

n: number of isolates

For *msp1* gene, K1, Ro33, and Mad20 allelic families were found with frequencies of 71% (97/136), 38% (52/136), 31% (42/136) respectively. In Samples with single allelic family, K1 was the most represented with 44% (61/136). K1+RO33 and K1+MAD20 allelic combination were higher among samples with two different allelic families with equal frequency of 11% (15/136). Samples with all three allelic families (K1+MAD20+RO33) represented 5% and were found only in Kedougou ($p = 0.008$). Multiclinal isolates found in all study sites were 36% (49/136) with a maximum in Kedougou (46%; 23/50) and minimum in Thiès (25%; 10/41), however no statistical difference was found ($p = 0.33$).

Concerning *msp2* gene, IC3D7 and FC27 allelic families were identified with frequencies of 83% (113/136) and 48% (65/136) of the isolates, respectively. The frequency

of samples with only the IC3D7 allelic family was more predominant with 52% (71/136) than those harboring only the FC27 allelic family with 17% (23/136). The frequency of multiclinal isolates found in all localities was 31% (42/136) with the highest value in Kedougou (34%, 17/50) and the lowest in Thiès (27%, 11/41), however the difference was not statistically significant between localities ($p=0.865$).

Multiplicity of infection, heterozygosity and genetic differentiations

The multiplicity of infection (MOI) and heterozygosity (He) in different localities are shown in table 3. The number of *msp1* and *msp2* genotypes per isolates ranged from 1 to 5. A higher value of MOI was found in Pikine 2.97. MOI found in all localities was 2.56 with statistically different between areas ($p=0.024$).

Table 3: Multiplicity of infection and heterozygosity of *P.falciparum* populations

	Localities		
	Pikine	Kédougou	Thiès
MOI <i>msp1</i>+<i>msp2</i>	2.97	2.51	2.21
He <i>msp1</i>	0.632	0.637	0.495
He <i>msp2</i>	0.509	0.457	0.394

MOI, multiplicity of infection; He, expected heterozygosity

For *msp1* gene, heterozygosity (He) and genetic differentiation (Fst) observed in all study areas were 0.588 and 0.011 respectively. We found approximately the same value of He in Kedougou (0.637) and Pikine (0.632).

Regarding *msp2* gene, He and Fst found in whole study sites were 0.453 and 0.017 respectively. A low He value was observed in Thies (0.394) while a high value was noticed in Pikine with 0.509.

Discussion

The present study aimed to provide a current overview on *P. falciparum* population structure in Senegal by analyzing the most polymorphic regions of *msp1* and *msp2* genes on isolates from areas presenting different malaria transmission patterns. This recent information on the molecular epidemiology of the most virulent plasmodial species in this country could inform malaria program of monitoring, control and to adapt if necessary the interventions to local malaria epidemiology settings.

In this study, K1 and IC3D7 allelic families were most predominant in all study sites. The strong presence of both allelic families has been already reported by studies carried out in West Africa^{8,15,35}, Western Uganda³⁶ and Iran³⁷. Twenty-two alleles for *msp1* gene and twenty alleles for *msp2* gene were identified. Similar data were found in areas where malaria transmission is low^{13,38}. However, a larger allele's numbers were observed in high malaria transmission areas in Kedougou, Senegal²³ and in Burkina Faso¹⁴ suggesting that malaria endemicity affects the circulating strains numbers.

P. falciparum multiclonal isolates for *msp1* and *msp2* allelic families found in all study areas were relatively high with the higher in Kedougou. Similar findings were reported in hyperendemic areas^{13,14,33,39}. The lower multiclonal isolates were found in Thies reflecting the low endemicity level of this study area which is agreement with other studies performed in hypoendemic areas^{16,40,41}. These differences show that multiclonal isolates are more common in high malaria transmission area¹⁶.

MOI is an indicator of malaria transmission level because it has been to be higher in high malaria transmission areas and decreased when this latter decline^{16,42}. Our results show moderate to high MOI values and a significant difference on MOIs between these localities ($p=0.024$). Therefore, our findings found in Thies and Kedougou are

in phase with this hypothesis. In this last locality, Niang et al, found the same results in 2017⁴³. However, our most important MOI value was obtained in Pikine, an area with particular malaria epidemiological characteristics linked to floods during raining season (artificial water collection reservoirs) which increase opportunities for mosquito breeding growth². These urban characteristics, reported by many studies performed in African cities^{44,45,46,47,48,49}, could explain the high MOI found in this locality.

Also, low to moderate genetic diversity with heterozygosity were found in our study, ranging from 0.394 to 0.637. The lowest He value was found in Thies (He=0,394) and the highest He values were found in Kedougou (He=0.637) and Pikine (He=0.632). This variability between genetic diversity levels observed within country could be explained by local malaria epidemiology settings. Similar results were reported in Mali where the He values increase with malaria transmission gradient north to South⁸. The low genetic diversity of *P. falciparum* population found in Thies is consistent with clonal expansion reported previously in this area⁵⁰ suggesting a high self-fertilization rate between genetically identical parasites during the sexual stages in mosquito. This low genetic diversity was also reported in countries where malaria decline, due to scale up of interventions^{9,51}. However, moderate heterozygosity was found in Kedougou and Pikine indicating a reduction in clonal expansion and an increase of genetic diversity in these localities. Likewise, it suggest that genetic diversity of *P. falciparum* is greater in high malaria transmission areas and decreases when transmission regresses^{4,5,52}.

Concerning Fst, we found a very low genetic differentiation (Fst *msp1*= 0.011; Fst *msp2*= 0.017) between *P. falciparum* population within country. Ahoüdi et al,²² had already reported this little genetic divergence (Fst *msp2* = 0.012) in parasites populations in Senegal. These data reveal a gene flow between parasite populations facilitated by extensive human migration events between endemic regions and then causing the vector's displacement. Likewise, a lack of genetic differentiation was reported by others studies, on sites not far as ours studies areas, located approximately from 61 km to 685km^{8,22}. Similar trends have been observed in Mali⁸ and elsewhere of the world such as in Latin American countries, Malaysia and South-East Asia where malaria transmission declines^{16,53,54,55,56}. Overall, the low to moderate genetic diversity and little genetic differentiation in *P. falciparum* population found in

Senegal shows that the effectiveness of the malaria controls scaling up since 2006². Nonetheless, disparities exist on malaria transmission levels in different areas.

The main limitation of this study was the use of msp genotyping which, as others marker based on DNA fragment size, could reduce the genetic diversity evaluation of the parasites strains. Nevertheless, msp1 and msp2 genes are robust polymorphism markers and can be used successfully to characterize genetic *P. falciparum* strains populations^{12,13,14,15}.

Conclusion

This study gives current data on genetic diversity of *P. falciparum* population in Senegal. Low to moderate genetic diversity was found on parasite strains reflecting the decline of malaria transmission as well as interventions effectiveness. A high gene flow of parasites between localities shows rapid genes diffusion on *P. falciparum* population in this country. Our findings also confirm the disparities on malaria transmission level within Senegal. Thus, to achieve the malaria elimination objectives, Senegalese national malaria control program (NMCP) should readjust the malaria control strategies linked to the malaria epidemiological local patterns of sentinel sites like Pikine and Kedougou due to their endemicities.

Conflict of interest

The authors declare that they have no competing interests.

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