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NO INFLUENCE OF AGE ON INFECTION COMPLEXITY AND ALLELIC DISTRIBUTION IN *PLASMODIUM FALCIPARUM* INFECTIONS IN NDIOP, A SENEGALESE VILLAGE WITH SEASONAL, MESOENDEMIC MALARIA

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Abstract. We have shown previously that in Dielmo, a Senegalese village with intense perennial *Plasmodium falciparum* transmission, the infection complexity and the distribution of some allelic types harbored by asymptomatic carriers was age-dependent. We report here an investigation of these parameters in Ndiop, a village located 5 km from Dielmo, where malaria is mesoendemic and seasonal, and where immunity is acquired at a very low rate, as indicated by the lifelong distribution of *P. falciparum* clinical attacks. Blood was collected from 143 and 125 inhabitants, including 122 individuals sampled in both surveys, during two cross-sectional surveys at one-month intervals during the 1994 transmission season. *Plasmodium falciparum* parasites were genotyped for three polymorphic single copy genes. Genetic diversity was very large, with 17, 43, and nine distinct alleles detected for the merozoite surface protein-1 (MSP-1), MSP-2, and glutamate-rich protein loci, respectively. These figures, similar to those previously observed in Dielmo, indicate that the parasite genetic diversity is not directly related to the inoculation rate, at least in the range of transmission intensity studied here. The complexity of the asymptomatic infections (average number of distinct genotypes per isolate) was more than two-fold lower in Ndiop than in Dielmo and importantly, did not decrease with age. Likewise, the allele distribution was not influenced by age, contrasting with the observations made in Dielmo. This indicates that the number of parasite types per isolate and the influence of age on complexity and allele distribution depend on the level of endemicity, consistent with the interpretation that they reflect acquired anti-parasite immunity.

In humans living in regions endemic for malaria, immunity to *Plasmodium falciparum* is acquired as a result of natural exposure to multiple infections over many years. In holo- or hyper-endemic areas, immunity develops at a younger age than in areas where transmission is less intense. Recent studies conducted in Dielmo and Ndiop, two neighboring Senegalese villages with different transmission conditions, outlined the influence of transmission on the rate of acquisition of immunity. Interestingly, this comparison showed a different incidence rate of malaria attacks with age but little difference in the total number of attacks over an entire lifetime.[1]

The characteristics of malaria immunity are assumed to reflect the need to be infected with a large number of antigenically diverse parasite populations to develop an effective anti-parasite immunity. It is usually considered that immunity to *P. falciparum* has two components: an anti-disease immunity, which is believed to develop rapidly and an anti-parasite immunity, which is acquired slowly and leads to a marked decrease in parasite densities.[2–4] In many endemic regions, the presence of blood-stage parasites is not synonymous with disease. Asymptomatic carriage, interpreted as reflecting acquired clinical immunity and incomplete anti-parasite immunity, can be observed in up to 90% of individuals in holoendemic areas, as demonstrated by highly sensitive detection techniques such as the polymerase chain reaction (PCR).[5]

To gain a better understanding of the factors that have an impact on the development of immunity in different endemic areas, it is necessary to study the parasite population to which people are actually exposed. To date, few studies have been carried out on the circulation and the genetic diversity of parasite strains in the field.[4,6–8] Molecular analysis of parasites collected in Dielmo during cross-sectional or longitudinal surveys indicated that parasite polymorphism was very large in this village. Most symptomatic and asymptomatic subjects were infected by multiple *P. falciparum* genotypes. Interestingly, the number of clones present in asymptomatic infections decreased at the age where an efficient immunity is in place, as indicated by low parasite densities and reduced clinical attack rates.[4] Furthermore, the distribution of some merozoite surface protein-1 (MSP-1) alleles was influenced by age.[9] These results are consistent with the interpretation that the acquired anti-*P. falciparum* immunity reduces parasite density, limits the number of parasite genotypes infecting an individual at any given time, and controls parasites against which a strong immune response has been mounted.

The extent of parasite diversity may vary with transmission intensity, being at least in part generated by intragenic recombination and independent chromosome assortments during meiosis in the mosquito. The work reported here was undertaken to analyze parasite diversity in Ndiop. We have carried out the genotyping of parasites collected from individuals of all age groups during two cross-sectional surveys made at one-month intervals during the 1994 transmission season. Genotyping of peripheral blood *P. falciparum* parasites was done using a PCR-based methodology. We analyzed three single-copy genes, the glutamate rich protein (GLURP) gene, located on chromosome 10,[10] the MSP-1 gene located on chromosome 9,[11] and the MSP-2 gene located on chromosome 2.[12] These highly polymorphic loci are convenient typing markers[4,8,9,13] because they possess numerous alleles. The GLURP alleles are identified by size

polymorphism of one domain, due to variable copy number of repeats.[10] Based on variable nucleotide sequence and copy number of repeats of block 2, the various MSP-1 alleles can be grouped into three major allelic families designated MAD20, R033, and K1 according to reference clones. Likewise, there are two major MSP-2 allelic families, designated FC27 and 3D7, which differ in the nucleotide sequence and copy number of repeats of the central domain of the gene. Since the duration and intensity of transmission as well as the kinetics of acquisition of immunity differ in Ndiop and in Dielmo, the comparison of the parasite populations and molecular characteristics of infections in both settings can provide novel clues to understand host-parasite interactions. In particular, it is likely to differentiate parameters related to age from those due to immunity and/or cumulated exposure to parasites.

MATERIALS AND METHODS

Study site and blood collection. Ndiop, a village of about 350 inhabitants situated in Saloum, in the Sahelo-Soudanian region of Senegal, is located 5 km from Dielmo and has a markedly different malaria transmission pattern. Since 1993, a longitudinal epidemiologic and entomologic follow-up has been carried out. The patterns of transmission over four consecutive years has been recorded, indicating an annual entomologic inoculation rate ranging from seven to 63 infective bites/person/year, depending on the year (17 for 1994) and a strictly seasonal transmission by *Anopheles arabiensis* and *An. gambiae*.[14] In contrast, transmission in Dielmo is perennial, varying from 89 to 350 bites/person/year.[2,15] Fingerprick blood samples were collected from permanent residents of the village after informed consent was obtained from the individuals or their parents. The protocol was approved by the Ministere de Cooperation et du Developpement and the Ministere de la Sante Publique of Senegal. The samples studied here were collected from September 12 to 18, 1994 (143 samples) and from October 10 to 15, 1994 (125 samples). Transmission 2–3 weeks before blood collection (i.e., at the time parasites were supposedly inoculated) was estimated at 1.7 and 3.3 infective bite/person/week for the September and October samples, respectively. All samples were collected and stored as described.[13] Parasite density was determined by microscopic examination of thick blood smears. Two hundred oil-immersion fields were systematically examined on each slide; the average sensitivity threshold was estimated to be two parasites per microliter of blood.[16]

Clinical status. There were 87 and 79 asymptomatic inhabitants in the September and October groups, respectively, who had not received any antimalarial in the preceding seven days and who did not present clinical symptoms on the day of blood collection or in the preceding or following week. Based on data from regions with similar endemicity[17] and from a preliminary determination of the parasite density fever threshold in Ndiop (Tall A, Rogier C, Spiegel A, unpublished data), individuals with clinical symptoms and a parasite density > 3,500 trophozoites/μl on the day of blood sampling or within the next four days were considered as having a clinical malaria attack. Using this definition, there were 17 clinical malaria cases in the September group, with 15 in 0–14-year-old children and eight cases in October, seven of whom were less than 14 years old. In addition, there were four and two febrile subjects in September and October, respectively, who carried *P. falciparum* parasites at a very low density. Since the clinical episode did not require any anti-malarial treatment, they were considered as nonclinical malaria and were excluded from the comparisons of asymptomatic versus clinical malaria. This comparison also excluded the following subjects because we could not define the parasites carried by these individuals as new or old infections: 1) seven febrile individuals in September and 11 febrile subjects in October who carried *P. falciparum* parasites but had received an anti-malarial treatment at any time during the 15 days before blood donation; 2) 10 and 14 individuals in September and in October, respectively, who did not present clinical symptoms but who had received antimalarials in the preceding seven days.

Extraction of DNA and PCR genotyping. The DNA was extracted from saponin-lysed, thawed red blood cell pellets with proteinase K, followed by phenol/chloroform extractions, as described.[4] Two microliters of DNA (corresponding to 1 μl of blood) were amplified in a Hybaid thermal reactor (Cera-Labo, Ecquevilly, France) in a final volume of 50 μl containing 200 μM of each deoxynucleotide (Pharmacia, Saint-Quentin en Yvelines, France), 1 μM of each primer, and 2.5 units of *Taq* polymerase (Pharmacia) in 75 mM KCl, 2.25 mM $MgCl_2$, and 15 mM Tris-HCl (pH 9 at room temperature). The primary PCRs were done as described previously.[4] The nested family-specific PCRs were carried out with 1 μl of the primary PCR sample (diluted if necessary). Amplification was done for 17 cycles (5 min at 94°C, 1.5 min at the appropriate annealing temperature, and 2 min at 72°C). The optimal stringency condition for annealing the family-specific primers was determined for each allelic family. The sequence of the various primers and the annealing temperatures used are listed in Table 1. For all reactions, parasite clones, monomorphic parasite lines, or cloned PCR fragments were used as positive controls. The species-specific nested PCR were done as described, using ribosomal DNA-derived primers.[19] The products of the genotyping PCR were analyzed for size polymorphism on a 2% equivalent low-melting agarose gel containing 0.5% multipurpose agarose and 0.75% infinity agarose enhancer (Appligene Oncor, Illkirch, France). The DNA was visualized under ultraviolet light after being stained with ethidium bromide. For Southern blot analysis of MSP-1 and MSP-2 allelic families, 5 μl of each PCR product were loaded onto a 4% agarose gel in the presence of 0.5 μg/ml of ethidium bromide. After electrophoresis, the DNA was visualized with ultraviolet light and photographed. The DNA was then transferred to a Hybond N^+ nylon membrane (Amersham, Les Ullis, France) as recommended by the manufacturer. The nylon membranes were prehybridized at 65°C in 6× SSC (20× SSC = 3 M NaCl, 0.3 M trisodium citrate), 2.5% nonfat milk, and 0.1% sodium dodecyl sulfate (SDS) for 1 hr and incubated overnight in the same conditions with a ^{32}P-radiolabeled probe (derived from cloned DNA fragments of each allelic family). The membranes were then washed three times at 65°C with decreasing concentrations of SSC (6× SSC, 2× SSC, 0.5× SSC, and 0.1× SSC). After the 2× SSC (nonstringent conditions), 0.5× SSC (medium stringency), or 0.1× SSC (high

TABLE 1

Oligonucleotide sequences and corresponding annealing temperature used for polymerase chain reaction (PCR) amplification of the *Plasmodium falciparum* genetic loci examined*

Target	Primer	Annealing temperature (°C)	Oligonucleotide sequences
MSP-1† (primary PCR)	P1‡	55	5′-CAC ATG AAA GTT ATC AAG AAC TTGTC-3′
	P2‡		5′-GTA CGT CTA ATT CAT TTG CACG-3′
K1	K1	65	5′-GAA ATT ACT ACA AAA GGT GCA AGTG-3′
	K2		5′-AGA TGA AGT ATT TGA ACG AGG TAA AGTG-3′
MAD20	M1	67	5′-GAA CAA GTC GAA CAG CTG TTA-3′
	M2		5′-TGA ATT ATC TGA AGG ATT TGT ACG TCT TGA-3′
RO33	R1	68	5′-GCA AAT ACT CAA GTT GTT GCA AAGC-3′
	R2		5′-AGG ATT TGC AGC ACC TGG AGA TCT-3′
MSP-2† (primary PCR)	1	55	5′-ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA-3′
	4		5′-ATA TGG CAA AAG ATA AAA CAA GTG TTG CTG-3′
3D7§	A1	58	5′-GCA GAA AGT AAG CCT TCT ACT GGT GCT-3′
	A2		5′-GAT TTG TTT CGG CAT TAT TAT GA-3′
FC27§	B1	58	5′-GCA AAT GAA GGT TCT AAT ACT AAT AG-3′
	B2		5′-GCT TTG GGT CCT TCT TCA GTT GAT TC-3′
GLURP† (primary PCR)	E	55	5′-ATG AAT TTG AAG ATG TTC ACA CTG AAC-3′
	F		5′-AAA TAT TAC TAT ATC CTT TGC TAT TCC-3′
GLURP†	G	57	5′-CTG AAC CAA ATC AAA ATA ACG-3′
	H		5′-TTC TTC TGG TTT TAT AGT TTC-3′

* MSP-1 = merozoite surface protein-1; GLURP = glutamate-rich protein.
† Conserved region.
‡ Ranford-Cartwright and other.[18]
§ Ntoumi and others.[4]

stringency) washes, membranes were autoradiographed at −80°C using X-OMAT film (Eastman Kodak, Rochester, NY). The same membrane was used for successive probings with each family-specific probe. The probe was removed from the membranes by incubation at 45°C in 4 M NaOH for 30 min, then in 0.1× SSC, 0.1% SDS, and 0.2 M Tris HCl, pH 7.5, for 15 min and washed in 2× SSC at 60°C. Before the next hybridization, removal of the probe was verified by autoradiography of the membranes at −80°C using X-OMAT film.

Allele distribution, complexity of infection, and statistical analysis. The prevalence of each allelic family was estimated by calculating the percentage of fragments assigned to one family (by PCR with family-specific primers) within the overall number of fragments detected for that locus in the group considered. Comparisons of the distribution of MSP-1 and MSP-2 allelic families by age or by clinical group were made using chi-square tests. Yates' corrections were applied when needed. Temporal variations in the prevalence of individual alleles (within an allelic family) were studied using the O'Quigley and Schwartz test.[20]

The complexity of infection (number of bands per infected person), a quantitative variable, was calculated as the average number of distinct fragments per PCR positive sample. It was estimated by dividing the total number of fragments detected in the typing reaction by the number of positive samples for that reaction. It is technically difficult to determine the exact number of different genotypes in such isolates because distinct parasites may differ by one, two, or three loci investigated. The complexity of infections was therefore calculated for each typing reaction (MSP-1, MSP-2, and GLURP) independently. An alternative, more accurate estimate of the overall complexity of the isolates was provided by combining the three typing reactions, namely by using the highest number of bands detected in one carrier (whatever the locus). Complexity of infections was analyzed using a generalized estimating equation approach that allows the analysis of repeated measures and can be implemented for Poisson responses, using the SPIDA statistical package (SPIDA Version 6; Statistical Computing Laboratory, Eastwood, New South Wales, Australia).[21] We used an exchangeable correlation structure where the correlation between observations made in the same person at different times is assumed to be the same. With this model, the estimated odds ratio can be considered as an estimation of individual relative risk (to have another band).[22] The differences were tested by the Wald test.[23] The explanatory variables, i.e., age, hemoglobin phenotype, month, and clinical status, were included together in the model and their interaction terms were also tested.

RESULTS

Prevalence. The blood samples analyzed were collected during two successive cross-sectional surveys: the first one (September 12–18, 1994) was obtained from 143 Ndiop villagers and the second one (October 10–15, 1994) from 125 individuals, including 122 individuals from the first survey. Examination of blood smears by microscopy indicated that approximately 36% and 34% of the samples were positive for *P. falciparum* parasites in September and October, respectively. This was a substantial underestimate since the nested species-specific PCR-based ribosomal DNA, the most sensitive technique reported so far, indicated a 77% prevalence of *P. falciparum* parasites for the September samples (Table 2). This discrepancy is due to the low parasite density of many asymptomatic carriers in this village. This interpretation was substantiated by the nested PCR genotyping reactions, based on amplification of polymorphic regions of three single copy genes: MSP-1, MSP-2, and GLURP. The

TABLE 2

Prevalence of *Plasmodium falciparum* parasites in different age groups recruited in September 1994 estimated by three different techniques

Age (years)	No. of samples analyzed	Blood slides		No. (%) of positive species-specific PCRs‡	No. of positive primary PCRs§	No. (%) of positive samples nested PCRs¶
		No. (%) of positive blood slides*	Range of parasite density†			
0–4	36	12 (33)	8–52,800	24 (66)	7	28 (78)
5–9	36	14 (39)	1–143,200	29 (80)	6	30 (83)
10–19	30	9 (25)	2–90,000	30 (100)	2	25 (83)
20–39	21	6 (29)	2–1,840	12 (57)	1	17 (81)
>40	20	11 (55)	4–18,080	15 (75)	0	16 (80)
Total	143	52 (36)	1–143,200	110 (77)	16	116 (81)

* No. of positive blood slides refers to the number of samples with thick blood smears positive for *P. falciparum* asexual or sexual stages by microscopic examination.
† No. of trophozoites/μl of blood.
‡ No. of positive samples for species-specific polymerase chain reactions (PCRs) based on a ribosomal DNA nested PCR.
§ No. of positive primary PCRs: no. of isolates positive for any one of the merozoite surface protein-1 (MSP-1), MSP-2, or glutamate-rich protein (GLURP) reactions.
¶ No. of nested PCR samples positive for at least one of the loci typed (MSP-1 and/or MSP-2 and/or GLURP).

results of the three genotyping reactions indicated a slightly higher prevalence than the species-specific PCR (81 versus 77%, respectively). Thus, a very large proportion of individuals of all age groups living in Ndiop carried *P. falciparum* parasites during this transmission period, most of them at a density below the microscopic or single PCR detection threshold. Similar figures were obtained for the samples collected in October 1994, in which a prevalence of 83% was estimated from the genotyping amplifications.

Typing strategy: comparison of nested PCRs with family-specific primers and hybridization. The nested PCR typing strategy used was based on a primary PCR using external, highly conserved primers flanking a polymorphic domain of the gene and a second reaction driven by an internal set of primers. For GLURP, the second set of primers was also derived from highly conserved regions of the gene (Table 1); the various alleles, which differ by the copy number of repeats, were identified by size polymorphism. For the MSP-1 block 2 and MSP-2 central domain, in which individual allelic types differ both by size and sequence, family-specific primers were used in the secondary PCR, as indicated in Table 1. For MSP-1, primer pairs specific for each allelic family were used, namely K1 + K2, M1+ M2, or R1 + R2. For MSP-2, we used homologous and heterologous primer combinations to amplify 3D7-types (A1 + A2), FC27-types (B1 + B2), or hybrid types (A1 + B2 or A2 + B1).[4] This allowed a simultaneous typing of the alleles by size polymorphism and identification of the allelic family and/or of hybrid sequences.

The validity of this approach is illustrated in Figure 1, where the products of the four nested MSP-2 secondary PCRs carried out using genomic DNA from the 3D7 parasite clone (lanes 1) and from a parasite line with a FC27-type MSP-2 gene (lanes 2) have been analyzed. Staining with ethidium bromide indicated the presence of a product with the homologous primer pair, as predicted. The assignment to a specific allelic family was confirmed by hybridization using family-specific probes under nonstringent and stringent conditions as described previously.[4] Figure 1 shows that only the homologous gene family was detected and confirms the absence of a PCR product when using the three heterologous primer combinations. Similar results were obtained for MSP-1. Since the specificity of both approaches was similar and the nested PCR presents the additional advantage of orient-

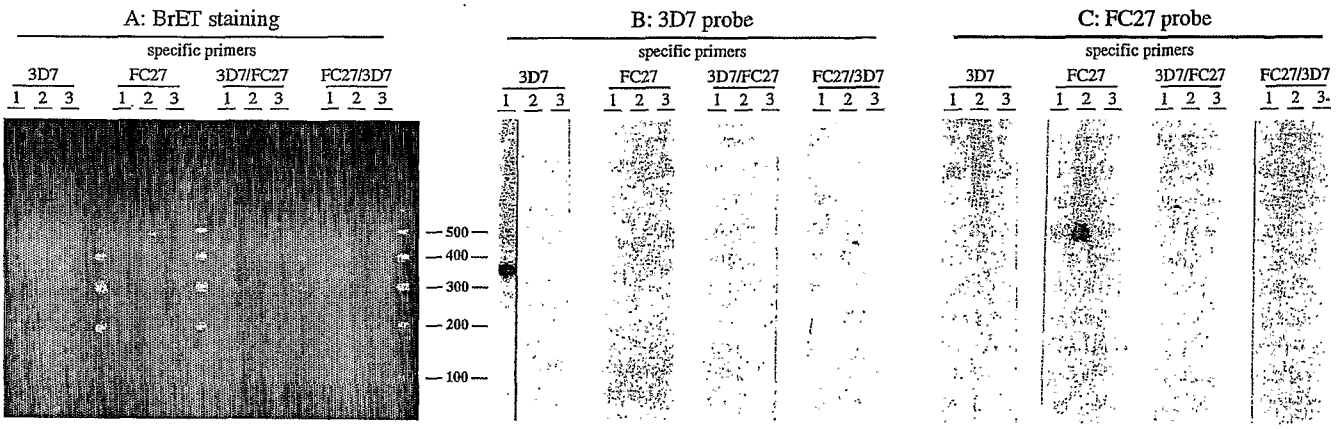

FIGURE 1. Analysis of the merozoite surface protein-2 (MSP-2) locus of reference DNAs by a nested family-specific polymerase chain reaction (PCR). **A,** analysis of the PCR products by electrophoresis on agarose gels and visualization by staining with ethidium bromide (BrET). The nested family-specific PCRs were carried out using A1 + A2 (3D7 specific), B1 + B2 (FC27 specific), A1 + B2 (3D7/FC27 specific), and B1 + A2 (FC27/3D7) primers after a first PCR using the conserved primers 1 + 4. The gel was loaded as indicated on top of the lanes. Molecular mass markers (100-basepair ladder; Superladder-Low, Advanced Biotechnologies, Epsom, Surrey, United Kingdom) were added on the right of each series. Lanes 1, 3D7; lanes 2, FC27; lanes 3, no DNA added. **B,** Southern blot analysis of the gel shown in **A.** The PCR products were transferred onto a Nylon N^+ membrane (Amersham) and incubated with a ^{32}P-labeled 3D7-specific probe labeled as indicated in the Materials and Methods. Shown is the autoradiogram after several washes at high stringency. **C,** same as in **B,** but after dehybridization and incubation with a FC27-specific probe. Values are in basepairs.

ing the hybrid genes, distinguishing 3D7/FC27 hybrids from FC27/3D7 ones, which otherwise generate similar hybridization profiles,[4] all isolates were typed by nested PCRs using family-specific primers. For a subset, typing was also conducted by hybridization. Concordant results were observed.

Population diversity. The major allelic families described for MSP-1 block 2 and for the central MSP-2 domain were detected in the village both in September and October 1994. In September, 127 MSP-1 fragments were detected in the parasite population, with 60, 59, and eight belonging to the R033, K1, and MAD20 allelic families, respectively. All the R033 fragments were of the same size, and were considered as a single allele. Based on size polymorphism, eight distinct K1 alleles and six MAD20 alleles were observed (Table 3). Of 101 MSP-2 fragments detected in the parasite population in September, 43 were typed as FC27 (nine distinct alleles), 47 belonged to the 3D7 allelic family (15 distinct alleles), and 11 hybrids were detected (nine distinct types). For the GLURP typing, 83 fragments were detected in September, representing nine distinct alleles.

In the October samples, 122 MSP-1 fragments were amplified, with 63, 56, and three belonging to the R033, K1, and MAD20 allelic families, respectively. Here a single R033 allele was also identified. There were 10 distinct K1 and two MAD20 alleles (Table 3). Of 67 MSP-2 fragments detected in the parasite population in October, 31 were typed as FC27 (10 distinct alleles), 26 belonged to the 3D7 allelic family (nine distinct alleles), and 10 hybrids were detected (eight distinct types). For the GLURP typing, 64 fragments were detected, representing nine distinct alleles.

Combining the data from both sets of samples indicated that allelic polymorphism was very large, with 17 distinct MSP-1 block 2 alleles differing by size and/or allelic type, as many as 43 distinct alleles MSP-2 alleles, and nine distinct GLURP alleles.

The prevalence of the individual alleles fluctuated from September to October. For the MSP-1 block 2 locus, eight of 10 and two of six distinct K1-type alleles and MAD20-type alleles were present both in September and October. However, only the minor differences in frequency of the K1-type individual alleles reached statistical significance ($P < 0.05$, by the O'Quigley and Schwartz test). Seven of 12 FC27-type and eight of 16 3D7-type MSP-2 alleles were found both in September and October, but the variation of the prevalence of individual alleles was not statistically significant ($P > 0.05$). The low prevalence of individual hybrid MSP-2 alleles precluded any comparison in both sets of samples. All nine GLURP alleles were observed in both months, with slight but nonstatistically significant variations in the prevalence of individual alleles ($P > 0.05$).

Distribution of alleles by age. The various MSP-1 and MSP-2 allelic families, individual alleles, and the various individual GLURP alleles were similarly distributed throughout all age ranges studied. Figure 2 illustrates the distribution of the various MSP-1 and MSP-2 allelic families in two age groups using the age of 15 years as a cut-off value since above this age, the clinical attack rate progressively decreases in this village.[1] There was no preferential carriage of any allelic family in any age group both in September and in October. The same conclusion was reached

TABLE 3

Distribution of individual MSP-1, MSP-2, and GLURP alleles in the various groups considered for the cohorts recruited in Ndiop in September and October 1994*

	Month of sampling	No. of samples analyzed	MSP-1					MSP-2					GLURP	
			No. of bands	No. of distinct alleles				No. of bands	No. of distinct alleles				No. of bands	No. of distinct alleles
				R033	K1	MAD20			3D7	FC27	3D7/FC27	FC27/3D7		
Total population	Sep 1994	143	127	1	8	6		101	15	9	6	3	83	9
	Oct 1994	125	122	1	10	2		67	9	10	3	5	64	9
Asymptomatic individuals	Sep 1994	87	87	1	8	5		65	10	8	6	2	50	7
	Oct 1994	79	72	1	10	1		38	7	9	1	2	46	7
Clinical malaria	Sep 1994	17	22	1	5	1		19	8	4	1	1	24	9
	Oct 1994	8	10	1	4	0		11	3	5	1	2	10	6

* The number of bands refers to the total number of fragments identified for the locus analyzed. Individual MSP-1 and MSP-2 alleles were identified as indicated in the Materials and Methods. The number of distinct alleles refers to the number of individual alleles identified for the allelic family considered (MSP-1 and MSP-2) and for the GLURP locus. For definitions of abbreviations, see Table 1.

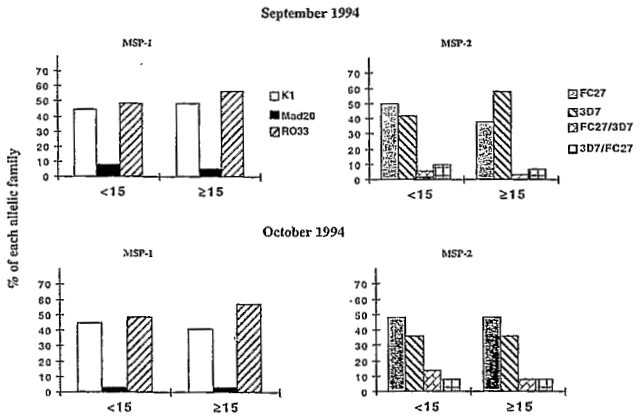

FIGURE 2. Frequency of the merozoite surface protein-1 (MSP-1) and MSP-2 allelic families by age in September and October 1994. There was no statistically significant difference in the age distribution of MSP-1 allelic families in September ($P > 0.5$) or in October ($P > 0.5$). The distribution of the MSP-2 allelic families was not statistically significantly different in both age groups in September ($P > 0.2$) and in October ($P > 0.5$). For both loci, the allelic family distribution in both months was similar ($P > 0.5$).

when analyzing the distribution of individual alleles for the three loci investigated here. Likewise, no association of any individual allele with age was observed when other age groups were considered.

Distribution of alleles in clinical malaria and asymptomatic subjects. We next considered whether there were specific features associated with parasites causing clinical malaria as compared with those carried without symptoms. The parasites infecting febrile individuals with parasite densities > 3,500 trophozoites/μl were compared with those present in asymptomatic individuals (see Materials and Methods for the definition of groups).

As indicated in Table 3, the parasites associated with a malaria attack showed a remarkably large polymorphism. There was no statistically significant difference in the distribution of MSP-1 and MSP-2 allelic families and individual alleles or GLURP individual alleles in samples from those with clinical malaria compared with asymptomatic *P. falciparum* carriers. Some variations were found in the number of different alleles represented in each group, but these were due to the limited number of clinical malaria samples and were not statistically significant.

Infection complexity. The typing reactions analyze single copy loci, and therefore generate only one PCR fragment per haploid blood stage parasite genome. We observed, as in many other endemic regions,[4] numerous samples from which several fragments were amplified, indicating that the isolate contained several distinct parasites. The complexity of the infection was similar in the September and in October 1994 surveys. Table 4 shows that multiple bands were observed in 40–44% of the MSP-1 and MSP-2 PCR-positive samples and in 15–18% of the GLURP PCR-positive samples. When the three typing reactions were combined, 50–52% of the PCR-positive samples were typed as mixed infections. The mean number of fragments per carrier was 1.5 for MSP-1, 1.55–1.6 for MSP-2, and 1.2 for GLURP. Combining the three reactions resulted in an average of 1.75 and

TABLE 4

Complexity of infections genotyped for the MSP-1, MSP-2, and GLURP loci in the various groups considered for the cohorts recruited in September and October in Ndiop*

	Month of sampling	No. of samples analyzed	% of multiple band patterns in PCR-positive isolates				Complexity Mean no. of fragments per carrier			
			MSP-1	MSP-2	GLURP	Combined	MSP-1	MSP-2	GLURP	Combined
Total population	Sep 1994	143	41	43	18	52	1.5	1.6	1.2	1.75
	Oct 1994	125	44	40	15	50	1.5	1.55	1.2	1.65
Asymptomatic individuals	Sep 1994 and Oct 1994	166	41	38	13	48	1.5	1.5	1.2	1.65
Clinical malaria	Sep 1994 and 1994	25	55	64	25	80	1.75	2.15	1.4	2.3

* The complexity was also estimated by combining the three loci. The % of multiple bands refers to the % of individuals typed as carrying more than one band for each locus as indicated and for at least one of the loci when the three typing reactions were combined. The mean number of bands/carrier indicates the average number of distinct alleles detected per polymerase chain reaction (PCR)–positive isolate. Individual MSP-1, MSP-2, and GLURP alleles were identified as indicated in the Materials and Methods. For definitions of abbreviations, see Table 1.

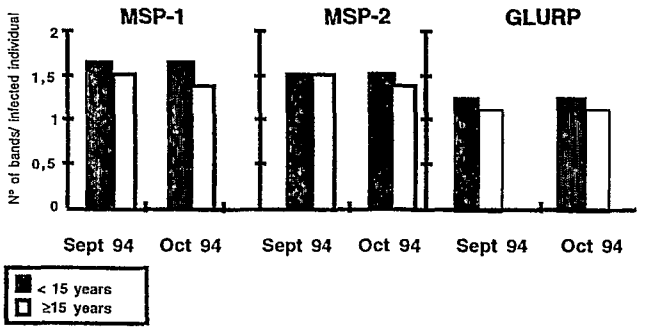

FIGURE 3. Distribution of the complexity of infections detected by merozoite surface protein-1 (MSP-1), MSP-2, and glutamate-rich protein (GLURP) typing by age in Ndiop in September and October 1994 using the age of 15 years as a cut-off value.

1.65 fragments per carrier in September and October, respectively.

In asymptomatic *P. falciparum* carriers (i.e., individuals carrying *P. falciparum* parasites without clinical symptoms, as defined above), 41%, 38%, and 13% of the MSP-1-, MSP-2-, and GLURP-positive samples were typed as containing more than one fragment. As indicated in Table 4, a larger proportion of samples from those with clinical malaria contained multiple genotypes. The three-locus typing indicated 80% of the mixed infections in clinical cases as opposed to 48% in the asymptomatic individuals ($P < 0.01$). Interestingly, the infection complexity was also higher in the clinical cases than in asymptomatic individuals. The mean number of fragments in asymptomatic carriers was 1.5, 1.5, and 1.2 for the MSP-1 block 2, MSP-2, and GLURP typing reactions, respectively, and 1.65 when the three loci were combined. The average number of fragments per clinical malaria sample was higher: 1.75 for MSP-1 (32 fragments/18 positive samples), 2.15 for MSP-2 (30 fragments/14 positive samples), and 1.4 for GLURP (34 fragments/24 positive samples). Combining the three reactions resulted in a an average complexity of 2.3 fragments/clinical case. This figure was statistically different from the complexity of the infection in asymptomatic carriers ($P < 0.05$). In both groups, most multiple infections contained 2–3 MSP-1 or MSP-2 alleles. There was one sample with five distinct MSP-1 alleles and one with four fragments. A maximum of four distinct MSP-2 alleles was detected in two samples. Eighty-one percent of multiple allele carriers harbored parasites belonging to different MSP-1 family types and 76% harbored parasites belonging to different MSP-2 family types. The GLURP typing showed only three carriers with three distinct fragments, with the other mixed infections showing two alleles.

Figure 3 shows that age did not influence the average number of alleles present in asymptomatic individuals for any of the three loci investigated. No difference was found when other age groups were considered.

DISCUSSION

It is now well established that infections in humans are frequently composed of a complex mixture of parasite clones with different genetic and phenotypic characteristics.[4,7,13] The number and relative ratio of the various clones present at any time point vary substantially from individual to individual and for one individual may fluctuate with time. The PCR analysis of parasite infecting asymptomatic individuals exposed to intense transmission showed that the parasite population has a rapid turnover rate.[24,25] This dynamic characteristic of the *P. falciparum* infections complicates the interpretation of the molecular typing at the level of the individual. However, it does not preclude an analysis of the molecular characteristics of the infection at the population level. The purpose of the work reported here was to analyze the parasite population in the village of Ndiop. For that purpose, a substantial fraction of the inhabitants of Ndiop (42% and 36% for the September and October surveys, respectively) was enrolled. The blood samples studied were collected over a short period of time to reduce potential temporal heterogeneity.

In an analysis of the genetic characteristics of parasite populations, the PCR typing methodology provides the advantage of a great sensitivity and specificity, and allows alleles with similar or identical serologic properties to be rapidly distinguished by size polymorphism. The single copy genes MSP-1, MSP-2, and GLURP present a large allelic polymorphism, allowing easy detection of parasite diversity. However, the approach used here underestimates the actual number of distinct alleles present in the peripheral circulation at the time of sampling since alleles with identical size may present sequence differences that remain undetected.[26] It also complicates the measurement of allele frequency since distinct parasite clones carrying the same allelic form, which may coexist in one isolate, are not differentiated in such an analysis. Despite these limitations, the strategy has provided invaluable insights on parasite diversity in field isolates.

The first remarkable result of our analysis is that the parasites infecting inhabitants of Ndiop in September 1994 and/or October 1994 showed a very large polymorphism: 17 different alleles were detected for MSP-1, 43 for MSP-2, and nine for GLURP. As indicated above, this represents a minimal estimate. For MSP-1 and MSP-2 at least (GLURP has not yet been investigated in Dielmo), these figures are similar to those observed in the samples collected during the 1992 transmission season in Dielmo.[9] A recent analysis of a cross-sectional survey conducted in Dielmo in October 1994 indicated that about twice as many MSP-1 and MSP-2 alleles circulated at that time in Dielmo compared with Ndiop (Konate L and others, unpublished data). The large parasite diversity in Ndiop is notable since there was a significant difference in the transmission intensity in both villages. Since every inoculation is preceded by sexual reproduction of parasites in the mosquito, the inoculation rate is predicted to reflect the rate of parasite mating. Sexual reproduction, which generates new genotypes[27–31] and novel alleles by intragenic recombination,[32] is likely to play a significant role in the generation of parasite diversity. The observation that the *P. falciparum* populations of Ndiop and Dielmo have a similar degree of polymorphism indicates that in the range of transmission intensity studied here, a 3–10-fold increase in the transmission intensity does not translate into a parallel increase in allele number. A precise analysis of the influence of transmission intensity on the extent of parasite diversity

is complicated by the marked annual variations of the entomologic inoculation rate and by the differences in the duration of transmission in different endemic areas.[14,15,33] Additional factors such as migration (travel to and from the village) could increase diversity of a parasite population through introduction of foreign alleles. The movements of Ndiop villagers, which are recorded in detail, are limited, and it is thus unlikely that they could have contributed substantially to the high diversity of parasites observed in Ndiop.

In Ndiop, FC27-type and 3D7-type alleles represented 44.5% and 43% of the MSP-2 fragments, respectively. These figures are similar to those reported for The Gambia[7,33] and Papua New Guinea,[8] but differ from those observed in Dielmo.[4,9] In Ndiop, K1-type alleles accounted for 46% of the MSP-1 fragments, Mad20 for 4%, and R033 for 50%. The higher prevalence of R033 and K1 types differs from that reported for samples collected in Dielmo.[9] This suggests considerable heterogeneity in parasite populations, be it geographic or temporal. Indeed, this comparison concerns samples collected in different places but also during different transmission seasons (1994 for Ndiop, this work; 1992 for Dielmo;[4,9] and 1990–1992 for The Gambia[7,33]). The analysis of parasites collected in Dielmo during the same week as the October 1994 Ndiop survey analyzed here showed a striking geographic heterogeneity of parasite populations (Konate L and others, unpublished data).

There were minor fluctuations in the parasite population in Ndiop over the period of the study (as indicated in Table 3), but only the fluctuation in K1-type MSP-1 block 2 alleles reached statistical significance. This might be partly due to a relatively small sample size when one considers the large allelic polymorphism, which was unexpected when this study was designed. In addition, given that rapid turnover of peripheral blood parasites has been shown to occur in asymptomatic individuals during the transmission season,[25,32] some fluctuations in the parasite populations are likely to be observed. Indeed, different parasite genotypes were seen in the paired September and October samples collected from the same 52 persons who remained asymptomatic throughout the study period. Finally, the administration of anti-malarial treatments to 64 individuals in the interval between both surveys is also likely to contribute to changes in allelic frequencies by removing the parasites from the circulation and potentially preventing their transmission. Nonetheless, our results show that the overall genetic characteristics of the parasites circulating in Ndiop were similar in September 1994 and October 1994. This is consistent with the analysis of Conway and others, which indicated very good genetic stability of the MSP-1 and MSP-2 serotypes in The Gambia over several years.[7,34] An analysis of additional cross sectional surveys conducted in Ndiop during the following months is underway to study potential temporal fluctuation in the parasite population in the absence of transmission.

No significant differences were found in the distribution of MSP-1 and MSP-2 allelic families in asymptomatic individuals and clinical malaria cases. This is in contrast to data reported by Engelbrecht, who observed that parasites carrying a FC27 genotype were twice more frequent in symptomatic patients than in asymptomatic carriers living in Papua New Guinea.[35] This discrepancy might reflect geographic variations in host/parasite interactions, different sample size, or spatial heterogeneity in the parasites collected over a large region in Papua New Guinea.

Multiple bands were observed in 41% and 38% of the MSP-1- and MSP-2-positive asymptomatic samples collected in Ndiop. Up to five distinct MSP-1 and four MSP-2 fragments per carrier were observed, with an average of 1.5 MSP-1 and MSP-2 fragments per carrier. This is different from an average of 3.3 MSP-1 and 3 MSP-2 bands per carrier observed in Dielmo.[9] Such differences were also found when comparing complexity in epidemiologic settings with different transmission intensities.[33,35] This indicates that the number of distinct clones harbored by asymptomatic individuals is influenced by the inoculation rate; the higher the inoculation rate, the larger the number of genotypes present in asymptomatic *P. falciparum* carriers at any time. Surprisingly, symptomatic carriage tended to increase the infection complexity. This was statistically significant when comparing the complexity estimated combining the three typing reactions. Multiple bands were observed in 80% of the PCR-positive clinical samples, with a mean number of 2.3 fragments per patient as opposed to 48% of the PCR-positive isolates from asymptomatic carriers, which contained an average of 1.65 fragments. This is different from our observations in Dielmo, where complexity estimated using MSP-2 typing was reduced in symptomatic cases compared with asymptomatic individuals (unpublished data) and from data from Tanzania, where asymptomatic individuals living in hyperendemic areas harbored an average of five distinct MSP-2 genotypes, while there was a mean of 3.4 genotypes in symptomatic cases.[36] It must be noted that the results from Ndiop concern a small number of clinical cases and that asymptomatic individuals in this village usually carry very low parasite densities, which may result in underestimating minor parasite populations undetected by the PCR technique used.

Since a similar technology for analyzing parasite polymorphism and identical protocols for the epidemiologic and entomologic monitoring were used for the studies conducted in Dielmo and in Ndiop, direct comparison of the genetic characteristics of parasites in both villages can be made. We have previously reported that in Dielmo, where inhabitants are exposed to several hundreds of infective bite/year, the average number of clones present in asymptomatic individuals is lower in adults than in children[4] and that MSP-1 block 2 allelic type distribution is influenced by age.[9] Interestingly, these features were not observed for the samples obtained for both surveys in Ndiop, where neither age-dependent variation in allelic frequency nor in infection complexity could be observed. The age-dependent reduction in the complexity and modification of MSP-1 allele distribution observed in Dielmo was interpreted as a reflection of the specific antiparasite immunity acquired at approximately 10–15 years of age in this village.[4,9] The lack of influence of age on complexity or allelic type distribution observed here for Ndiop supports this interpretation since there is no evidence of acquired efficient immunity in Ndiop, where clinical incidence rates show only a slow decrease with age.[1] Thus, the reduction in complexity of infection observed in Dielmo at approximately 10–15 years of age is probably due to immunity resulting from intense exposure to infections by diverse par-

asites and not to aging *per se*. In summary, the parasite populations in Dielmo and Ndiop showed a similar degree of allelic diversity, but the different transmission conditions result in different characteristics of the asymptomatic carriage in both villages.

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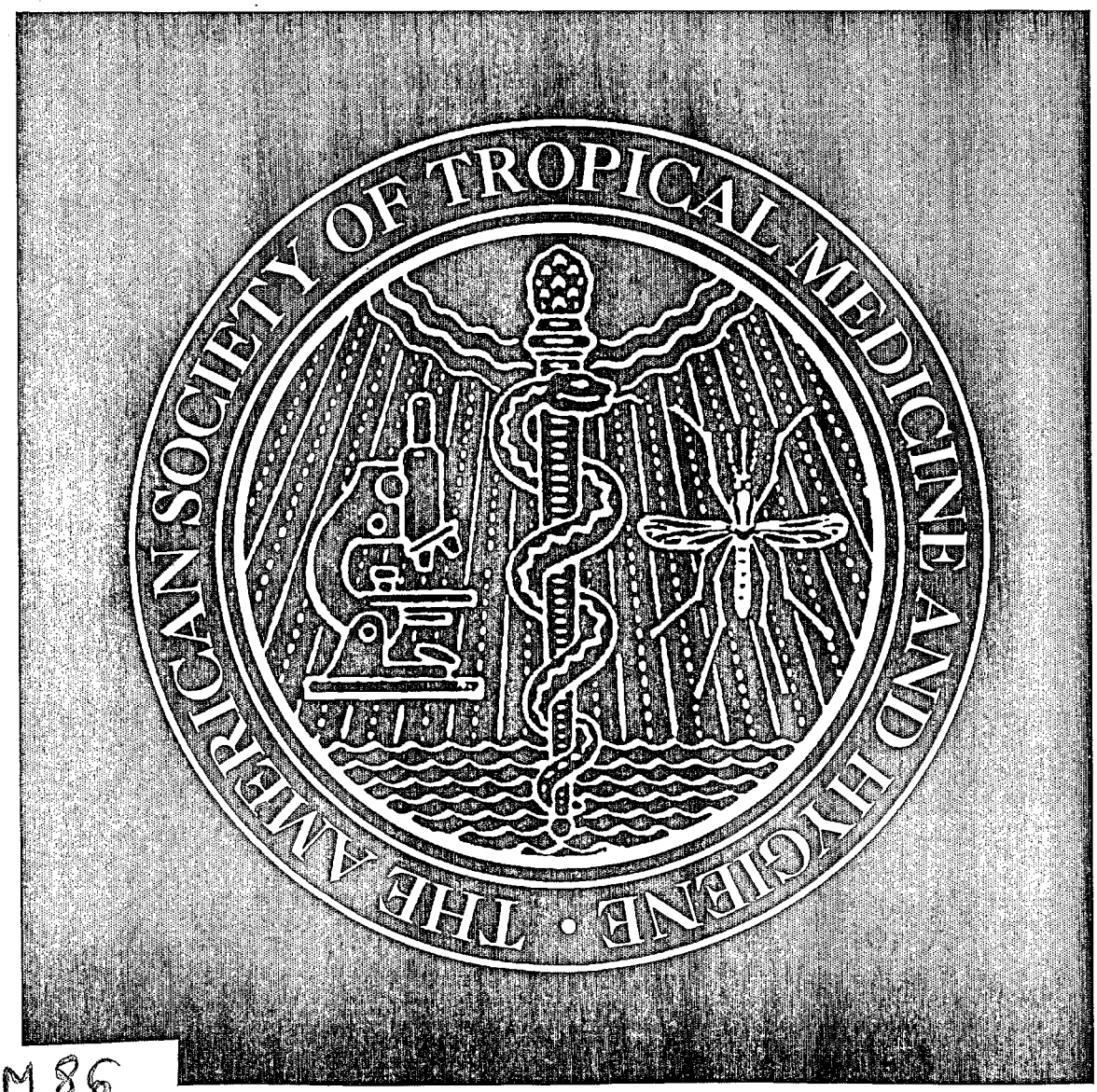

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