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# A cohort study of *Plasmodium falciparum* diversity during the dry season in Ndiop, a Senegalese village with seasonal, mesoendemic malaria

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**Abstract**

Prolonged carriage of *Plasmodium falciparum* in humans during the dry season is critical for parasite survival, as the infected subjects constitute a major reservoir in the absence of transmission. Yet, very little is known about the host/parasite interactions contributing to parasite persistence. In order to study the characteristics of *P. falciparum* infections during the dry season, we have genotyped parasites collected from untreated, asymptomatic individuals during 3 cross-sectional surveys conducted during the dry season in Ndiop, a Senegalese village with seasonal, mesoendemic malaria. Monthly entomological surveillance did not detect any transmission during that period. Parasite prevalence decreased markedly in the children aged < 7 years after 7 months of undetected transmission, but was stable in older children and adults throughout the dry season. In all chronically infected individuals, infection complexity remained stable, but there were substantial fluctuations of individual genotype(s), reflecting complex dynamics of multiple-clone infections during chronic asymptomatic parasite carriage. This fluctuation resulted in changes in the *msp1* and *msp2* allelic distribution within the cohort after 7 months of undetected transmission, contrasting with the stability observed during the preceding rainy season in that village.

**Keywords:** malaria, *Plasmodium falciparum*, genotypes, genetic diversity, *msp1*, *msp2*, Senegal

## Introduction

The immunity acquired by individuals living in endemic areas is a premunition (SERGENT & PARROT, 1935), which efficiently prevents the more or less severe clinical manifestations in *Plasmodium falciparum*-infected individuals and substantially reduces parasite loads, but is not a sterile immunity. The consequence is that asymptomatic infections are frequent. Prolonged parasite carriage is critical for parasite survival, as at the end of the dry season the infected subjects constitute a major, if not the only, reservoir for the subsequent transmission. However, very little is known about the 'armed peace' established between the host and the persisting parasites during this period.

Studies conducted in several endemic areas have demonstrated that *P. falciparum* populations are highly diverse, with a large number of circulating serotypes and serotype combinations (BABIKER *et al.*, 1991; CONWAY & MCBRIDE, 1991; FELGER *et al.*, 1994; NTOUMI *et al.*, 1995, 1997; CONTAMIN *et al.*, 1996; ROBERT *et al.*, 1996; ZWETYENGA *et al.*, 1998; KONATÉ *et al.*, 1999). Recently, DAUBERSIES *et al.* (1996), BABIKER *et al.* (1998) and ROPER *et al.* (1998) analysed the molecular characteristics of persisting infections during the dry season in regions of hypoendemic, unstable malaria. These studies showed a substantial proportion of chronic, asymptomatic infections with fluctuating genotypes in multiply infected hosts. The characteristics of persisting parasite carriage in regions where malaria is more intense, and where individuals progressively acquire a protective immunity, are largely unknown. The present study was undertaken to investigate the age-dependent dynamics of chronic *P. falciparum* carriage at the village level in Ndiop, a Senegalese village, where malaria transmission is moderate and strictly seasonal.

A longitudinal epidemiological and entomological follow-up has been conducted in Ndiop since 1993. The surveillance includes a daily home visit to each person and the permanent presence of a medical team within the village so as to diagnose, record and treat any pathological episode (ROGIER & TRAPE, 1995; TRAPE & ROGIER, 1996). The duration of the dry season is usually 7–8 months. The entomological inoculation rate is monitored all year round, so that precise longitudinal records on transmission intensity are available. These records showed that transmission is strictly seasonal with marked year-to-year variations of both the inoculation rate and the vector species (FONTENILLE *et al.*, 1997). In order to study the characteristics of *P. falciparum* blood-stage infections in this setting, about 40% of the villagers of all age-groups were recruited in a series of sequential cross-sectional surveys conducted from September 1994 to May 1995. Parasites were genotyped using a polymerase chain reaction (PCR)-based methodology investigating allelic polymorphism of 3 single-copy loci: the polymorphic block 2 of the *msp1* gene coding for the merozoite surface protein 1, the central domain of the *msp2* gene encoding the merozoite surface protein 2, and one repetitive domain derived from the *glurp* gene, coding for the glutamic acid-rich merozoite-associated protein (CONTAMIN *et al.*, 1995). The specific sequence and length polymorphism of the regions targeted by the PCRs used is large enough to allow detection of genetically distinct parasites present within a population and to estimate the minimal number of distinct genotypes present in a blood sample. Such an analysis conducted during the 1994 transmission season showed the absence of an age-dependent infection complexity (ZWETYENGA *et al.*, 1998), contrasting with the observations made in holoendemic areas (NTOUMI *et al.*, 1995, 1997; BECK *et al.*, 1997; KONATÉ *et al.*, 1999). A large genetic diversity was observed, with numerous alleles for each locus investigated. Importantly, the *msp1* block 2, *msp2* and *glurp* individual allele frequencies were stable during the transmission season, reflecting circulation of individual genotypes from one individual to the other within the village (ZWETYENGA *et al.*, 1998).

With the aim of investigating the characteristics of chronic parasite carriage during the dry season, we have conducted a similar *msp1* block 2 and *msp2* genotyping of the samples collected from untreated individuals from the same cohort during the subsequent dry season, namely in December 1994, January and May 1995. We have studied here the age-dependence and the dynamics of the individual infections.

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## Materials and Methods

*Study site and blood collection*

Ndiop is a village of about 350 inhabitants situated in Saloum, in the Sahelo–Soudanian region of Senegal. Since 1993, a longitudinal epidemiological and entomological follow-up has been carried out (ROGIER & TRAPE, 1995). The patterns of transmission over 4 consecutive years have been recorded, showing an annual entomological inoculation rate (EIR) ranging from 7 to 63, depending on the year (17 for 1994), and a strictly seasonal transmission where the main vectors are *Anopheles arabiensis* and *An. gambiae* (FONTENILLE *et al.*, 1997). In the period of interest for this work, transmission was monitored through monthly entomological surveys consisting of 12 man-night captures per month. Malaria transmission occurred from August to October 1994, with an average of 7·5, 8·5 and 1 infected bites/person/month, respectively. The 1994 dry season started early November 1994 and lasted until mid-June 1995. No transmission was detected from November 1994 to August 1995. No single mosquito was captured from 6 December 1994 to 15 June 1995.

Finger-prick blood samples were collected from permanent residents of the village. The samples studied here were part of a longitudinal follow-up of 146 individuals (42·5% of the villagers), who donated blood on successive occasions during a 1-year follow-up. There were 52 children aged < 7 years, 41 children aged 7–14 years and 53 aged >15 years. Blood was collected on 12–18 September 1994 (143 individuals), on 10–15 October 1994 (125 individuals), on 13–17 December 1994 (131 individuals), on 9–29 January 1995 (66 individuals), and on 15–20 May 1995 (85 individuals). The age-group distribution was similar for all surveys. Informed consent was obtained from the donors or from the parents. Daily detailed clinical data and treatment courses are available for every subject all through this period. All blood samples were collected and stored as described previously (TRAPE *et al.*, 1994).

*DNA extraction and PCR genotyping*

The DNA was extracted from saponin-lysed, thawed red blood cell pellets with proteinase K, followed by phenol–chloroform extractions, as previously described (CONTAMIN *et al.*, 1995). Then, 2 μL 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, Saint-Quentin en Yvelines, France) 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 previously described (ROBERT *et al.*, 1996). The product(s) of the primary PCR were examined by agarose gel electrophoresis and ethidium bromide staining. The nested family-specific PCRs were carried out with 1 μL of the primary PCR reaction sample or with a diluted aliquot in order to adjust all nested reactions to a similar template amount. 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) as previously described (ZWETYENGA *et al.*, 1998). The products of the genotyping PCR were analysed 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.

*Prevalence, complexity of infection and statistical analysis*

The prevalence was estimated as the percentage of nested PCR-positive isolates within each group considered. A *msp1* block 2 and/or *msp2*-positive reaction was interpreted as an indication for the presence of *P. falciparum* parasites in that sample, without discriminating gametocytes from trophozoites. Assignment of a PCR fragment to a specific allelic family was based on the result of the secondary PCR using family-specific primers. The distribution of the various *msp1* block 2 and *msp2* allelic families was estimated by dividing the total number of fragments assigned to one family detected in the typing reactions by the total number of fragments for the locus considered. Within each allelic family, alleles were differentiated by size polymorphism. Comparison of the distribution of prevalence and multiple infections by age was made using $\chi^2$ tests. Yates' corrections were applied when needed.

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. We have considered here the overall 2 loci complexity of the isolates, using the highest number of bands detected in each carrier (whatever the locus). Complexity of infections was analysed 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, NSW, Australia, 1992) (ZEGER & LIANG, 1986). We used an exchangeable correlation structure in which 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) (ZEGER *et al.*, 1988). We tried to apply an m-dependence correlation structure to model the correlation of one individual observation with the previous one and the next previous one and so on. This did not reveal any deviation from the exchangeable correlation hypothesis.

## Results

P. falciparum *prevalence and infection complexity*

The parasite prevalence, as evaluated using the *msp1* block 2 and *msp2* nested PCR was 75%, 68% and 59% in December 1994, January 1995 and May 1995, respectively (131, 66 and 85 individuals recruited, respectively). This is to be compared to a prevalence of 78% and 82% during the preceding transmission season, in September and October 1994, respectively (143, and 125 individuals recruited, respectively). The decrease in prevalence observed during the dry season was statistically significant ($P < 0·01$).

In order to follow the course of natural infections during the dry season, we excluded from the analysis reported here 31 individuals recruited in this cohort who had received an anti-malarial treatment after the October 1994 survey. Twenty villagers were treated at the end of the rainy season (before the December survey) and 11 persons received antimalarials between January and May 1995. They were all febrile and carried low parasite densities. There were 17 children aged <7 years and 14 aged ≥7 years. All individuals were clinically cured. However, subsequent PCR analysis conducted for this work showed that post-treatment cross-sectional blood samples from 19 of these individuals (11 children aged <7 years and 8 aged ≥7 years) were PCR-positive, a finding similar to the observations reported by BABIKER *et al.* (1998) and ROPER *et al.* (1998). As treatment had altered the 'natural' course of infection, we excluded the treated individuals from the analysis of chronic carriage reported here. The decreased parasite prevalence noted above at the end of the rainy season for the total population was no longer observed for the untreated group. Parasite prevalence was 76%, 61% and 69% in December 1994, January 1995 and May 1995, respectively ($P > 0·3$).

Parasite prevalence by age (<7 years, ≥7–14 years and ≥15 years) is shown in Figure 1A. Prevalence was similar in the 3 age-groups in December 1994. There was no

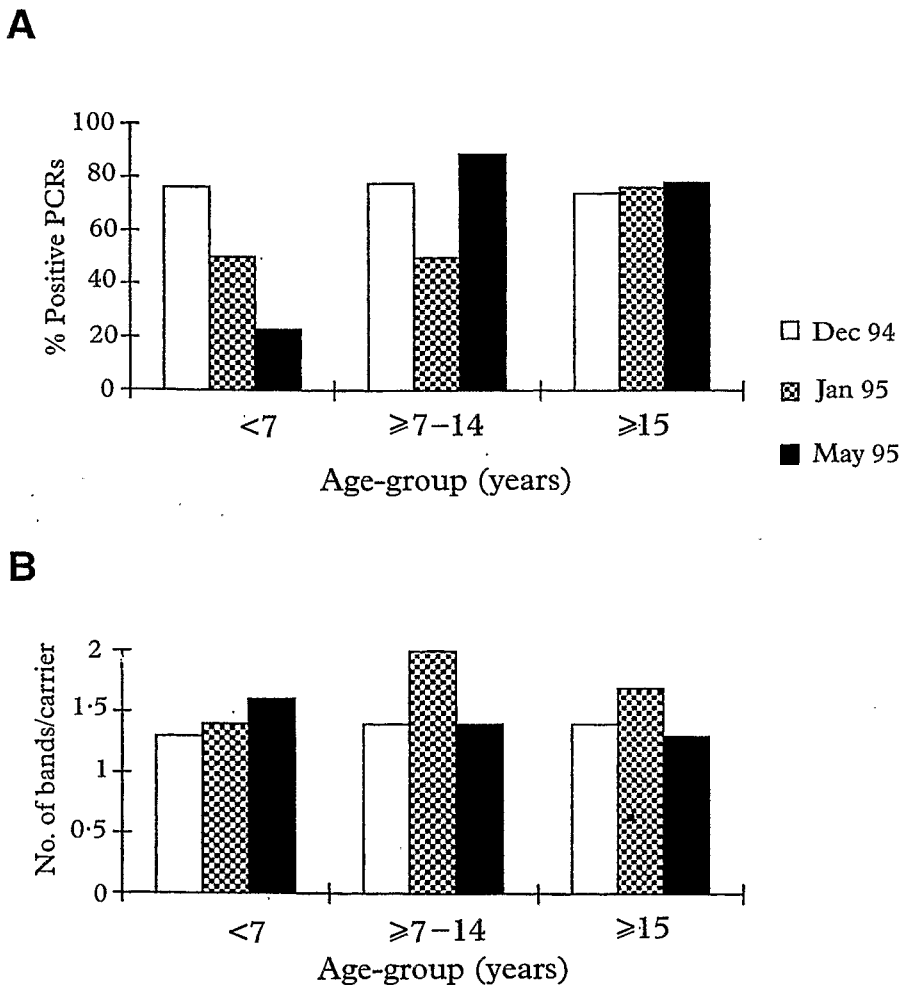


Fig. 1. *P. falciparum* prevalence and infection complexity by age in the untreated asymptomatic individuals recruited in Ndiop (Senegal) from December 1994 to May 1995. The numbers of untreated villagers were 114, 65 and 77 in December 1994, January 1995 and May 1995, respectively. There were 38, 21 and 24 children aged <7 years; 30, 22 and 27 children aged ⩾7–14 years; and 46, 22 and 26 individuals aged ⩾15 years at these time points, respectively. Parasite prevalence (A) and infection complexity (B) were estimated using the *msp1* and *msp2* genotyping.

significant difference in parasite prevalence in the 2 older age-groups for the 3 dry season cross-sectional surveys. However, there was a markedly decreased prevalence in May 1995 in the group aged <7 years. Prevalence was 23% in May as compared to 76% and 50% in this age-group for the December and January surveys, respectively, and as opposed to 89% and 78% for the groups of villagers aged ⩾7–14 years and ⩾15 years, respectively, during the May 1995 survey ($P < 0{\cdot}001$). Thus, there was a specific, marked decrease in prevalence within the untreated, asymptomatic children aged <7 years after 7 months of undetectable transmission.

Figure 1B illustrates the complexity by age in these untreated villagers throughout the dry season. There was no statistically significant difference in isolate complexity in each age-group for the 3 sampling times and between age-groups. A complexity of 1·3–1·6 *msp1* block 2 and/or *msp2* alleles/isolate was observed in the youngest age-group; this figure was 1·4–2·0, and 1·3–1·7 in the *P. falciparum* carriers aged ⩾7–14 years and >15 years, respectively. These data indicate that there was no decrease in complexity with time of interrupted transmission.

*Individual follow-up during the dry season*

Among the untreated individuals sampled during the 3 dry season cross-sectional surveys, blood samples were available for 45 individuals on all 3 occasions, allowing a longitudinal analysis of parasite carriage. As observed by others (BABIKER, 1998), alternating patent and sub-patent infections were detected in many individuals. This is due to the fact that asymptomatic Ndiop villagers usually carry a very low parasite density, which fluctuates above and beneath the detection level (ROGIER & TRAPE, 1995). Table 1 illustrates this phenomenon, showing the fluctuations of PCR patency as estimated using individual reactions or using the combined *msp1* and/or *msp2* reactions. Among the 34 villagers with a *msp1* PCR-positive blood sample in December 1994, 27 had a PCR-positive blood sample in one or more subsequent survey. Seventeen of these were *msp1*-positive in May 1995, as well as 5 villagers with a sub-patent PCR in December 1994. The same fluctuating patency was observed for the *msp2*-based PCR detection. Seven out of the 16 *msp2*-positive subjects in December 1994 still had an *msp2*-positive blood sample in May 1995, whereas 12 additional villagers who had an *msp2*-negative sample earlier had an *msp2*-positive blood sample in May 1995. As several samples had discordant *msp1* vs. *msp2* positivity, combining both reactions provides a more accurate picture of the infection dynamics. Table 1 shows that among the 37 villagers with an *msp1* and/or *msp2* PCR-positive blood sample in December 1994, 24 still had a PCR-positive blood sample in May 1995. Six

**Table 1. Fluctuation of PCR-patent infections during the dry season in the cohort of 45 villagers in Ndiop (Senegal) who did not receive any antimalarial treatment and completed the longitudinal follow-up**

| | Number of isolates genotyped | | |
|---|---|---|---|
| PCR pattern | *msp1* | *msp2* | Combined *msp1/msp2* |
| Dec+ Jan− May− | 7 | 7 | 5 |
| Dec+ Jan+ May− | 10 | 2 | 8 |
| Dec+ Jan+ May+ | 6 | 5 | 19 |
| Dec+ Jan− May+ | 11 | 2 | 5 |
| Dec− Jan+ May− | 2 | 9 | 1 |
| Dec− Jan+ May+ | 2 | 10 | 5 |
| Dec− Jan− May+ | 3 | 2 | 1 |
| Dec− Jan− May− | 4 | 8 | 1 |

individuals with a PCR-sub-patent infection in December were positive later on. Interestingly, 7/14 of the previously positive individuals who had a PCR-negative blood sample in May 1995 were children aged <7 years. In the <7-years group, 7/9 children were PCR negative in May. In contrast, most of the villagers aged >7 years, namely 26/36 (77%), remained infected in May 1995.

The individual longitudinal follow-up of the *msp1* and *msp2* genotypes showed substantial fluctuations of the parasite type hosted by the infected villagers over time. Table 2 shows that fluctuating genotypes were observed for 25 and 19 individuals with longitudinal blood samples successfully typed for *msp1* and *msp2*, respectively. There were 16 individuals who persistently carried the same specific *msp1* allelic form during the study; only 4 of those were single band, 'identical' *msp1* genotype. The other 12 had a persistent allele within an otherwise fluctuating *msp1* profile. Interestingly in 15 of 16 cases with a persisting *msp1* allele, the allele in question was RO33 (for which no size polymorphism was detected in this survey). Only 4 individuals were found with a persisting *msp2* allele within an otherwise complex multiple-band *msp2* pattern. There were 2 cases of a persisting 410 bp FC27 allele, 1 of a persisting 380 bp FC27 type and 1 of a persisting 240 bp 3D7 allele. No single individual hosted the same single band *msp2* genotype throughout the dry season.

Combined genotyping indicated that the villagers with a single identical *msp1* allele throughout the dry season had otherwise distinct *msp2* genotypes, indicating that they carried persistent multiple-clone infections, which happened to have the same *msp1* allelic form. The analysis shown in Table 2 indicated that 31 villagers with complete combined genotyping carried fluctuating

**Table 2. Longitudinal fluctuation of individual allelic *msp1* and/or *msp2* genotypes during the dry season in the cohort of 45 villagers in Ndiop (Senegal) who did not receive any antimalarial treatment and completed the longitudinal follow-up**

| | Locus genotyped | | |
|---|---|---|---|
| PCR pattern | *msp1* | *msp2* | Combined *msp1/msp2* |
| Fluctuating genotypes | | | |
| Total | 25 | 19 | 31 |
| With 1 persistent allele | 12 | 4 | 16 |
| Identical genotype | 4 | 0 | 1 |
| Single-locus positive PCR | 12 | 18 | 13 |
| Negative PCR | 4 | 8 | 1 |

infections; there were 16 individuals with a persisting allelic form. These data show that the individuals with a PCR-positive blood sample after 7 months of undetected transmission had an *msp1* block 2 and/or *msp2* multiple-band PCR profile different from the pattern observed for the earlier survey(s).

These allelic fluctuations resulted in a significant modification of the parasite population as a whole during the dry season. This is illustrated in Figure 2, which shows the distribution of the 3 *msp1* block 2 allelic families (K1, Mad20 and RO33), of the 2 major *msp2* allelic families (3D7 and FC27) and of the 2 *msp2* hybrid allelic families during the period under study. The prevalence of *msp1* and *msp2* allelic families in the May 1995 populations differed significantly from the September and October 1994 populations ($P < 0{\cdot}01$ and $<0{\cdot}01$, respectively).

## Discussion

The work described here investigated the natural course of parasite carriage during the dry season at the village level in a mesoendemic region, where individuals progressively acquire a premunition. As drug treatment obviously interferes with the 'armed peace' described by SERGENT & PARROT (1935), we restricted our analysis to the villagers who had not taken any antimalarial. The design of the longitudinal epidemiological survey is such that drug intake is strictly controlled and regularly monitored by in-vivo drug level measures (TRAPE *et al.*, 1994; ROGIER & TRAPE, 1995). We therefore were in a position to analyse villagers of all age-groups who did not receive or take any antimalarial medication from October 1994 to June 1995 in order to study the infection dynamics and the potential influence of age on parasite persistence.

After 7 months of undetected transmission, the *P. falciparum* prevalence, as estimated by nested PCR, decreased specifically in younger children, whereas it remained stable throughout the period studied in subjects aged ≥7 years. Since PCR obviously has a detection limit and investigates a small volume of blood, we cannot conclude that the children aged <7 years who were PCR-negative in May 1995 had totally cleared their infection but rather that, after 7 months of undetected transmission, young children in this village had a parasite density much lower than older children and adults, resulting in a larger proportion of PCR-negative blood samples. Interestingly, the infection complexity in the few young children who remained infected in May 1995 did not differ from that in the older age-groups (Fig. 1B). In all age-groups, infection complexity was similar to that recorded in asymptomatic individuals whatever the age-group during the preceding rainy season (ZWETYENGA *et al.*, 1998). As the number of infected children studied here is low, a definitive statement on this point awaits confirmation on a larger sample size. At this stage, the present data indicate that the children who became PCR-negative upon prolonged interrupted transmission fended off their multiple-clone infection more efficiently than older children or adults. This raises the intriguing possibility that the mechanisms controlling parasite density in young children might differ from those operating in individuals with a longer history of parasite exposure.

The longitudinal follow-up of individuals showed alternating patent and sub-patent infections over the dry season, as well as fluctuations of parasite types. This is reminiscent of the genotype fluctuations reported for some chronically infected Sudanese individuals (BABIKER *et al.*, 1998; ROPER *et al.*, 1998). The extent of fluctuations however seems larger in Ndiop, as only 1 Ndiop chronically infected villager carried the same genotype throughout the dry season. Persistence of specific individual alleles was readily observed, but this usually occurred within a fluctuating profile. We also detected, as did BABIKER *et al.* (1998), novel alleles

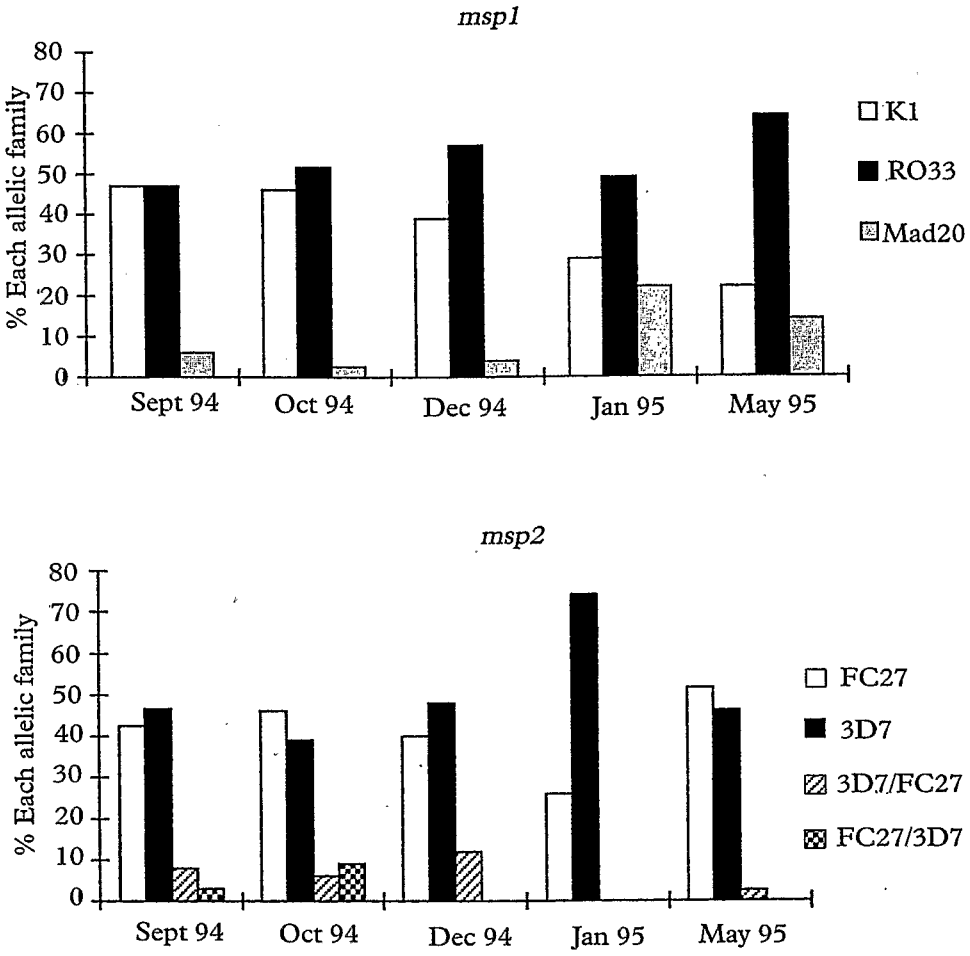


Fig. 2. Comparison of the frequency of the *msp1* block 2 and *msp2* allelic families in the isolates collected in Ndiop (Senegal) from September 1994 to May 1995.

during persistent infections in a proportion of villagers. Therefore, fluctuations in Ndiop seem more pronounced and more generalized than in the Sudanese situation. This difference probably reflects more complex infections in Ndiop due to absence of drug treatment of the villagers studied here and to a higher inoculation rate in Ndiop, where an average of 20 infective bites/year are received by each inhabitant (FONTENILLE *et al.*, 1997), as compared to less than 1 infective bite/person/year in Sudan (BABIKER, 1998).

We think that the fluctuations of parasite genotypes observed during the dry season in Ndiop are unlikely to be due to the inoculation of novel parasite types. No single mosquito was captured from early December 1994 to mid-June 1995. While this obviously does not rule out the possibility of an ongoing low-grade transmission during that period, it excludes the possibility of inoculation of novel parasites to such a large proportion of villagers. This interpretation is substantiated by the observation of a decreased prevalence in younger children, the most susceptible group to novel inoculations. Likewise, the observed fluctuations cannot be attributed to import of novel types due to inoculation outside the village, as the records of villagers' movements exclude such a major impact. Detection of novel genotypes in the absence of recent inoculation is more likely to be due to detection in a blood sample of genotypes that were already present earlier in the individual but which were PCR sub-patent or sequestered at the time of blood sampling. Day-to-day genotype variation, depending on which parasite type was present in the peripheral circulation at the time of blood collection, was reported in subjects exposed to a large number of infective bites in holoendemic areas such as Dielmo (Senegal) (DAUBERSIES *et al.*, 1996) or Tanzania (FARNERT *et al.*, 1997). Under these conditions, the frequent inoculation of novel parasites results in a high turn-over rate, the average period during which a specific genotype was detected being 2 weeks (DAUBERSIES *et al.*, 1996). The situation investigated here is quite different, as novel inoculations are unlikely or at minimum infrequent. We do not know whether the fluctuations observed here reflect stochastic events due to sequential sampling of an infection with fluctuating genotype ratios or whether they indicate a substantial shift in the dominant types during prolonged chronic carriage. This second scenario predicts that persistence of parasites in a semi-immune host would result in a substantial modification of the local parasite population surviving the dry season in humans. If so, the parasites inoculated during the next transmission season should differ from those circulating the year before. Preliminary analysis collected in September 1995, when transmission had resumed, indicated that indeed *msp1* and *msp2* allelic frequencies differ from those observed during the 1994 transmission season.

The fluctuating patent and sub-patent parasitaemia, and the fluctuating genotypes observed during chronic infections here and in Sudan (BABIKER *et al.*, 1998; ROPER *et al.*, 1998), indicate that most infected individuals host more parasite types/clones than detected by the nested PCRs at any given time point and hence that both parasite prevalence and complexity are underesti-

mated by the technical approach used (ARNOT, 1998). More frequent blood sampling could help solve in part this issue, but there are obvious limitations to the sampling regimen, in particular if close clinical and entomological monitoring is carried out for long periods in a community.

The results reported here show a decreased parasite prevalence in younger children after several months of interrupted transmission and predict a reduced anti-disease immunity at the onset of transmission in the younger age-group. This unexpected finding is interesting, as a decreased concomitant immunity might contribute to the higher risk for younger children of developing clinical malaria once transmission and circulation of many strains including novel ones have resumed.

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