The epidemiology of multiple *Plasmodium falciparum* infections

5. Variation of *Plasmodium falciparum msp1* block 2 and *msp2* allele prevalence and of infection complexity in two neighbouring Senegalese villages with different transmission conditions

Lassana Konaté[1]*, Joanna Zwetyenga[1], Christophe Rogier[2], Emmanuel Bischoff[1], Didier Fontenille[3], Adama Tall[2], André Spiegel[2], Jean-François Trape[4] and Odile Mercereau-Puijalon[1]** *[1]Unité d'Immunologie Moléculaire des Parasites, Institut Pasteur, Paris, France; [2]Laboratoire d'Epidémiologie and [3]Laboratoire ORSTOM de Zoologie Médicale, Institut Pasteur, Dakar, Sénégal; [4]Laboratoire de Paludologie, ORSTOM, Dakar, Sénégal*

Abstract

To investigate the impact of transmission on the development of immunity to malaria and on parasite diversity, longitudinal surveys have been conducted for several years in Dielmo and Ndiop, 2 neighbouring Senegalese villages with holo- and mesoendemic transmission conditions, respectively. We analysed *Plasmodium falciparum msp1* block 2 and *msp2* genotypes of isolates collected from 58% of the Dielmo villagers during the same week as those studied recently from Ndiop. Allele frequencies differed in both villages, indicating considerable microgeographical heterogeneity of parasite populations. The complexity of the infections, estimated using individual or combined *msp1* and *msp2* genotyping, in Dielmo was more than double that in Ndiop and it was age-dependent in Dielmo but not in Ndiop. Thus, this study confirmed the influence of age on the complexity of asymptomatic malaria infections in a holoendemic area. The age distribution of complexity in Dielmo substantiates the interpretation that the number of parasite types per isolate reflects acquired antiparasite immunity. This cross-sectional survey also confirms that the sickle cell trait has no impact on complexity but influences the distribution of *P. falciparum* genotypes.

Keywords: malaria, *Plasmodium falciparum*, multiple infection, genotypes, transmission, immunity, sickle cell gene, children, Senegal

Introduction

In inter-tropical Africa, the intensity and duration of malaria transmission show marked geographical variations. Differences in exposure to *Plasmodium falciparum* parasites result in different clinical manifestations of severe malaria (SNOW *et al.*, 1997), different age incidence of disease, and different rates of acquisition of premunition (TRAPE & ROGIER, 1996). Such observations call for the adaptation of control measures to the local context. This, however, necessitates a better understanding of the dynamics of the transmission and disease patterns, as well as of the factors contributing to malaria mortality and morbidity, including local parasite diversity.

Allelic polymorphism, antigenic variation and sexual reproduction (which generates novel gene combinations and hence novel mosaics of characters, including surface serotypes) contribute to inter- and intra-population diversity. It is usually admitted that the long duration required to achieve immune protection in endemic areas reflects the need to develop a progressively enlarging panel of antibody specificities, eventually enabling recognition of numerous serologically diverse isolates (DAY & MARSH, 1991). There is good evidence that, in semi-immune individuals, antigenic diversity is an obstacle to acquired immune defence mechanisms. In experimental malaria in humans, a primary infection by one strain elicited an immune response protecting against that strain but not against infection by another parasite strain (JEFFERY, 1966). The finding that the successive clinical episodes experienced by Senegalese children living in a holoendemic area were associated with the rapid, apparently uncontrolled growth of recently inoculated parasites, the genetic characteristics of which differed in each episode and differed from those that each child carried without symptoms (CONTAMIN *et al.*, 1996), supports the view that parasite diversity is one of the factors which contribute to the occurrence of clinical attacks. The actual targets of the protective immune mechanisms preventing clinical attacks and reducing parasite loads are still obscure, as are the respective roles of immune response to merozoite surface antigens and to red blood cell surface antigens. However, whatever the target antigen, all field studies indicate that 'wild' *P. falciparum* populations are highly diverse and thus that the immune system of individuals living in endemic areas faces numerous serotypes and serotype combinations (FORSYTH *et al.*, 1989; CONWAY & MCBRIDE, 1991; FELGER *et al.*, 1994; NTOUMI *et al.*, 1995, 1997a; ROBERT *et al.*, 1996).

Molecular typing studies conducted recently indicate that numerous parasite types are controlled during asymptomatic infections. Longitudinal analysis of parasites carried by asymptomatic individuals living in holoendemic areas showed a rapid turnover of parasites with different genetic characteristics in the peripheral circulation (DAUBERSIES *et al.*, 1996; FÄRNERT *et al.*, 1997), suggesting that the acquired immunity restricts growth of a large number of parasites with distinct genotypic and phenotypic characteristics. This interpretation is substantiated by the observation that the complexity of infection (number of distinct parasite types/isolate) in a holoendemic area drops at the age when efficient immunity is in place (NTOUMI *et al.*, 1995, 1997a) and by the lack of such an age-dependent reduction in a mesoendemic village where premunition is acquired at a much slower rate (ZWETYENGA *et al.*, in press). A further indication that infection complexity reflects acquired immunity is the negative correlation of number of distinct parasite types with clinical malaria and the reduction of complexity observed in children immunized with the multi-epitope vaccine SPf66 (BECK *et al.*, 1997).

To investigate the impact of transmission on the development of immunity and on parasite diversity, longitudinal surveys have been conducted for several years in Dielmo and Ndiop, 2 neighbouring Senegalese villages with different transmission conditions. In Dielmo, transmission is perennial, due to the presence of a constantly irrigated stream, providing breeding sites all the

Address for correspondence: Odile Mercereau-Puijalon, Unité d'Immunologie Moléculaire des Parasites, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris, France; phone +33 (0)1 45 68 86 23, fax +33 (0)1 40 61 31 85, e-mail <omp@pasteur.fr>
*Present address: Université Cheikh Anta Diop, Département de Biologie Animale, Dakar, Sénégal.
**Present address: Institut de Médecine Tropicale du Service de Santé des Armées, Marseille, France.

year round. The entomological inoculation rate (EIR) during 1990–1996 fluctuated between 89 and 350 *P. falciparum*-infective bites/person/year (TRAPE *et al.*, 1994; FONTENILLE *et al.*, 1997a). In Ndiop, transmission is strictly seasonal, none being detected during the dry season which lasts 6–9 months. The intensity of transmission varied during 1993–1996 from 7 to 63 infective bites/person/year (FONTENILLE *et al.*, 1997b). These distinct transmission conditions (differing in intensity and duration) result in a quite different age incidence of clinical attacks, but little difference in the overall number of clinical attacks during an entire lifetime (TRAPE & ROGIER, 1996).

Transmission intensity is predicted to influence genetic diversity in the parasite population since each mosquito inoculation is preceded by meiosis, possibly generating novel chromosome assortments and new alleles through intragenic recombination in heterozygous zygotes. Parasite diversity has been studied in Dielmo by NTOUMI *et al.* (1995, 1997a) during cross-sectional surveys conducted over 3 weeks in July–August 1992, a period of intense transmission, using polymerase chain reaction (PCR) methodology based on amplification of the *msp1* block 2 and *msp2* central domain (CONTAMIN *et al.*, 1996; ROBERT *et al.*, 1996). It has been analysed recently in Ndiop (ZWETYENGA *et al.*, 1998), using samples collected from 45% and 38% of the villagers during 2 cross-sectional surveys conducted in September and October 1994, respectively. The genetic diversity was very large in both villages. However, the comparison of parasite diversity between villages was limited by the potential confounding effect of time, because these surveys had been conducted 2 years apart. The work reported here was undertaken to provide the basis for a comparison of the extent of parasite diversity and of the molecular characteristics of the infection in both villages at the same time, using a strictly identical methodology for both surveys. For this purpose, we analysed *msp1* block 2 and *msp2* diversity in a cross-sectional survey recruiting 58% of the inhabitants of Dielmo during 10–15 October 1994, the same week that the October 1994 Ndiop cross-sectional survey was conducted by ZWETYENGA *et al.* (1998). The molecular typing carried out on the parasites collected during this second cross-sectional survey from Dielmo also provided an opportunity to compare the populations of Dielmo 2 years apart and to reassess some molecular characteristics of *P. falciparum* carriage observed during the 1992 Dielmo survey, such as age-dependent complexity of the infection (NTOUMI *et al.*, 1995), and age or haemoglobin type-dependent allele distribution (NTOUMI *et al.*, 1997a).

Materials and Methods

Study site

Dielmo (13°45′N, 16°25′W) is situated in the district of Fatick in Senegal, about 280 km from Dakar. The population is 250, with a majority of Serer and Mandinka people. Epidemiology of malaria in Dielmo has been described in detail by TRAPE *et al.* (1994). Transmission is perennial, with marked seasonal and annual variation (TRAPE *et al.*, 1994; FONTENILLE *et al.*, 1997a). In 1994, the average EIR was 120 *P. falciparum*-infective bites/person/year. The EIR in the 2–3 weeks preceding collection of the blood samples studied here was 2·9 *P. falciparum*-infective bites/person/week, compared with 5·7 in the 1992 Dielmo survey (NTOUMI *et al.*, 1997a). Ndiop, 5 km from Dielmo, has a population of 350, with a majority of Wolof and Fula people. Both villages are involved in farming, but there is limited exchange between the villages. The epidemiology of malaria in Ndiop has been described by TRAPE & ROGIER (1996) and FONTENILLE *et al.* (1997b). Transmission is strictly seasonal, with substantial year-to-year variation (FONTENILLE *et al.*, 1997b). In 1994, the average EIR was 17 *P. falciparum*-infective bites/person/year and transmission occurred from August to the end of October. In the 2–3 weeks preceding the October blood collection from the 125 persons examined by ZWETYENGA *et al.* (1998), the inhabitants had received an average of 1·7 *P. falciparum*-infective bites/inhabitant/week.

Recruitment for this study used the same strategy as that for the cross-sectional studies conducted during the same period in Ndiop, namely sampling a substantial proportion of the villagers (58% of the Dielmo population) from all age groups (1–84 years) and selecting inhabitants who had spent >50% of their life time, ≥2 of 3 previous years, and ≥5 preceding months in the village. One hundred and forty-four villagers were included in this study, including 129 asymptomatic individuals. There were 5 subjects with clinical malaria, who received antimalarial medication on the day of blood collection or the next day. Ten additional subjects were not included in the analysis of asymptomatic infections, because they had received a 3 d course of quinine during the previous 8 d and/or were symptomatic (without requiring antimalarial treatment in the previous or next 15 d) on the day of blood donation±3 d. Details of the cohort recruited here and those studied for the 1992 cross-sectional Dielmo survey (NTOUMI *et al.*, 1997a) and the October 1994 Ndiop survey (ZWETYENGA *et al.*, 1998) are given in Table 1.

Table 1. Characteristics of the cohorts studied in Senegal

	Dielmo 1994[a]	Dielmo 1992[b]	Ndiop 1994[a]
No. of subjects			
Total	144	77	125
Asymptomatic for malaria	129	77	79
Aged ≤10 years	50	26	61
Aged >10 years	94	51	64
Haemoglobin AS	19	20	27
Haemoglobin AA	125	57	98
No. of compounds	22	21	23
Monthly entomological infection rate (mean)	25	9	8

[a]October 1994 (Ndiop data from ZWETYENGA *et al.*, 1998).
[b]July–August 1992 (NTOUMI *et al.*, 1997a).

Blood was collected using a capillary tube, centrifuged, and the red blood cell pellet was frozen in liquid nitrogen in the village (without additive) and stored thereafter at –80°C. Informed consent was obtained from the donors or from their parents.

Extraction of deoxyribonucleic acid and PCR genotyping

The strategy used for PCR typing was exactly the same as that used in the cross-sectional 1994 surveys in Ndiop by ZWETYENGA *et al.* (1998), namely a nested PCR consisting of a primary PCR driven by primers derived from conserved flanking regions and a series of secondary PCRs using family-specific primers. In order to allow comparison with the data from Ndiop, both sets of samples were analysed by a standardized method using the same electrophoresis system, cloned alleles serving as internal size and specificity standards, and running a subset of samples from both villages on the same gels to calibrate for determination of the retardation factor (R_F) and calculation of the apparent molecular mass (fragment size).

In brief, the deoxyribonucleic acid (DNA) was extracted from saponin-lysed, thawed red blood cell pellets with proteinase K, followed by phenol/chloroform extraction. The primary PCR was done with 2 μL of DNA (corresponding to 1 μL of blood) as template, amplified as described by ZWETYENGA *et al.* (1998) using the conserved primers A+B and 1+4 for *msp1* and *msp2*, respectively. Family-specific primers were used for the

secondary reactions carried out using a maximum of 1 μL of the primary PCR. For *msp1*, primer pairs specific for each allelic family were used, namely K1+K2, M1+M2 or R1+R2. For *msp2*, the 2 homologous and 2 heterologous primer combinations were used to amplify specifically 3D7 types (A1+A2), FC27 types (B1+B2), or hybrid types (A1+B2 or A2+B1). This strategy allowed allele typing by size polymorphism and family type. The PCR conditions and the sequence of the primers used are described by ZWETYENGA *et al.* (1998). For all reactions, parasite clones, monomorphic parasite lines or cloned PCR fragments were used as positive controls. The size of the PCR products was determined, to detect possible 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.

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 among the overall number of fragments detected for that locus in the group considered. Comparisons of the distribution of *msp1* and *msp2* allelic families by age and by haemoglobin type were made using χ^2 tests. Yates's correction was applied when needed. The distribution of individual alleles was analysed using the O'QUIGLEY & SCHWARTZ (1986) and FLEISS (1981) tests, correcting for multiple comparisons using the correction factor of FISHER (1988). Methods are fully described by NTOUMI *et al.* (1997a).

The complexity of infection 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 samples positive in that reaction in the group under consideration. The complexity of infections was calculated for each typing reaction (*msp1*, *msp2*) independently, as well as by combining both typing reactions, the highest number of bands detected in each carrier (whatever the locus) being used to estimate complexity. Complexity of infection by age group was analysed using the Mann–Whitney *U* test and the non-parametric Kruskal–Wallis test.

Results

Parasite diversity

The October 1994 cross-sectional survey was conducted during a period of intense transmission. Most villagers were infected with *P. falciparum*: 94% and 93% of the 144 samples studied generated an *msp1* and an *msp2* PCR product, respectively. A large number of alleles was detected for each locus: 33 for *msp1* block 2 (19 K1 types, 12 Mad 20 types and 2 RO33 types); these figures were about twice those observed in the 1992 Dielmo survey (Table 2). This probably reflected the fact that the number of isolates studied in the 1994 survey was twice that in the 1992 survey and confirmed that diversity in this village was quite large. The number of alleles observed in the October 1994 Dielmo survey

Table 2. Number of distinct *msp1* and *msp2* alleles of *Plasmodium falciparum* observed in the cross sectional surveys

			msp1					*msp2*			
			No. of alleles					No. of alleles			
Survey	No. of subjects	No. with positive PCR[a]	K1	Mad 20	RO33	Total	No. with positive PCR[a]	3D7	FC27	Hybrids	Total
Dielmo 1994[b]	144	136	19	12	2	33	135	20	11	16	47
Dielmo 1992[c]	77	67	8	5	4	17	60	8	7	7	22
Ndiop 1994[b]	125	81	10	2	1	13	43	9	10	8	27

[a]Polymerase chain reaction.
[b]October 1994 (Ndiop data from ZWETYENGA *et al.*, 1998).
[c]July–August 1992 (NTOUMI *et al.*, 1997a).

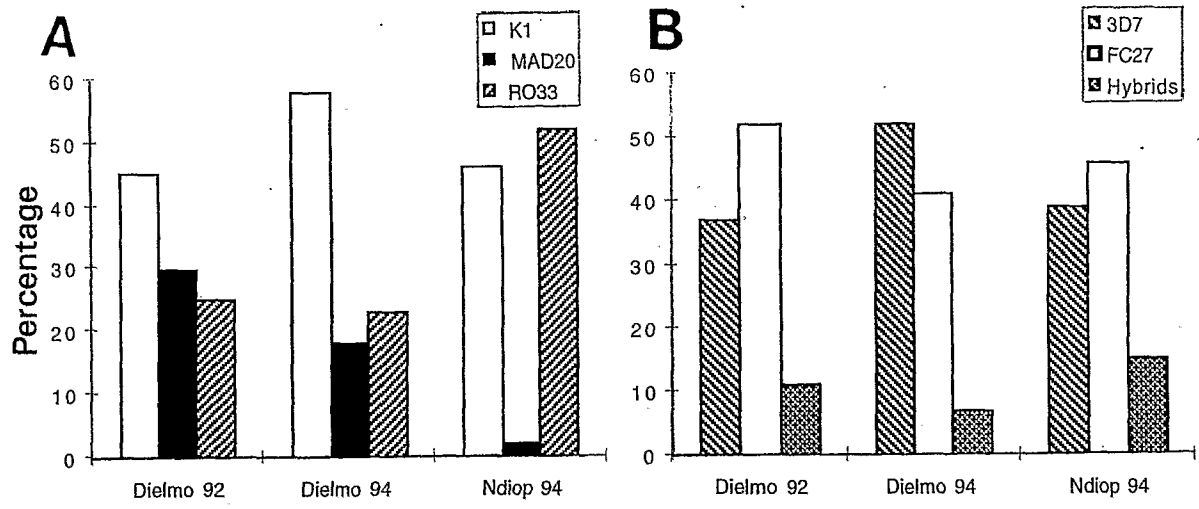

Fig. 1. Comparison of the frequency of (A) the *msp1* block 2 and (B) the *msp2* allelic families in the *Plasmodium falciparum* isolates collected in Dielmo in October 1994 (94) (n=144) and in July–August 1992 (92) (n=77; NTOUMI *et al.*, 1997a) and in Ndiop during the October 1994 survey (94) (n=125; ZWETYENGA *et al.*, 1998). The distribution of the families was statistically significantly different in the 3 surveys. Parasites were typed as described in the Materials and Methods section. Assignment of a fragment to a specific allelic family was based on the result of the secondary nested polymerase chain reaction using family-specific primers.

was approximately twice that observed in the October 1994 survey in Ndiop.

The proportions of individual *msp1* block 2 and *msp2* allelic families in the 1994 isolates were different from those observed in the 1992 survey (Fig. 1). In both series, K1-type *msp1* alleles predominated, but the numbers of Mad 20 types and RO33 types were different ($P<0{\cdot}01$). Similarly, the ratio of 3D7-types to FC27-type *msp2* alleles differed in 1992 and 1994 ($P<0{\cdot}01$). The parasite population in the October 1994 Dielmo survey also differed ($P<0{\cdot}001$) from that examined at the same time in Ndiop (Fig. 1), indicating that different parasite populations were circulating in both villages. This is further illustrated in Figs 2 and 3, which compare the distribution of individual *msp1* and *msp2* bution of the individual 3D7 and FC27 type alleles (Fig. 3), the abundant alleles in one village being minor in the other (e.g., the 380 and 410 bp FC27-type alleles and the 290 and 320 bp 3D7-type alleles, prevalent in Ndiop and barely or not detected in Dielmo).

Complexity of the infections

The vast majority of the October 1994 Dielmo isolates contained more than one *msp1* block 2 or *msp2* fragment; 81% and 80% of the samples contained multiple *msp1* and *msp2* genotypes, respectively (Table 3). Combining the results of *msp1* and *msp2* genotyping showed that 90% of the isolates contained more than one parasite genotype. This very high percentage of isolates with multiple *msp1* and/or *msp2* fragments was

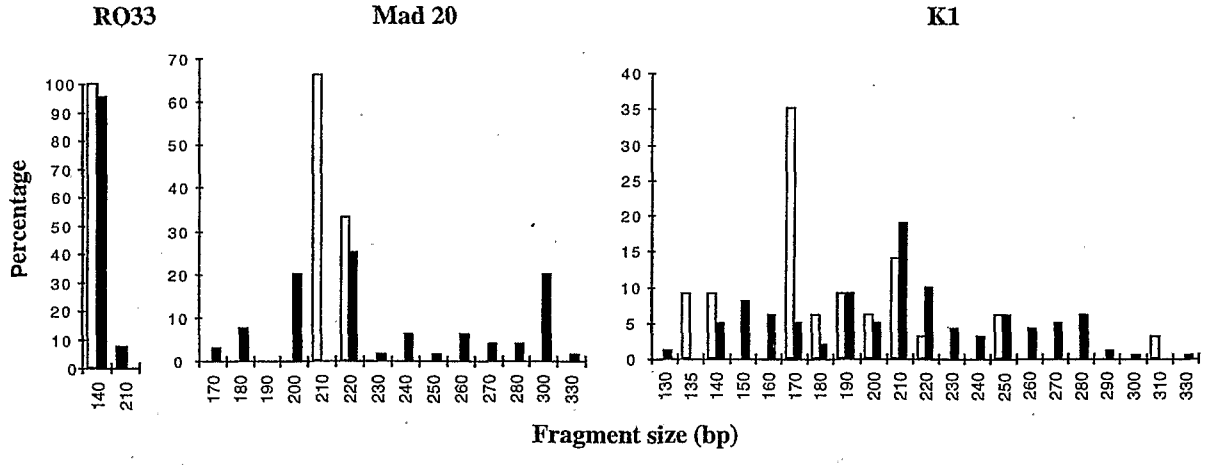

Fig. 2. Comparison of the distribution of individual *msp1* alleles in the October 1994 cross-sectional surveys conducted simultaneously in Dielmo (■) and Ndiop (□). Within each allelic family, alleles were differentiated by size polymorphism. In the Dielmo survey, there were 383 *msp1* fragments, including 244 K1 types, 70 Mad 20 types and 89 RO33 types. In Ndiop there were 112 fragments, including 46 K1 types, 3 Mad 20 types and 63 RO33 types; bp=base pairs.

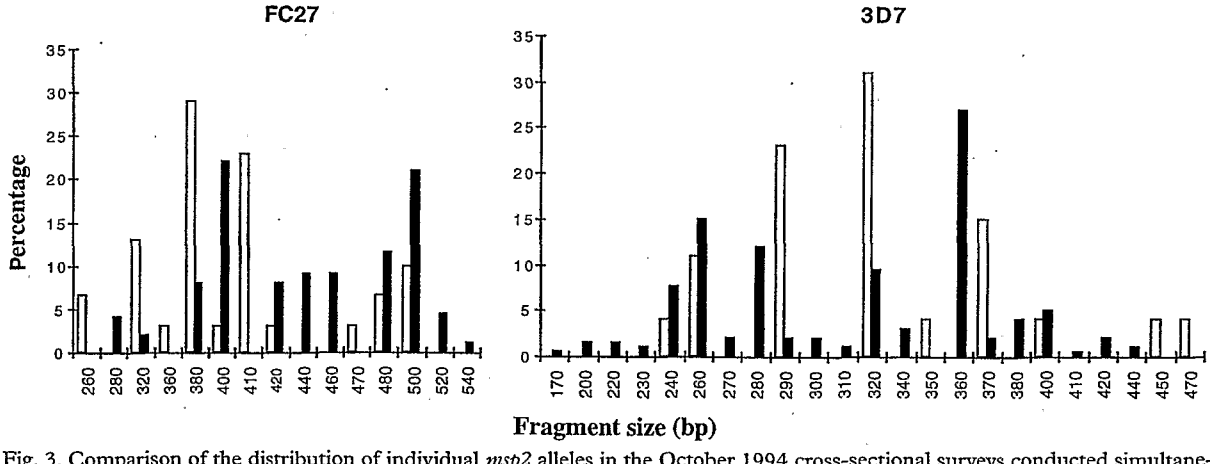

Fig. 3. Comparison of the distribution of individual *msp2* alleles in the October 1994 cross-sectional surveys conducted simultaneously in Dielmo (■) and Ndiop (□). Whithin each allelic family, alleles were differentiated by size polymorphism. In the Dielmo survey, there were 410 *msp2* fragments, including 169 FC27 types, 211 3D7 types and 30 hybrid types. In Ndiop, there were 67 fragments, including 31 FC27 types, 26 3D7 types and 10 hybrid types. The individual hybrid types were not compared in view of their very low frequency.

alleles, respectively, in the October 1994 Dielmo and Ndiop surveys. The distributions of individual K1-type or Mad 20-type alleles in the samples collected during the same week from Ndiop and Dielmo villagers were remarkably different (Fig. 2). In particular, the most frequently detected fragments in one village were almost absent from the other one: e.g., the 210 bp Mad 20 fragment, which represented 70% of the Mad 20 types in the Ndiop isolates, was not detected in Dielmo. Similarly, the 170 bp K1 type, the most abundant K1 type in Ndiop, was a minor allele in Dielmo. A similar conclusion was drawn from the analysis of *msp2* alleles in both villages. There was limited overlap in the distri-similar in both Dielmo surveys but was twice that observed in the October 1994 survey in Ndiop (and also twice that seen in September 1994 in Ndiop, which was very similar to the October 1994 figure; ZWETYENGA *et al.*, 1998).

Calculation of the complexity of the infection (average number of fragments derived from a single copy locus per isolate) in the whole cohort indicated an average of 2·8 and 3·1 *msp1* block 2 and *msp2* fragments per isolate, respectively. This was very similar to the figures observed in the 1992 survey and, as previously noticed (ZWETYENGA *et al.*, 1998), about twice the complexity observed in Ndiop (Table 3). Complexity in Dielmo

Table 3. Complexity of *Plasmodium falciparum* infection in all subjects stratified by haemoglobin type, estimated using individual or combined *msp1* block 2 and *msp2* genotyping

	No. of subjects	*msp1* Positive by PCR[a] (%)	*msp1* Multiple infections (%)	*msp1* No. of bands /carrier[b]	*msp2* Positive by PCR (%)	*msp2* Multiple infections (%)	*msp2* No. of bands /carrier[b]	*msp1+msp2* Positive by PCR (%)	*msp1+msp2* Multiple infections (%)	*msp1+msp2* No. of bands /carrier[b]
Whole cohort										
Dielmo 1994[c]										
Total	144	94	81	2·8 (1–9)	93	80	3·1 (1–12)	100	90	3·4 (1–12)
AA	125	97	79	2·8 (1–9)	94	79	3·1 (1–12)	100	90	3·5 (1–12)
AS	19	79	93	2·7 (1–5)	90	82	2·6 (1–6)	100	89	2·9 (1–6)
Dielmo 1992[d]										
Total	77	87	85	3·3 (1–8)	78	76	3·0 (1–6)	92	92	3·7 (1–8)
AA	57	93	87	3·7 (1–7)	79	82	3·2 (1–6)	95	100	3·8 (1–7)
AS	20	70	71	3·4 (1–8)	75	60	2·2 (1–5)	85	65	3·1 (1–8)
Ndiop 1994[c]										
Total	125	65	44·5	1·5 (1–3)	34	39	1·55 (1–4)	74	53	1·6 (1–4)
AA	98	65	48	1·5 (1–3)	33	38	1·4 (1–3)	74	55	1·6 (1–3)
AS	27	67	28	1·3 (1–2)	40	45	1·9 (1–4)	74	45	1·7 (1–4)

[a]Polymerase chain reaction.
[b]Mean (range in parentheses).
[c]October 1994 (Ndiop data from ZWETYENGA *et al.*, 1998).
[d]July–August 1991 (NTOUMI *et al.*, 1997a).

and Ndiop differed in all the criteria indicated in Table 3. The percentage of multiple infections, the average number of bands per isolate, and the maximum number of bands detected in each isolate were consistently lower in Ndiop than in Dielmo.

The complexity of infection did not differ between individuals with haemoglobin AA or AS in any of the 3 surveys, whatever the criterion used to assess it (Table 3): the percentage of multiple infections, the average number of fragments per isolate, and the range of number of fragments per isolate did not differ significantly in the isolates collected from AA and AS individuals, in both Dielmo and Ndiop.

The influence of age on the complexity of asymptomatic *P. falciparum* infections is illustrated in Table 4. As previously reported, the numbers of both *msp1* and *msp2* fragments were reduced in the older age group of the 1992 Dielmo asymptomatic individuals (NTOUMI *et al.*, 1997a). This contrasts with the infections of asymptomatic Ndiop villagers, in which there was no influence of age on infection complexity (Table 4; ZWETYENGA *et al.*, 1998). Analysis of the 129 asymptomatic *P. falciparum* infections in the Dielmo 1994 survey showed

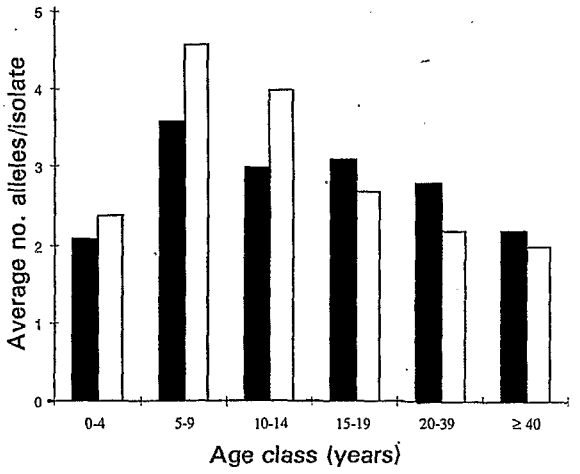

Fig. 4. *Plasmodium falciparum* infection complexity according to age, estimating using the *msp1* (■) and *msp2* (□) genotyping of isolates from 129 asymptomatic individuals recruited in Dielmo in October 1994 (see the Materials and Methods section). The number of individuals in each age class was as follows: 0–4 years, 14; 5–9 years, 25, 10–14 years, 18; 15–19 years, 19; 20–39 years, 22; ≥40 years, 31.

Table 4. Complexity of *Plasmodium falciparum* infection in asymptomatic subjects stratified by age, estimated using individual or combined *msp1* block 2 and *msp2* genotyping

	No. of subjects	*msp1* Positive by PCR[a] (%)	*msp1* Multiple infections (%)	*msp1* No. of bands /carrier[b]	*msp2* Positive by PCR (%)	*msp2* Multiple infections (%)	*msp2* No. of bands /carrier[b]	*msp1+msp2* Positive by PCR (%)	*msp1+msp2* Multiple infections (%)	*msp1+msp2* No. of bands /carrier[b]
Asymptomatic subjects										
Dielmo 1994[c]										
Total	129	96	82	2·8 (1–11)	92	77	3·0 (1–12)	100	89	3·4 (1–12)
≤10 years	41	98	85	3·0 (1–7)	93	82	3·7 (1–12)	100	88	3·8 (1–12)
>10 years	88	93	80	2·7 (1–11)	92	75	2·7 (1–6)	100	90	3·2 (1–11)
Dielmo 1992[d]										
Total	77	87	85	3·3 (1–8)	78	76	3·0 (1–6)	92	92	3·7 (1–8)
<10 years	26	92	96	4·4 (1–8)	88	91	4·0 (1–6)	92	96	4·7 (1–8)
>10 years	51	84	79	2·7 (1–6)	73	67	2·3 (1–6)	92	91	2·8 (1–6)
Ndiop 1994[c]										
Total	79	60	45	1·5 (1–3)	33	31	1·45 (1–4)	70	47	1·55 (1–4)
≤10 years	26	54	50	1·5 (1–2)	30	25	1·5 (1–4)	65	47	1·5 (1–4)
>10 years	53	64	41	1·5 (1–3)	34	33	1·4 (1–3)	72	47	1·6 (1–3)

[a]Polymerase chain reaction.
[b]Mean (range in parentheses).
[c]October 1994 (Ndiop data from ZWETYENGA *et al.*, 1998).
[d]July–August 1991 (NTOUMI *et al.*, 1997a).

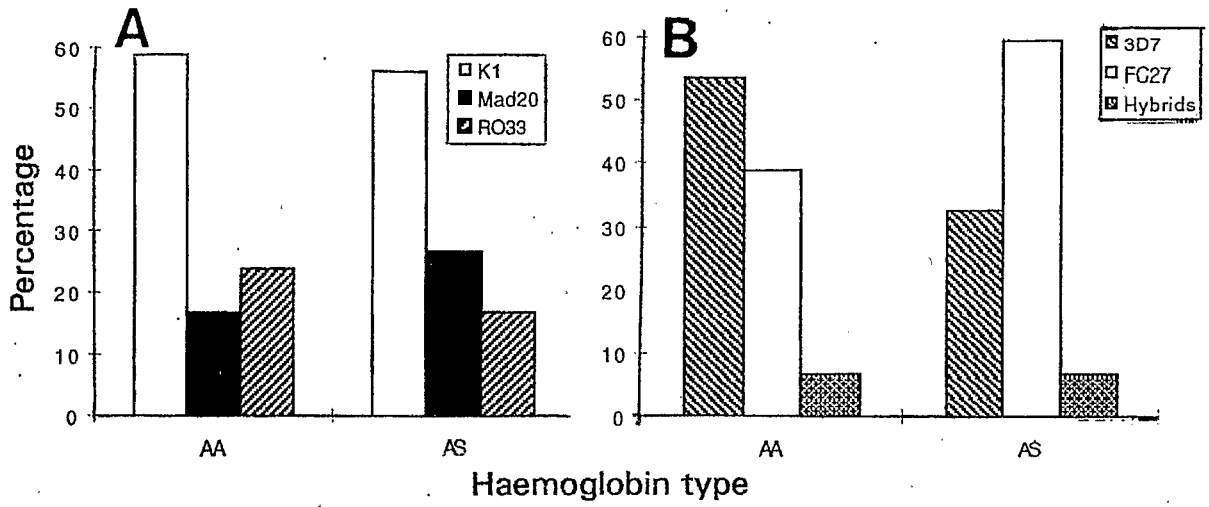

Fig. 5. Frequency of the *msp1* and *msp2* allelic types in 144 subjects recruited in Dielmo in October 1994, according to haemoglobin type (125 AA, 19 AS). Typing of the parasites was done as described in the Materials and Methods section. Assignment of a fragment to a specific allelic family was based on the result of the secondary nested polymerase chain reaction using family-specific primers.

that the average number of *msp1* bands per isolate was slightly lower in the older age group, but this difference was not significant (P=0·53). On the other hand, the *msp2* complexity was influenced by age, with an average of 3·7 and 2·7 *msp2* alleles per isolate in the younger and older age group, respectively (P=0·002). Similarly, the range of number of *msp2* bands detected in each isolate was lower in the older age group, with a maximum of 12 bands in the children and a maximum of 6 in the older individuals. Combined *msp1* and *msp2* genotyping also indicated a reduced complexity in the older age group (P=0·016). Similar conclusions were reached when using the age of 15 years as cut-off (as used by NTOUMI *et al.*, 1995) (data not shown).

Further stratification by age showed an influence of age on complexity. The numbers of both *msp1* and *msp2* genotypes per isolate were age-dependent (Fig. 4) (Mann–Whitney U test, P<0·0003 and P<0·0001, respectively; non-parametric Kruskal–Wallis test, P<0·001 and P<0·0001, respectively). The number of distinct *msp1* and *msp2* genotypes per isolate in the 0–4 years old children was lower than that in the 5–9 or 10–14 years old subjects (Mann-Whitney U test, P<0·005), and of the same order as that in adults. The number of distinct *msp2* genotypes per isolate was significantly larger in the children aged 5–9 and 10–14 years, differing from those in the younger and older age groups (Mann–Whitney U test, P<0·05). The age-dependence of *msp1* complexity was slightly different, the rate being highest in the 5–9 years old children, but it was not statistically significantly different from that for the 10–14 and 15–19 years old individuals. Therefore, the *msp1* complexity in 5–19 years old individuals differed from that in the younger and older age groups (Mann–Whitney U test, P<0·05). Similar results were observed when analysing the entire cohort of 144 subjects independent of the clinical status of the blood donor (data not shown).

Influence of age and haemoglobin type on allele distribution

There was no effect of age on the distribution of *msp1* block 2 or *msp2* allelic families (data not shown), but there was an influence of the haemoglobin type on the distribution of the genotypes. The distribution of the 3 *msp2* types differed in the AA and the AS individuals (Fig. 5) (χ^2=7·58, P=0·022). There was no statistically significant difference in the distribution of the 3 *msp1* allelic families.

The tests of O'QUIGLEY & SCHWARTZ (1986) and FLEISS (1981) were used to analyse the distribution of individual alleles by age or haemoglobin type. There was a trend for a biased distribution of K1-type *msp1* alleles in sicklers (AS) compared to non-sicklers (AA), but this did not reach statistical significance after correcting for multiple comparisons. Because of the large number of alleles, no individual allele distribution by age or by haemoglobin type was statistically significant.

Discussion

This second cross-sectional survey conducted 2 years after the first confirmed the very large genetic diversity of *P. falciparum* parasites in Dielmo. The number of distinct *msp1* block 2 and *msp2* alleles observed among the 144 isolates collected over a 5 d period in October 1994 was large: 33 *msp1* and 47 *msp2* allelic forms were detected. This is twice the number observed for both loci during the 1992 survey, when the sample size was half that studied here. This linear relationship indicates that, though 58% of the villagers were investigated in the October 1994 survey, the sample size was not large enough to include the total parasite population of the village at that time. This suggests a remarkably large parasite diversity in Dielmo, especially as the method used underestimates allelic polymorphism, since alleles of identical size but with point mutations are not differentiated from each other, and since alleles present in a complex blood infection at very low frequency are likely to remain undetected (CONTAMIN *et al.*, 1996).

There were marked differences between the Dielmo parasite populations studied in 1992 and 1994. These differences are difficult to interpret in view of the quinine treatment administered to 119 villagers on 13 August 1992 (a few days after the 1992 survey) and to 105 villagers on 12–18 July 1994 (2 months before the 1994 survey), as part of an experiment investigating the time elapsing between cure and new blood infection (DIEYE *et al.*, 1997). The magnitude of the impact of the drug treatment on the overall parasite population (in both mosquitoes and humans) is uncertain, as some carriers may have already transmitted their infection before being given quinine. An analysis of samples collected in 1994 before drug administration is planned to address this question.

The potential consequences of treatment on temporal fluctuation of allele frequency do not preclude a comparison of the parasite populations present in Dielmo and in Ndiop in October 1994, nor an analysis of some of the molecular characteristics of the infection in Dielmo and the comparison with those observed in Ndiop at the same time. Interestingly the complexity of the infections in the 1992 and 1994 Dielmo surveys was similar. This suggests that the quinine treatment did not have a

major impact on concomitant immunity. The EIR was high in Dielmo during the 2 months between the summer 1994 quinine treatment and blood collection for this study. Transmission was such that each inhabitant is predicted to have received approximately 100 *P. falciparum*-infective bites during that period.

The trend for an influence of the sickle cell trait on *P. falciparum* genotype distribution observed during the 1992 survey has been confirmed here, with some qualification. The analysis of the 1992 isolates indicated an imbalanced distribution of *msp1* allelic families and of some individual *msp1* and *msp2* alleles when comparing AA and AS individuals. In the present study, the distribution of *msp2* allelic families did differ between AA and AS subjects, but there was no statistically significant effect of the sickle cell trait on the *msp1* allelic family distribution or on the distribution of any individual allele. The large number of alleles detected for both loci in this second survey reduced the power of the analysis. In addition, the proportion of AS individuals recruited in the 1994 cohort was low (19 AS and 125 AA) compared to the 1992 cohort (20 AS and 57 AA). The observation for the second time in Dielmo that the distribution of *P. falciparum* genotypes was influenced by the haemoglobin type is particularly interesting in view of the major qualitative differences observed in the parasite population over this period of 2 years and supports the hypothesis of an influence of the haemoglobin type on parasite fitness. Current analysis using additional markers, both involved and not involved in host–parasite interactions at the blood-stage level, and analysis of strain-specific immune responses should help clarify this. This study also confirmed that the haemoglobin type did not influence infection complexity in either Dielmo or Ndiop, contrasting with the observation made in Gabon on AA and AS children by NTOUMI *et al.* (1997b). The reason for this discrepancy is still obscure.

The influence of age on multiplicity of infection in Dielmo, originally estimated using *msp2* genotypes (NTOUMI *et al.*, 1995), was confirmed in this second survey. We extended our previous observations on the complexity of infection in different age groups, showing that the 0–4 years old children had less complex asymptomatic infections than older ones. This is not surprising, as young children are those whose immunity is the least developed and who in addition receive the most drug treatment. The age group with the highest complexity was the 5–9 years old children, who in this village had a risk of clinical malaria substantially lower than that of the 0–4 years old group (TRAPE & ROGIER, 1996). The 10–14 years old children still had a complexity higher than the older age group. Thus, the 5–14 years age group, in which clinical immunity is in place and antiparasitic immunity is being developed, is the group hosting the highest number of different genotypes, consistent with the notion that cumulative exposure to numerous *P. falciparum* types is required to achieve efficient parasite clearance. The picture that emerges is that low complexity of infection in young children reflects their poor immunity, whereas in adults it reflects the efficiency of clearance mechanisms. The interpretation that infection complexity reflects the acquired antiparasite immunity in older groups is consistent with the absence of such age dependence in Ndiop, where the antiparasite immunity is obviously less efficient than in Dielmo.

The main objective of studying parasites collected in Dielmo during October 1994 was to provide a basis for comparison with the parasites collected during that period in Ndiop, which is located 5 km away. We were interested in comparing parasite diversity in both villages and we analysed both sets of samples in parallel, with the same methods. Two important conclusions can be drawn from this analysis. The first is that infection complexity, which was remarkably similar in the 1992 and 1994 Dielmo surveys, was twice as high as that observed in Ndiop. There are 3 indicators of this: (i) the percentage of isolates shown to contain multiple genotypes (53% in Ndiop from combined genotyping vs. 90–92% in Dielmo; see Table 3); (ii) the average number of distinct alleles per isolate (1·6 in Ndiop for combined genotypes compared to 3·4–3·7 in Dielmo); and (iii) the maximum number of bands per isolate, which was also much higher in Dielmo (up to 12 distinct bands) than in Ndiop (maximum 4 bands). The 3 variables were remarkably consistent in both Dielmo surveys, as they were in the September 1994 and October 1994 surveys in Ndiop (ZWETYENGA *et al.*, 1998). Therefore, it looks as if infection complexity in asymptomatic subjects reflects the accumulation of strains which completed the pre-erythrocytic phase and successfully commenced the erythrocytic cycle. A lower number of infective bites translates into a lower number of 'clones' detected later on in the peripheral circulation.

The second noteworthy conclusion is the observation that the parasite populations circulating in Dielmo and in Ndiop at the same time were quite different, with alleles observed in numerous isolates from Ndiop being barely detected in Dielmo and vice-versa. This indicates that deployment of immune interventions such as vaccines necessitates prior appreciation of the diversity of the local parasite population, and that caution should be exercised when conducting comparisons between villages. Such microgeographical heterogeneity is reminiscent of that described in Papua New Guinea by FORSYTH *et al.* (1989), based on village-specific prevalence of S antigen serotypes. The basis for such a microgeographical heterogeneity remains to be determined. The notion that naturally-acquired immunity consists of a series of 'strain'-specific responses, with each individual host mounting his or her own repertoire of responses and thus resulting in a mosaic human population with a heterogeneous success rate for each individual strain, is compatible with the observation of extensive heterogeneity in parasite diversity within and between villages.

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Zonae Torridae Tutamen

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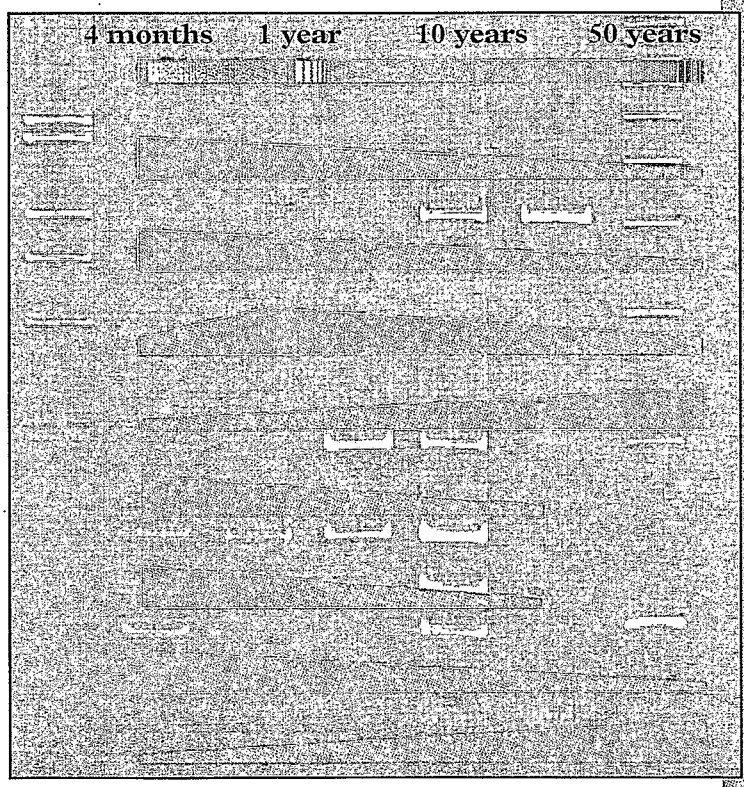

Royal Society of Tropical Medicine and Hygiene,
Manson House, 26 Portland Place,
London, W1N 4EY, UK
Telephone: +44 (0)171 580 2127
Fax: +44 (0)171 436 1389
e-mail: mail@rstmh.org
Web site: http://www.rstmh/org

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