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

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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 28% 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 & Roger, 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 immunity 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 a challenge but not against an inoculated 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 of each child carried without symptoms (CONTAMN 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 that the immune system of individuals living in endemic areas faces numerous serotypes and serotype combinations (Forsyth et al., 1989; Conway & McBride, 1991; Pelgret 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; Farnert 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 lower progression to age-dependent reductions 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 SP66 (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...
year round. The entomological inoculation rate (EIR) during 1992–1994 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 life-time (TRAPE & ROGER, 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 the comparison 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 38% 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 Dielmo survey 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 surveys, 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). 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, 22 of 3 previous years, and 25 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 5 d and/or were symptomatic (without requiring antimalarial treatment in the previous or next 15 d) on the day of blood donation. 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

<table>
<thead>
<tr>
<th></th>
<th>Dielmo</th>
<th>Dielmo</th>
<th>Ndiop</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>144</td>
<td>77</td>
<td>125</td>
</tr>
<tr>
<td>Asymptomatic for malaria</td>
<td>129</td>
<td>77</td>
<td>79</td>
</tr>
<tr>
<td>Aged &lt;10 years</td>
<td>47</td>
<td>26</td>
<td>61</td>
</tr>
<tr>
<td>Aged &gt;10 years</td>
<td>44</td>
<td>31</td>
<td>64</td>
</tr>
<tr>
<td>Haemoglobin AS</td>
<td>19</td>
<td>20</td>
<td>27</td>
</tr>
<tr>
<td>Haemoglobin AA</td>
<td>125</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>No. of compounds</td>
<td>22</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>Monthly entomological infection rate (mean)</td>
<td>25</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

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

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

Extraction of desoxyribonucleic 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 (Rf) and calculation of the apparent molecular mass (fragment size). In brief, the desoxyribonucleic 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 steps followed allele typing by size polymorphism and family type. The PCR conditions and the sequence of the primers used are described by ZWETYENGA et al. (1997a).

The PCR product was visualized under ultraviolet light after being stained with ethidium bromide. Heterologous primer combinations were used to amplify parasite lines (A1+B2 or A2+B1). For each allelic family were used, namely Kl+K2, mSP1, and an mSP2 PCR product, respectively. A large number of alleles was detected for each locus: 19 K1 types, 12 Mad 20 types and 2 R033 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 was applied when needed. The distribution of individual alleles was analysed by 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 NTOMI 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

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

<table>
<thead>
<tr>
<th>Survey</th>
<th>No. of subjects</th>
<th>No. with positive PCR</th>
<th>No. of alleles</th>
<th>No. with positive PCR</th>
<th>msp1</th>
<th>msp2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dielmo 1994b</td>
<td>144</td>
<td>136</td>
<td>19</td>
<td>12</td>
<td>2</td>
<td>33</td>
</tr>
<tr>
<td>Dielmo 1992c</td>
<td>77</td>
<td>67</td>
<td>8</td>
<td>5</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>Ndiop 1994b</td>
<td>125</td>
<td>81</td>
<td>10</td>
<td>2</td>
<td>1</td>
<td>13</td>
</tr>
</tbody>
</table>

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A

![Graph A](image)

B

![Graph B](image)

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**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; NTOMI 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.

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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 haemoglobine type were made using χ² tests. Yates's correction was applied when needed. The distribution of individual alleles was analysed by using the O'QUIGLEY & SCHWARTZ (1986) and FLEISS (1981) tests, correcting
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<0.01). Similarly, the ratio of 3D7-types to FC27-type msp2 alleles differed in 1992 and 1994 (P<0.01). The parasite population in the October 1994 Dielmo survey also differed (P<0.001) 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 alleles, respectively, in the October 1994 Dielmo and 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).

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 similar in both Dielmo surveys but was twice that observed in the October 1994 survey in Ndiop and barely or not detected in Dielmo.

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...
VARIATION OF PLASMODIUM FALCIPARUM ALLELES

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

<table>
<thead>
<tr>
<th></th>
<th>No. of subjects</th>
<th>Positive by PCR (%)</th>
<th>msp1 Multiple infections (%)</th>
<th>No. of bands/carrier</th>
<th>Positive by PCR (%)</th>
<th>msp2 Multiple infections (%)</th>
<th>No. of bands/carrier</th>
<th>Positive by PCR (%)</th>
<th>msp1+msp2 Multiple infections (%)</th>
<th>No. of bands/carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cohort</td>
<td></td>
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<td>Dielmo 1994c</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>144</td>
<td>94</td>
<td>81</td>
<td>2.8 (1-9)</td>
<td>93</td>
<td>80</td>
<td>3-1 (1-12)</td>
<td>100</td>
<td>90</td>
<td>3.4 (1-12)</td>
</tr>
<tr>
<td>AA</td>
<td>129</td>
<td>97</td>
<td>79</td>
<td>2.8 (1-9)</td>
<td>94</td>
<td>79</td>
<td>3-1 (1-12)</td>
<td>100</td>
<td>90</td>
<td>3.5 (1-12)</td>
</tr>
<tr>
<td>AS</td>
<td>19</td>
<td>79</td>
<td>93</td>
<td>2.7 (1-5)</td>
<td>90</td>
<td>82</td>
<td>2.6 (1-6)</td>
<td>100</td>
<td>89</td>
<td>2.9 (1-6)</td>
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<tr>
<td>Ndiop 1994c</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>Total</td>
<td>77</td>
<td>87</td>
<td>85</td>
<td>3.5 (1-8)</td>
<td>78</td>
<td>76</td>
<td>3.0 (1-6)</td>
<td>92</td>
<td>92</td>
<td>3.7 (1-8)</td>
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<tr>
<td>AA</td>
<td>57</td>
<td>93</td>
<td>87</td>
<td>3.7 (1-7)</td>
<td>79</td>
<td>82</td>
<td>3-2 (1-6)</td>
<td>95</td>
<td>100</td>
<td>3.8 (1-7)</td>
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<tr>
<td>AS</td>
<td>20</td>
<td>70</td>
<td>71</td>
<td>3.4 (1-8)</td>
<td>75</td>
<td>60</td>
<td>2.2 (1-5)</td>
<td>85</td>
<td>65</td>
<td>3.1 (1-8)</td>
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</tbody>
</table>

*Polymerase chain reaction.
*bMean (range in parentheses).
*cOctober 1994 (Ndiop data from WETENGA et al., 1998).
*dJuly–August 1991 (NTONUI 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 studies, 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 (NTONUI 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; WETENGA et al., 1998). Analysis of the 129 asymptomatic P. falciparum infections in the Dielmo 1994 survey showed

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

<table>
<thead>
<tr>
<th></th>
<th>No. of subjects</th>
<th>Positive by PCR (%)</th>
<th>msp1 Multiple infections (%)</th>
<th>No. of bands/carrier</th>
<th>Positive by PCR (%)</th>
<th>msp2 Multiple infections (%)</th>
<th>No. of bands/carrier</th>
<th>Positive by PCR (%)</th>
<th>msp1+msp2 Multiple infections (%)</th>
<th>No. of bands/carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic subjects</td>
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<td></td>
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<tr>
<td>Dielmo 1994c</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>129</td>
<td>96</td>
<td>82</td>
<td>2.8 (1-11)</td>
<td>92</td>
<td>77</td>
<td>3-0 (1-12)</td>
<td>100</td>
<td>89</td>
<td>3.4 (1-12)</td>
</tr>
<tr>
<td>&lt;10 years</td>
<td>41</td>
<td>98</td>
<td>85</td>
<td>3.0 (1-7)</td>
<td>93</td>
<td>82</td>
<td>3-7 (1-12)</td>
<td>100</td>
<td>88</td>
<td>3.8 (1-12)</td>
</tr>
<tr>
<td>&gt;10 years</td>
<td>88</td>
<td>93</td>
<td>80</td>
<td>2.7 (1-11)</td>
<td>92</td>
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<td>2.7 (1-6)</td>
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<td>3.2 (1-11)</td>
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<td>Ndiop 1994c</td>
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<tr>
<td>Total</td>
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<td>87</td>
<td>85</td>
<td>3.3 (1-8)</td>
<td>78</td>
<td>76</td>
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<tr>
<td>&lt;10 years</td>
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<td>96</td>
<td>4.4 (1-8)</td>
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<td>92</td>
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<tr>
<td>&gt;10 years</td>
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<td>67</td>
<td>2.3 (1-6)</td>
<td>92</td>
<td>91</td>
<td>2.8 (1-6)</td>
</tr>
</tbody>
</table>

*Polymerase chain reaction.
*bMean (range in parentheses).
*cOctober 1994 (Ndiop data from WETENGA et al., 1998).
*dJuly–August 1991 (NTONUI et al., 1997a).
that the average number of mspl 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 mspl2 complexity was influenced by age, with an average of 3.7 and 2.1 mspl2 alleles per isolate in the younger and older age groups, respectively ($P=0.002$). Similarly, the range of number of mspl2 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 mspl1 and mspl2 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 NTOMI et al., 1995) (data not shown).

Further stratification by age showed an influence of age on complexity. The numbers of both mspl1 and mspl2 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 mspl1 and mspl2 genotypes per isolate in the 0–4 years old children was lower than that in the 5–9 or 10–14 years old children (Mann-Whitney U test, $P<0.005$), and of the same order as that in adults. The number of distinct mspl2 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 mspl1 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 mspl1 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).

**Haemoglobin type**

Fig. 5. Frequency of the mspl and mspl2 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 was a trend for a biased distribution of K1-type mspl 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 mspl1 block 2 and mspl2 alleles observed among the 144 isolates collected over a 5 d period in October 1994 was large: 33 mspl1 and 47 mspl2 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-infected bites during that period.

The influence of the sickle cell trait on P. falciparum genotype distribution observed during the 1993 survey has been confirmed here, with some qualification. The analysis of the 1992 isolates indicated an imbalanced distribution of some individual allelic families and a larger number of alleles detected for both loci in this cohort (20 AS and 57 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, even when the parasite fitness at the blood-stage level, and analysis of strain-specific immune responses should clarify this. The 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., 1992), 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 and in other areas of malaria substantially lower than that of the 0–4 years old group (TRAPE & ROGER, 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 immunity reflects the acquired 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 3·4–5·7 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 1995 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 a more thorough 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 FORSYTT et al. (1993), 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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References


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