HIGH RATE OF MIXED AND SUBPATENT MALARIAL INFECTIONS IN SOUTHWEST NIGERIA

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Abstract. The rate of malarial parasitemia in children and adults was assessed by microscopy and the polymerase chain reaction in a holoendemic area in Nigeria. A high rate of subpatent Plasmodium falciparum parasitemia (19.6%) was found. Plasmodium malariae and P. ovale infections were common in a rural area (26.1% and 14.8%) but were observed sporadically in individuals from an urban area. Simultaneous infections with P. falciparum, P. malariae, and P. ovale were frequent in the urban area (11.7% triple infections). The rate of triple infections was higher than expected from the prevalences of each species ($P < 0.00001$). Spleen enlargement was associated with mixed infections of P. falciparum and P. malariae (odds ratio [OR] = 5.9, 95% confidence interval [CI] 3.0–11.7) and less frequently observed in individuals without detectable parasitemia (OR = 0.06, 95% CI = 0.01–0.3). Spleen enlargement and titers of antibodies to schizonts were positively correlated with parasite densities. The results also suggest that in some individuals a long-lasting subpatent parasitemia might occur.

In areas holoendemic for Plasmodium falciparum infection, clinical malaria affects mostly young children. Adults have acquired a partial immunity after years of successive exposure to a variety of different parasite strains. An anti-disease immunity evolves in early childhood, while an anti-parasite immunity develops at the age of 15–20 years. It is yet unclear to what extent subpatent parasitemia contributes to acquired immunity and whether clinically asymptomatic infections reflect effective parasite control or may eventually convert to manifest disease.

The polymerase chain reaction (PCR) allows the detection of parasitemia below the threshold of conventional microscopy. Re-evaluations of the epidemiology of unstable malaria have now shown that the prevalence of submicroscopic P. falciparum infections is far higher than has previously been expected. Until the PCR came into use it had been difficult to assess correlations of submicroscopic parasitemia with particular features of malarial infections such as spleen size or antibody titers.

Although simultaneous infections with different Plasmodium species have been described in humans, detailed reports of prevalences in holoendemic areas were rare before species-specific PCR assays became available. The extent of interspecies interactions in mixed infections is currently the subject of discussion. In a recent study, it has been suggested that cross-immunity between P. falciparum and P. vivax antigens might occur. However, there is a controversy whether anti-disease- and anti-parasite immunity are in fact species-specific.

In a cross-sectional study in an urban and a rural setting of southwest Nigeria, we determined the rates of simultaneous P. falciparum, P. malariae, and P. ovale infections, including submicroscopic parasitemia. Furthermore, we looked at associations of different plasmodial infections with spleen size and with antibody titers against crude schizont antigen.

PATIENTS, MATERIALS, AND METHODS

Study group. The study was conducted in Ibadan, a town of approximately 4 million residents, and in Abanla, a small community approximately 20 km south of Ibadan in the rain forest zone of southwest Nigeria. Transmission of malaria parasites is intense and stable in this area. Ethical approval for this study was obtained from the Joint Ethical Committee of the University of Ibadan/University College Hospital.

Six hundred ninety-five individuals were enrolled. Subjects with clinical signs of severe malaria were not included in the study. One hundred two individuals from whom either parasite counts or PCR results were not available were excluded from further analysis. The remaining 593 individuals belonged to different subgroups. The first subgroup consisted of 230 children from a nursery and a primary school in Abanla (123 boys and 107 females, age range = 1–11 years). The second subgroup was composed of 59 children from Ibadan recruited from a nursery and a primary school (31 boys and 28 girls, age range = 3–8 years). The third and fourth subgroups were composed of 144 children seen in health centers in Ibadan (83 boys and 61 girls, age range = 0.8–11 years) and 160 healthy adults recruited from students and the staff of the Ibadan University College Hospital (79 males and 81 females, age range = 15–56 years), respectively.

Informed consent was obtained from adults and parents/guardians of children. Blood collection and clinical examination of the children took place during the dry season between December 1996 and May 1997.

Sample processing and parasite count. Two milliliters of venous blood were collected and Giemsa-stained thick films were prepared. Aliquots were used for the determination of hematologic parameters. Serum was stored at 4°C and the remaining blood was preserved with urea (4 M final concentration). In a first step, 100 oil-immersion fields on thick films were examined by microscopy to distinguish negative from positive samples. Samples positive for plasmodia were then subdivided into those with an average of one or less parasites per field and those with an average of more than one parasite per field. Samples found to be negative or to have less than one parasite per field had an additional 100 fields examined by a second investigator.
Extraction of DNA and the PCR. Genomic DNA was extracted from urea-preserved peripheral blood using the DNA-Easy Kit (Invitrogen, St. Louis, MO). The PCRs specific for *P. falciparum*, *P. malariae*, and *P. ovale* were done by nested amplification of a small subunit RNA gene fragment as described elsewhere. Samples differing in the results of parasite PCR and microscopy were re-examined microscopically (100 high-power fields). In addition, DNA was re-extracted and subjected to the PCR up to three times before definitely being classified as negative or positive.

Antibody titer. The titers of IgG antibodies against asexual *P. falciparum* antigens were determined in 547 individuals by indirect immunofluorescence microscopy using goat-derived anti-human globulin (Fluoline G; BioMerieux, San Diego, CA). Air-dried and acetone-fixed monolayers of erythrocytes infected by schizonts of the *P. falciparum* strain pOW (Ivory Coast) from continuous cultures served as antigen.

Clinical examination. Clinical examination of children was performed by a pediatrician of the University College Hospital. Rectal body temperature and body weight were documented. The spleen size was determined by palpation and categorized as being normal or enlarged. Individuals with clinical disease were either treated immediately or admitted to health posts or hospitals.

Statistical analysis. For statistical analysis, chi-square tests and chi-square tests for trend were performed to estimate differences in qualitative variables in the groups. The Kruskal-Wallis one-way analysis of variance was used for nonparametric comparisons. Geometric mean titers (GMTs) were calculated to compare antibody levels in subgroups.

RESULTS

With regard to *P. falciparum* parasitemia and based on the results of microscopy and species-specific PCR-arrays, individuals were divided into four groups (Table 1). Samples negative for *P. falciparum* by both PCR and microscopy were designated as negative (*n* = 187, 31.5% of all samples). Samples negative by microscopy, but positive by PCR were classified as subpatent (*n* = 116, 19.6%). The category low included those samples with an average of one parasite or less than one parasite per field (*n* = 142, 23.9%) and the category moderate included those with more than one parasite per field (*n* = 145, 24.5%). A specific PCR failed to amplify specific DNA fragments in three samples in which more than one *P. falciparum* parasite per field was observed. These samples were excluded from further analysis.

### Table 1

<table>
<thead>
<tr>
<th>Parasitemia level group</th>
<th>PCR</th>
<th>Microscopy</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Negative*</td>
<td>Negative</td>
<td>187</td>
</tr>
<tr>
<td>Subpatent</td>
<td>Positive</td>
<td>Negative</td>
<td>116</td>
</tr>
<tr>
<td>Low</td>
<td>Positive</td>
<td>1 P/F†</td>
<td>142</td>
</tr>
<tr>
<td>Moderate</td>
<td>Positive</td>
<td>&gt; 1 P/F†</td>
<td>145</td>
</tr>
<tr>
<td>Not classifiable‡</td>
<td>Negative</td>
<td>Positive</td>
<td>3</td>
</tr>
</tbody>
</table>

* One sample only *Plasmodium malariae* positive, one sample only *P. ovale* positive.
† P/F = parasites per high-power field.
‡ Excluded from further analysis.

### Table 2

|                  | Observed (O) | Expected (E) | (E - O)² | *P*
|------------------|--------------|--------------|----------|-----
| Pf only          | 323          | 331.6        | 0.2      | 0.673 |
| Pm only          | 41           | 20.4         | 10.0     | 0.0001 |
| Po only          | 1            | 11.2         | 10.8     | 0.0001 |
| Pf + Pm.         | 61           | 43.9         | 0.2      | 0.562 |
| Pf + Po.         | 12           | 24.2         | 6.2      | 0.011 |
| Pm + Po.         | –             | 1.5          | 1.5      | 0.981 |
| Pf + Pm. + Po.   | 27           | 3.2          | 177.0    | 0.00001 |
| Negative         | 185          | 153.9        | 6.3      | 0.0001 |

* Pf = Plasmodium falciparum; Pm = Plasmodium malariae; Po = Plasmodium ovale; – = not detected; χ² = 219.1, degrees of freedom = 7, *P* = 0.00001.

Infection rates. Four hundred three individuals (68.3%) were infected with *P. falciparum*, 69 (11.7%) with *P. malariae*, and 40 (6.8%) with *P. ovale*. In 185 subjects (31.4%), no parasites or parasite DNA were detected. Mixed infections with all three species were found more frequently (4.6%), while single (55.1%) or double (9.0%) infections were observed less frequently than expected from the prevalences of each of the species (χ² = 219.1, *P* < 0.00001) (Table 2). Only one *P. malariae* and one *P. ovale* infection were found without concomitant *P. falciparum* parasitemia.

Positivity rates of *P. falciparum* in children were comparably high in the rural area of Abanla (82.2%) and in Ibadan (school children = 79.7%, children from health centers = 74.6%) (Table 3). In contrast, marked differences in the infection rates of the other species were observed. *Plasmodium malariae* was found in 26.1% and *P. ovale* in 14.8% of the Abanla children. These species were identified only rarely in individuals from Ibadan. In Abanla, mixed infections were more frequent and exclusive infection with one species was less frequent than expected (χ² = 85.9, *P* < 0.00001). The adults from Ibadan had a lower prevalence of *P. falciparum* infections than children (38.4%). *Plasmodium malariae* was observed only in mixed infections together with *P. falciparum*, and *P. ovale* was found in one monoinfection and in two double infections.

Age dependence of species prevalences. In asymptomatic children from Abanla, prevalences of all plasmodial species increased with age (Figure 1). The rate of *P. falciparum* parasitemia increased almost linearly from 2 to 13 years (χ² trend = 13.9, *P* < 0.0005), whereas the occurrence of *P. malariae* (χ² trend = 67.3, *P* < 0.00001) and of *P. ovale* (χ² trend = 22.6, *P* < 0.00001) increased sharply, beginning at the age of five years. Accordingly, the rate of mixed infections increased and the parasite negativity rate decreased continuously with age. In children between 7 and 13 years, the prevalence of *P. malariae* was 76.3% and that of *P. ovale* was 39.5%. All children 7–13 years of age were found to be infected with plasmodia.

Parasitemia and antibody titer. Antibody titers were also age-dependent (*P* < 0.0001, by Kruskal-Wallis test). In the group with subpatent parasitemia, the GMT was between that of the negative and the low parasitemia groups. In children less than 6 years of age, antibody titers increased with the parasite density (*P* < 0.003, by Kruskal-Wallis test).
TABLE 3
Frequency of single and mixed infections in different subpopulations*

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Abanla n=230</th>
<th>Abanla n=95</th>
<th>Ibadan Health post n=142</th>
<th>Adults n=159</th>
<th>Adults n=159</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf. only</td>
<td>123 53.5%</td>
<td>42 71.2%</td>
<td>102 71.8%</td>
<td>56 35.2%</td>
<td></td>
</tr>
<tr>
<td>Pm. only</td>
<td>1 0.4%</td>
<td></td>
<td>4 2.8%</td>
<td>3 1.9%</td>
<td></td>
</tr>
<tr>
<td>Po. only</td>
<td></td>
<td></td>
<td>3 5.1%</td>
<td>2 1.3%</td>
<td></td>
</tr>
<tr>
<td>Pf. + Pm.</td>
<td>32 13.9%</td>
<td>2 3.4%</td>
<td>4 2.8%</td>
<td>3 1.9%</td>
<td></td>
</tr>
<tr>
<td>Pf. + Po.</td>
<td>7 3.0%</td>
<td>3 5.1%</td>
<td></td>
<td>2 1.3%</td>
<td></td>
</tr>
<tr>
<td>Pm. + Po.</td>
<td></td>
<td></td>
<td>3 5.1%</td>
<td>2 1.3%</td>
<td></td>
</tr>
<tr>
<td>Pf. + Pm. + Po.</td>
<td>27 11.7%</td>
<td></td>
<td></td>
<td>2 1.3%</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>40 17.4%</td>
<td>12 20.3%</td>
<td>36 25.4%</td>
<td>97 61.0%</td>
<td></td>
</tr>
</tbody>
</table>

* Pf. = Plasmodium falciparum; Pm. = Plasmodium malariae; Po. = Plasmodium ovale.

whereas in older children and adults, a slight decrease in titers was observed in the group with moderate parasitemia.

A total of 15.6% of all individuals without plasmodia had negative or borderline titers (Figure 2). In contrast, 13.5% of the subpatent parasitemia group, 3.6% of the low parasitemia group, and 1.6% of the moderate parasitemia group had negative or intermediate antibody titers ($\chi^2$ trend = 21.7, $P < 0.00001$). The proportion of higher antibody levels increased constantly from the negative parasitemia group to the moderate parasitemia group. Again, titers of the subpatent parasitemia group were between those of the group with negative parasitemias and those with low parasitemias.

**Parasitemia and spleen size.** Spleen enlargement was positively associated with parasite density ($\chi^2$ trend = 14.7, $P < 0.0005$). An enlarged spleen was found in 2.4% of non-infected children and in 7.5% of children with subpatent parasitemias (Figure 2). The proportion of individuals with enlarged spleens was highest in the two groups with microscopically detectable parasitemia (low = 20.6% and moderate = 18.5%).

A total of 51.9% of individuals with only *P. falciparum* infections had enlarged spleens (odds ratio [OR] = 0.7, 95% confidence interval [CI] = 0.4–1.3). Splenomegaly was strongly associated with additional infections by *P. malariae* or both of the other species (Table 4). Analysis revealed a higher OR of enlarged spleens in children with double infections of *P. falciparum* and *P. malariae* (OR = 5.9, 95% CI = 3.0–11.7) compared with those with triple infections (OR = 2.5, 95% CI = 1.9–4.9). Individuals without detectable parasitemias had a reduced risk of splenomegaly (OR = 0.06, 95% CI = 0.01–0.3).

**DISCUSSION**

The objective of this study was to estimate the prevalence of subpatent and mixed malaria infections by combined microscopy and PCR in subgroups in Nigeria and to determine differences of the antibody response and spleen size.

The sensitivity of *Plasmodium* species-specific PCR-assays depends on the primer design and differs from one locus to another. Because the PCR assay used in our study is designed to amplify a single copy gene fragment, an additional nested PCR was applied to increase sensitivity. The
sensitivity of that nested PCR has previously been studied in a limited dilution assay, detecting 2.5 parasites/µl of blood.21 The detection threshold of microscopy is approximately 10–20 parasites/µl.12 Thus, one can conservatively estimate a parasitemia of approximately 2.5–20 parasites/µl in individuals with subpatent infections.

In three parasite carriers identified by microscopy, parasite DNA could not be amplified by the nested PCR assay. In these cases, parasitemia was unambiguously higher than one parasite per field. A certain lack of concordance between microscopy and the PCR has previously been described.11 Mutations at the primer annealing sites of the parasite gene could be responsible for this observation.

*Plasmodium falciparum* and *P. ovale* infections were markedly more frequent in the rural area than in the urban area. This confirms findings in adolescents from the same area of Nigeria.13 In contrast to that study, however, our results show similar rates of *P. falciparum* infection in children from the urban and rural settings. The prevalence of plasmodia in adults from Ibadan was lower than that among children in the same area. Similar rates of infections in adults have also been reported in other cross-sectional and longitudinal studies from holoendemic areas in Africa.15

In our study population, mixed infections with *P. falciparum, P. malariae*, and *P. ovale* were common. The latter two were almost exclusively found concomitantly with *P. falciparum*. Double and triple infections were found more frequently than expected from the prevalences of each parasite species. This is in agreement with findings of others, but contradicts reports on lower rates of mixed infections in areas of high malaria transmission.17,18

It has been suggested that the course of an infection might be influenced by the simultaneous occurrence of several plasmodial species.19–21 Higher than expected prevalences of mixed infections have been explained by immunosuppressive effects caused by chronic *P. falciparum* infection and have also been attributed to differences in the individual exposure.7 Animal models indicate that the presence of a preceding infection with one *Plasmodium* species might be responsible for increased susceptibility to other *Plasmodium* species.22,23

A smaller rate of mixed infections than expected could be caused by interspecies interactions such as cross-specific immunity or natural resistance.24 Splenic enlargement caused by one parasite species inhibiting the replication of a second parasite could be such a mechanism.25 Intake of chloroquine for infections with chloroquine-resistant *P. falciparum* strains could also lead to higher prevalences of single *P. falciparum* infections. So far, it is not well defined which parasite- and host-dependent factors determine the risk of mixed infections and if the simultaneous occurrence of different species might be beneficial or promote disease.

In agreement with other findings, high prevalences of submicroscopic *P. falciparum* parasitemia identified only by the PCR were found. Reports on seroprevalences of plasmodial infections have also indicated that asymptomatic infection rates are high during the transmission season.27 Another study has shown that even in a region with unstable malaria, *P. falciparum* infections below the threshold of microscopic detection occur frequently.1 This supports the view that low parasitemia detectable only by the PCR is not an artifact caused by residues of a past infection and confirms the findings showing that only viable parasites can be recognized by the PCR.28,29

It has been suggested that antibodies against crude schizont antigens are not protective since high titers were not correlated with low parasite densities.30,31 In other studies, titers of antibodies against erythrocytic stages were found to be associated with the extent and duration of parasitemia.32,33 In our study population, antibody levels were also positively associated with the levels of parasitemia. The correlation of antibody titers with distinct levels of low-grade parasitemia could result from longer periods of that particular degree of parasitemia. With regard to the lack of protection conferred by these antibodies, higher degrees of antibody titers would then reflect the extent and duration of parasitemia prior to the current analysis.

Splenic enlargement was found in approximately 20% of children with microscopically detectable parasitemia, a rate lower than expected in holoendemic areas,34 but similar to that of previous studies in Nigeria.35 The rate of splenomegaly was lower in subjects with subpatent and lowest in those without parasitemia. Although *P. falciparum* infection is considered to cause spleen enlargement in endemic areas,33 we did not find a significant association of an exclusive *P. falciparum* infection with spleen size. Our data showed a strong association of simultaneous *P. falciparum/P. malariae* infections with splenomegaly. Because virtually all *P. malariae* infections occurred together with *P. falciparum* it cannot be determined whether *P. malariae* alone or the combined infection is the cause of this association.

Our cross-sectional study has the limitation of classifying individuals based on the point prevalence of an infection. It is known that in holoendemic areas, every individual is subjected to repeated malaria infections,6 and that premunition can exist in more than 90% of subjects.25 It is not clear whether subpatent parasitemia reflects control of infection or is an accidental finding of parasitemia that fluctuates during a chronic infection. A recent study has demonstrated daily dynamics of *P. falciparum* subpopulations in asymptomatic children, whereby in some individuals parasite densities varied below and above the detection threshold of microscopy.36 This would imply that each child, at different times, could have been classified in different parasite level groups. However, the associations of spleen size and antibody levels with parasitemia contradict that assumption and rather reflect a more constant and long-lasting condition.
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