Transmission-Dependent Tolerance to Multiclonal Plasmodium falciparum Infection

Anna Färnert,1,4 Thomas N. Williams,4,5 Tabitha W. Mwangi,4 Anna Ehlin,2 Greg Fegan,4,6 Alex Macharia,4 Brett S. Lowe,4 Scott M. Montgomery,2,3,7 and Kevin Marsh4,5

1Infectious Diseases Unit and 2Clinical Epidemiology Unit, Department of Medicine Solna, Karolinska Institutet, Stockholm, and 3Clinical Research Centre, Örebro University Hospital, Örebro, Sweden; 4Kenya Medical Research Institute/Wellcome Trust Programme, Centre for Geographic Medicine Research–Coast, Kilifi, Kenya; 5Nuffield Department of Medicine, John Radcliffe Hospital, Oxford, and 6Infectious Disease Epidemiology, Department of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, and 7Department of Primary Care and Social Medicine, Imperial College, London, United Kingdom

Whether the number of concurrent clones in asymptomatic Plasmodium falciparum infections reflects the degree of host protection was investigated in children living in areas with different levels of transmission on the coast of Kenya. The number of concurrent clones was determined on the basis of polymorphism in msp2, which encodes the vaccine candidate antigen merozoite surface protein 2. In a low-transmission area, most children had monoclonal infections, and diversity did not predict a risk of clinical malaria. In an area of moderate transmission, asymptomatic infections with 2 clones were, compared with 1 clone, associated with an increased risk of subsequent malaria. In a comparative assessment in a high-transmission area in Tanzania, multiclonal infections conferred a reduced risk. The different nonlinear associations between the number of clones and malaria morbidity suggest that levels of tolerance to multiclonal infections are transmission dependent as a result of cumulative exposure to antigenically diverse P. falciparum infections.
high-transmission settings, the highest diversity is, however, found in school-age children, who are relatively protected against clinical malaria; this suggests that patterns of immunity are more complex [4, 12]. Whether the number of concurrent clones in asymptomatic \textit{P. falciparum} infections reflects the degree of protection is not fully established. Among continuously exposed individuals, those who harbor few clones may be expected to have the most efficient immunity. In contrast, several studies have shown that multiclonal infections are associated with a reduced risk of malaria [13–16], whereas other studies have come to the opposite conclusion [17–19]. These conflicting findings may partly be attributed to age and transmission. Comparisons between studies are, however, restricted by differences in design and in molecular and data analyses.

With a view toward establishing the effect that transmission has on the diversity of \textit{P. falciparum} infections and the degree of host immunity, we studied the relationship between number of clones and malaria morbidity in 2 groups of children living in areas with different levels of transmission on the coast of Kenya [20]. The number of clones—determined by genotyping the highly polymorphic domain of \textit{msp2}, which encodes the vaccine candidate antigen merozoite surface protein 2 (MSP2) [21]—was investigated in relation to the risk of malaria in a cross-sectional survey with longitudinal follow-up. The study design allowed comparisons with a recent assessment conducted in a setting of high endemicity in Tanzania, in which an association between the number of clones and susceptibility to disease was found [16].

\textbf{METHODS}

\textbf{Study area and population.} The study sites, Ngerenya and Chonyi, are located 20 km north and south of the town of Kilifi on the coast of Kenya. Malaria transmission is stable, with a long rainy period from May through July and a short period in November. Ngerenya has low transmission, with 10 infective bites/person/year [22], and Chonyi has moderate transmission, with 22–53 infective bites/person/year [23]. Two cohorts of individuals have been followed since 1998 in the respective areas. The project was approved by the Kenya Medical Research Institute National Ethical Review Committee. Written informed consent was obtained from the guardians of all participants.

\textbf{Cross-sectional survey.} In October 2000, venous blood was collected from 1523 individuals, of whom 779 were <11 years old. Samples were available for DNA extraction from 748 children, 368 from Chonyi and 380 from Ngerenya.

\textbf{Surveillance of malaria morbidity.} Clinical episodes of malaria were continuously recorded by active and passive surveillance. All children were visited weekly by a fieldworker. In addition, the participants were encouraged to seek treatment whenever they fell ill. In the case of an axillary temperature \(\geq 37.5^\circ\text{C}\) or a history of fever, a blood smear was analyzed by microscopy. Children with fever and \textit{P. falciparum} parasites were treated, free of charge, with sulphadoxine-pyrimethamine, according to the national policies. Children with signs of severe malaria were admitted to Kilifi District Hospital.

\textbf{Case definition.} For the purpose of the study, clinical malaria was determined using 2 separate definitions: (1) fever (temperature \(\geq 37.5^\circ\text{C}\) or reported hot body) and the detection of \textit{P. falciparum} parasites and (2) fever and the detection of \(>2500\) \textit{P. falciparum} parasites/\(\mu\text{L}\) (a more specific definition for malaria in this setting) [20]. The observation period was 18 May 1999 to 26 August 2001.

\textbf{Asymptomatic status.} To clearly define children who were asymptomatic at the time of blood sampling, individuals with fever (with or without parasites) at the time of or within 1 week after the survey and afebrile children with \(>10,000\) parasites/\(\mu\text{L}\) were excluded. Because of the prophylactic effect of sulphadoxine-pyrimethamine [24], children treated within 4 weeks before the survey were excluded. This was considered a conservative approach, because the number of clones may be affected by ongoing disease [19, 25] and recent antimalarial treatment.

\textbf{Microscopy.} The presence of \textit{Plasmodium} parasites was investigated by light microscopy of Giemsa-stained thick and thin smears. Parasite densities were enumerated against 200 leukocytes, estimating 8000 leukocytes/\(\mu\text{L}\), or, if available, against measured leukocyte counts; in high-density samples, parasite densities were enumerated against 500 erythrocytes, estimating \(5 \times 10^4\) erythrocytes/\(\mu\text{L}\).

\textbf{msp2 genotyping.} Genomic DNA was extracted from packed erythrocytes (500 \(\mu\text{L}\)) by means of a QiaAmp Blood Mini kit (Qiagen), adding 100 \(\mu\text{L}\) of elution buffer. The polymerase chain reaction (PCR) method included initial amplification of the outer \textit{msp2} domain, followed by 2 separate nested reactions with primers targeting the 2 allelic variants, FC27 and IC [21]. PCR was performed in a total volume of 20 \(\mu\text{L}\) that included 1 \(\mu\text{L}\) of DNA, with 55 cycles in total and an annealing temperature of 58°C. The length of PCR products was determined by electrophoresis on 2% MetaPhor agarose gels (BMA), followed by ethidium bromide staining and UV transillumination. The number of \textit{msp2} alleles (FC27 plus IC alleles) determined the number of clones per infection.

Although in strict terms the term \textit{clone} indicates parasites derived from a common ancestor, it is here used to describe a parasite population with the same genotype. The true genetic complexity is, therefore, likely to be underestimated, and figures show the minimum number of clones.

\textbf{Statistical analysis.} Analyses were performed by means of SPSS (version 14; SPSS). Data were categorized to avoid assumptions about linearity, as follows: for number of clones, 0, 1, 2, 3, and \(\geq 4\); for parasite density, microscopy negative and
Table 1. Malarientropic Characteristics in 2 Areas of Kilifi District with Different Rates of Transmission of *Plasmodium falciparum*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Chonyi, moderate transmission&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ngerenya, low transmission&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All (n = 368)</td>
<td>Asymptomatic&lt;sup&gt;c&lt;/sup&gt; (n = 264)</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (95% CI)</td>
<td>5.36 (5.03–5.69)</td>
<td>5.66 (5.27–6.05)</td>
</tr>
<tr>
<td>Range</td>
<td>0.13–10.98</td>
<td>0.13–10.98</td>
</tr>
<tr>
<td>Female sex</td>
<td>180 (48.9)</td>
<td>131 (49.6)</td>
</tr>
<tr>
<td>Malaria episodes/person/year, mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever and presence of parasites</td>
<td>1.66 ± 1.69</td>
<td>1.19 ± 1.12</td>
</tr>
<tr>
<td>Fever and &gt;2500 parasites/µL</td>
<td>1.19 ± 1.45</td>
<td>0.82 ± 0.94</td>
</tr>
<tr>
<td>No episode&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever and presence of parasites</td>
<td>50 (19.1)</td>
<td>27 (9.9)</td>
</tr>
<tr>
<td>Fever and &gt;2500 parasites/µL</td>
<td>81 (30.9)</td>
<td>37 (13.6)</td>
</tr>
<tr>
<td>Parasite prevalence, % (95% CI)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>By microscopy</td>
<td>39.3 (34.2–44.4)</td>
<td>33.7 (28.0–39.5)</td>
</tr>
<tr>
<td>By PCR</td>
<td>47.0 (41.9–52.1)</td>
<td>36.7 (31.6–43.4)</td>
</tr>
<tr>
<td>Parasite density&lt;sup&gt;f&lt;/sup&gt;, parasites/µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>40–270,000</td>
<td>80–9920</td>
</tr>
<tr>
<td>Geometric mean</td>
<td>2238</td>
<td>1274</td>
</tr>
<tr>
<td>msp2 genotyping</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR positive, no.</td>
<td>173</td>
<td>97</td>
</tr>
<tr>
<td>&gt;1 allele</td>
<td>126 (72.8)</td>
<td>68 (70.1)</td>
</tr>
<tr>
<td>&gt;3 alleles</td>
<td>82 (47.4)</td>
<td>48 (49.5)</td>
</tr>
<tr>
<td>Mean (95% CI)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>2.84 (2.57–3.11)</td>
<td>3.01 (2.62–3.39)</td>
</tr>
</tbody>
</table>

**NOTE.** Data no. (%) of subjects, unless otherwise specified. CI, confidence interval; PCR, polymerase chain reaction; SD, standard deviation.

<sup>a</sup> Entomological inoculation rate of 22–53 infective bites/person/year [23].

<sup>b</sup> Entomological inoculation rate of 10 infective bites/person/year [22].

<sup>c</sup> Excluding individuals with an ongoing malaria episode at the time of the survey or who received antimarial treatment 4 weeks previously.

<sup>d</sup> Fever and reported hot body.

<sup>e</sup> No. of children who did not have any recorded episodes of malaria during the total observation time.

<sup>f</sup> Only among children who were microscopy positive.

<sup>g</sup> Mean no. of msp2 genotypes in PCR-positive children only.

PCR negative, microscopy negative and PCR positive, and microscopy positive into quintiles of their distribution; for age, 0–1.99, 2.00–4.99, 5.00–7.99, and 8.00–10.99 years; and for number of malaria episodes, 0, 1–2, 3–4, 5–6, 7–9, and ≥10. Logistic regression was used to assess associations in the cohorts for parasite prevalence, parasite densities, gametocytes, number of clones, and episodes. Odds ratios (ORs) were adjusted for age and sex. Association between number of clones and parasite density was analyzed by linear regression.

Association between the number of clones and malaria risk was investigated in strictly asymptomatic individuals. Cox regression was used to assess the time to the nearest episode before and after the survey, respectively. Hazard ratios (HRs) were adjusted for age, sex, and parasite density. Additional analyses included adjustments for time to previous episode and stratification of children into ≤5 and >5 years old. The risk of malaria was further analyzed by Poisson regression assessing the number of episodes during 6-month period before and after the survey, respectively. Incidence rate ratios (IRRs) were adjusted for age and sex. Association between the number of clones and the total number of episodes during the observation period was analyzed by multinomial logistic regression, using 1–2 episodes for comparison.

**RESULTS**

The present study included 748 children (0–10 years old) who participated in a cross-sectional survey in October 2000; 368 were from Chonyi, which has moderate transmission, and 380 were from Ngerenya, which has low transmission (table 1). At the time of the survey, 25 children had an ongoing episode of fever and detection of *P. falciparum*, 7 children developed a clinical episode during the following week, 161 children had an episode of fever and detection of *P. falciparum* with antimarial treatment within 4 weeks before the survey, and 15 children had parasite densities >10,000 parasites/µL. Three children had missing data. In total, 264 children from Chonyi and 273 from Ngerenya fulfilled the criteria of being asymptomatic.

**Parasite prevalence and densities.** The parasite prevalence by microscopy was 37.5% in Chonyi and 24.7% in Ngerenya.
Figure 1. Number of asymptomatic children 0–10 years old infected with different numbers of msp2 genotypes in a cross-sectional survey conducted in Ngerenya, which has a low level of transmission (n = 273), and in Chonyi, which has a moderate level of transmission (n = 264).

All samples were analyzed by PCR of the msp2 domain, resulting in detection of P. falciparum in an additional 62 children. In 30 microscopy-positive samples, the PCR assay failed to detect parasites despite duplicated analyses. Together, the 2 methods established a parasite prevalence of 49.0% and 31.6% in the Chonyi and Ngerenya groups, respectively. Among the asymptomatic children, 36.7% were positive by PCR in Chonyi, and 16.8% were positive by PCR in Ngerenya.

Parasite densities were higher among asymptomatic children in Chonyi (geometric mean ± 2 standard deviations [SDs], 1274 ± 2457 parasites/μL) than in Ngerenya (geometric mean ± 2 SDs, 558 ± 1612 parasites/μL), with more densities in the second to fifth quintiles of the distribution (ie, >240 parasites/μL) in Chonyi (P = .001). Gametocytes were detected in 43 children (5.7%), of whom 29 were asymptomatic; the frequency of gametocyte detection did not differ between the groups. Other Plasmodium species were distinguished in 29 children (3.9%), 18 in Chonyi and 11 in Ngerenya; 28 were P. malariae infections and 8 were P. ovale infections, of which 16 were mixed with P. falciparum.

**msp2 diversity.** Multiple msp2 genotypes (ie, clones) were detected in 70.1% of the asymptomatic children with parasitemia in Chonyi, compared with 41.3% in Ngerenya (figure 1). The mean number of clones was 3.01 (95% confidence interval [CI], 2.62–3.39; range, 1–9) in Chonyi and 1.70 (95% CI, 1.36–2.03; range, 1–6) in Ngerenya. Because the number of clones was not normally distributed, frequencies were used for comparisons (table 2). Infections with ≥3 clones were more prevalent in Chonyi (49.5%) than in Ngerenya (13.0%). The number of clones was positively associated with parasite density.
Table 2. Number of msp2 Clones (msp2 Genotypes) of Plasmodium falciparum in 2 Areas with Different Rates of Transmission

<table>
<thead>
<tr>
<th>No. of clones</th>
<th>Chonyi (Subjects, no. (%))</th>
<th>Ngerenya (Subjects, no. (%))</th>
<th>Unadjusted OR (95% CI)</th>
<th>P</th>
<th>Adjusteda OR (95% CI)</th>
<th>P</th>
<th>Adjusteb OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29 (29.9)</td>
<td>27 (58.7)</td>
<td>1.00 (reference)</td>
<td></td>
<td>1.00 (reference)</td>
<td></td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>20 (20.6)</td>
<td>13 (28.3)</td>
<td>0.70 (0.29–1.67)</td>
<td>.420</td>
<td>0.75 (0.31–1.84)</td>
<td>.530</td>
<td>0.59 (0.22–1.58)</td>
<td>.298</td>
</tr>
<tr>
<td>3</td>
<td>12 (12.4)</td>
<td>2 (4.3)</td>
<td>0.18 (0.04–0.87)</td>
<td>.034</td>
<td>0.19 (0.04–0.95)</td>
<td>.043</td>
<td>0.17 (0.03–1.00)</td>
<td>.049</td>
</tr>
<tr>
<td>≥4</td>
<td>36 (37.1)</td>
<td>4 (8.7)</td>
<td>0.12 (0.04–0.38)</td>
<td>&lt;.001</td>
<td>0.12 (0.04–0.37)</td>
<td>&lt;.001</td>
<td>0.13 (0.04–0.46)</td>
<td>.002</td>
</tr>
<tr>
<td>Total</td>
<td>97 (100)</td>
<td>46 (100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Adjusted for sex and age.

b Adjusted for sex, age, and parasite density.

(regression coefficient β, 0.173 [95% CI, 0.05–0.30]; P = .013). Also, after adjustment for parasite density, the number of clones was higher in all age groups in Chonyi (P = .049).

The highest number of clones was found in the 5.00–7.99-year-old group in both cohorts (figure 2). Among the 25 children with ongoing fever and detection of parasites, the mean number of clones was 2.83 (95% CI, 2.13–3.54) in Chonyi and 2.30 (95% CI, 1.34–3.26) in Ngerenya, which were significantly different from those among the asymptomatic children. Gametocyte prevalence was not associated with number of clones. Children infected with multiple species had a high number of P. falciparum clones (mean, 3.58), as has been previously noted [14]. Notably, the only 2 children from Chonyi who were concurrently infected with 3 species (P. falciparum, P. ovale, and P. malariae) had 7 clones.

Of the 2 dimorphic types of msp2, IC alleles were detected in 88.7% of asymptomatic infections in Chonyi and in 86.9% in Ngerenya, and FC27 alleles were found in 69.1% and 41.3%, respectively (figure 2). Infections composed of both types were more prevalent in Chonyi (57.7%) than in Ngerenya (28.3%). In Ngerenya, children with ongoing fever and detection of parasites at the survey had more FC27 types (75.0%).

**Malaria morbidity.** Malaria morbidity was higher in Ngerenya than in Chonyi (mean ± SD, 2.89 ± 2.59 vs 1.66 ± 1.69 episodes per person-year; mean ± SD, 2.30 ± 2.22 vs 1.19 ± 1.45 episodes of fever and >2500 parasites/μL per person-year). The number of episodes was higher for all age groups in Ngerenya except for the 0–1.99-year-old group, in which the frequency was higher in Chonyi (0.83 vs 1.01 episodes per year). In Ngerenya, 46.9% had ≥5 episodes of fever and detection of parasites over the study period, compared with 18.2% in Chonyi (age-adjusted P < .001), and 55.7% and 25.4% had ≥3 episodes of fever and >2500 parasites/μL, respectively (age-adjusted P < .001). The mean number of episodes for children who were excluded due to ongoing or recent malaria was 4.78 (SD, 3.27) in Ngerenya and 2.87 (SD, 2.22) in Chonyi. To allow comparison with a recent study in Tanzania [16], fever included temperature ≥37.5°C and reported hot body, explaining the higher morbidity found here than in previous reports in which only measured temperature was used [20].

**Association between number of clones and malaria episodes with treatment before the survey.** In Chonyi, children infected with ≥3 clones had, compared with children infected with 1 clone, a longer time to a previous episode of fever and detection of parasites (adjusted HR, 0.40 [95% CI, 0.17–0.94]; P = .035). Time to a malaria episode with >2500 parasites/μL was longer in children infected with ≥4 clones (adjusted HR, 0.17 [95% CI, 0.07–0.39]; P < .001) (figure 3A). Diversity was not associated with the number of episodes 6 months before the survey (table 3). The presence of parasites at the survey was not associated with the time to or the number of episodes before the blood sampling.

![Figure 2](https://via.placeholder.com/150)

**Figure 2.** Mean number of msp2 genotypes in the children positive by polymerase chain reaction in Ngerenya and Chonyi, by the 2 respective allelic types FC27 and IC.
Figure 3. Kaplan-Meier estimates of the time to the last episode of malaria and treatment before the survey (A) and the time to the first subsequent episode of malaria (fever and \(\geq 2500\) Plasmodium falciparum parasites/\(\mu L\)) after the survey in asymptomatic children infected with different numbers of \(msp2\) genotypes (adjusted for age and parasite density).

In Ngerenya, \(P. falciparum\)-negative children had fewer episodes during the previous 6 months (IRR, 0.44 [95% CI, 0.30–0.65]; \(P<.001\)). Children infected with 2 clones were, compared with children infected with 1 clone, more likely to have a recent episode of fever and detection of parasites (adjusted HR, 2.55 [95% CI, 1.16–5.60]; \(P = .020\)) and more episodes within 6 months before the survey (adjusted IRR, 1.68 [95% CI, 1.07–2.80]; \(P = .048\)) (table 3), likely reflecting subgroups of children with different exposure. The short time to previous episodes among children with parasitemia may also suggest incomplete parasite clearance after treatment due to partial sulphadoxine-pyrimethamine resistance in the less-immune population.

**Association between number of clones and risk of subsequent clinical malaria.** The number of clones in the asymptomatic children at the survey was further assessed in relation to their prospective risk of malaria by both case definitions. The definition of fever and \(\geq 2500\) parasites/\(\mu L\) was considered most specific in the prospective risk analysis, because it minimizes the effect of asymptomatic background parasitemia.
After an age-stratified analysis, children of 5 years old, who only had borderline significant risk of subsequent malaria when infected with 2 clones (adjusted HR, 4.23 [95% CI, 1.69–10.54]; P = 0.002) (figure 3B), as well as a higher number of episodes during the subsequent 6 months (table 3). In an age-stratified analysis, children ≤5 years old had higher risk of subsequent malaria when infected with 2 clones (adjusted HR, 13.61 [95% CI, 1.25–147.73]; P = .032), compared with children >5 years old, who only had borderline significant risk (adjusted HR, 3.97 [95% CI, 0.94–16.79]; P = .061). No significant associations were found with a subsequent risk of malaria among children infected with ≥3 clones, even when stratifying for age. Assessment of risk as the total number of episodes during the entire observation period revealed that children infected with ≥4 genotypes were more likely to not have experienced any episodes of fever and have ≥2500 parasites/µL (adjusted OR, 5.38 [95% CI, 1.35–21.52]; P = .017). No other associations were found between the number of clones and the total number of episodes.

In Ngerenya, children who were negative for parasites at the survey had an increased prospective risk of malaria (table 3), especially those >5 years old (adjusted HR, 4.39 [95% CI, 1.38–14.03]; P = .012). No associations were found between the number of clones and the time to subsequent malaria, the number of episodes 6 months after the survey, or the number of episodes during the entire observation period.

When the data for Chonyi and Ngerenya were combined and adjusted for area, infection with 2 clones was again associated only with time to subsequent malaria episode (adjusted HR, 2.69 [95% CI, 1.36–5.31]; P = .004).

**DISCUSSION**

*Plasmodium falciparum* has extensive antigenic and genetic polymorphism and, infections are often composed of several distinct parasite populations. The ability to control multiclonal infections is, therefore, likely to be an important component of protective immunity. We have assessed how the diversity of *P. falciparum* infection relates to the host’s susceptibility to disease in areas of different transmission.

Characterization of *P. falciparum* infections by *msp2* genotyping in children living in 2 closely located areas on the coast of Kenya revealed that the number of infecting clones was highest in the area of most-intense transmission, in line with previous reports [2–6]. In Chonyi, which has moderate transmission, 70% of infections were multiclonal and 50% were composed of ≥3 clones. The highest diversity was detected in children for whom a long time has elapsed since receiving previous antimalarial treatment, suggesting accumulation of infections with time in partially immune individuals. Also, multiple clones are more likely to be transmitted via single mosquito bites in areas of higher transmission [7]. In contrast, in Ngerenya (the area of low transmission) most infections were monoclonal, and only 6 children had ≥3 clones, reflecting very limited diversity and accumulation. The 2 closely located populations have the same ethnic origin, customs, and housing. The only apparent...
Figure 4. Prospective risk of malaria in relation to the number of clones in asymptomatic children in 2 areas of low and moderate transmission in Kilifi, Kenya (present study), and in an area of high transmission in Rufiji, Tanzania [16]. The risk is assessed by Cox regression as the time to the first malaria episode after the survey. The hazard ratios (HRs) are adjusted for age and parasite density.
An identical study design and analyses, including the same genotyping method [21] and statistical approach (Cox regression with the same adjustments), were used in our previous study in a high-transmission area in Tanzania [16]. There, children who harbored 2–3 clones or were parasite negative had a reduced risk of subsequent malaria, whereas children with 1 clone had the highest risk (figure 4), suggesting that multiclonal infections are a marker of protection. In both studies, associations between the number of clones and malaria risk were nonlinear and could not be revealed when clones were modeled as continuous data. Assumptions about linearity should indeed be avoided because host-parasite interactions may be level dependent, with regard to both pathogenesis and protection [28].

Moreover, only clearly asymptomatic children were included, to avoid the influence of ongoing clinical malaria or recent treatment [19, 25]. This may have underestimated the associations because the most vulnerable children are likely to be excluded, as reflected by their higher malaria morbidity. Inclusion of all children, however, did not reveal any associations between the number of clones and risk in either of the studies, demonstrating the need for distinct selection criteria in population-based assessments.

Transmission accounts for the main difference between the settings, suggesting that intensity and diversity of exposure lead to different levels of tolerance to multiclonal infections (figure 4). In the low-transmission area (Ngerenya), rarer exposure restricts acquisition of protective immunity, most infections lead to disease, and antigenic diversity does not appear to be of overall importance. In the moderate-transmission area (Chonyi), individuals are immune to 1 or a few clones, but additional clones lead to disease. Susceptibility may vary, however, because of individual differences in exposure. In high-transmission areas (Tanzania), continuous exposure to antigenically polymorphic parasites leads to tolerance to multiclonal infections, which maintain cross-protective immune responses.

The rate of acquiring and clearing *P. falciparum* infections is dependent on age [12]. The reduced risk conferred by multiclonal infections in high-transmission settings have mainly been found in school-age children [13, 14, 29], whereas younger children and infants were at increased risk [17, 18, 29, 30], suggesting a gradual acquisition of tolerance to multiple clones with age. Here, in the moderate-transmission area, infections with 2 clones generated the highest risk in children ≦5 years. There was, however, no reduced risk in older children, suggesting that they do not reach the same level of tolerance as in high-transmission settings.

The diversity of *P. falciparum* infections reflects simultaneous acquisition of new infections and immunity. The persistence of antigenically diverse parasites is also likely to affect the diversity of antibody responses. Analysis of anti-MSP2 antibodies in this area showed a reduced malaria risk, especially for antibodies directed against the same allelic types as the concurrent parasites [31]. Detailed assessments of antigen-specific antibody responses in relation to infecting clones may help us to further understand the importance of persistent parasites per se.

In conclusion, associations between the number of concurrent clones in asymptomatic *P. falciparum* infections and the prospective risk of malaria are dependent on transmission level. The same diversity is both a marker of risk and a marker of protection in different areas and is of no apparent importance in a low-transmission area. Tolerance to multiclonal infections is thus a result of the intensity and antigenic repertoire of exposure. Understanding how and when immunity to antigenically diverse *P. falciparum* is acquired under different transmission conditions is a prerequisite for the development of future control interventions, such as antimalarial vaccines or preventive treatment.

Acknowledgments

We thank the children and their guardians for their participation; the staff at the outpatient department; Sophie M. Uyoga, Moses Mosobo, Mohammed Shafi, and Spencer Polley for technical assistance; Edna Ogada for data management; and Margaret Mackinnon, Faith Osier, and Charles Mbogo for most valuable comments on the manuscript.

References

increase in malaria morbidity in Ghanaian children during the transition into the high transmission season. Parasitology 2001; 123:113–23.


