

# Molecular Evaluation of the Natural History of Asymptomatic Parasitemia in Ugandan Children

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We assessed the prevalence and natural history of malarial parasitemia by use of microscopy and polymerase chain reaction (PCR) in 314 asymptomatic children in Kampala, Uganda. The prevalence of asymptomatic parasitemia was 17% by microscopy and 47% by PCR. Children with parasitemia identified by microscopy had a 5-fold higher rate of subsequent symptomatic malaria, compared with children without detectable parasitemia. Children with parasitemia identified by PCR alone had a similar rate of subsequent symptomatic malaria, compared with children without detectable parasitemia. Among microscopy-positive children who later developed symptomatic malaria, 47% had strains identical to those identified at enrollment, and the proportion of symptomatic episodes due to persistent strains remained high for 3 months. Among the PCR-positive/microscopy-negative children, only 17% had identical genotyping patterns at the onset of symptomatic malaria, with most of these episodes occurring during the first month. Asymptomatic parasitemia detected by microscopy, but not by PCR, strongly predicted subsequent clinical malaria, often due to persistent infection.

It has been known for more than a century that some individuals can harbor malaria parasites without fever or other classic signs and symptoms of malaria [1, 2]. Asymptomatic parasitemia is common in areas of sub-Saharan Africa where malaria is endemic, and, with the advent of sensitive molecular methods, it is now known that, in certain areas, >90% of individuals may be parasitemic at any given time [3, 4]. Studies of asymptomatic parasitemia have helped to improve our understanding of the dynamics of infection and the development of antimalarial immunity. In areas of high transmission, parasite genetic diversity is extensive, and asymptomatic individuals may harbor complex infections with frequent turnover of specific strains [5].

It is generally believed that repeated infection leads to the development of strain-specific immunity, which reduces parasite burden, thereby protecting against symptomatic malaria [6].

Despite the high prevalence of asymptomatic parasitemia, our understanding of its natural history is limited. Children with asymptomatic parasitemia have been shown to have more-complex infections than do children with symptomatic malaria [7, 8]. It has been suggested that asymptomatic infections protect against developing clinical malaria and that such protection is enhanced by the diversity of infecting strains [9]. In prospective studies of patients with asymptomatic parasitemia, the development of symptomatic malaria often coincided with infection by novel parasite strains and abrupt increases in parasite density [10, 11]. These results suggest that the parasites causing symptomatic malaria are those against which an individual has not yet mounted an effective immune response.

Available studies of asymptomatic malarial parasitemia have often been limited to small numbers of selected patients in areas of high endemicity. We are unaware of any population-based studies that used molecular techniques to investigate the natural history of asymptomatic parasitemia. We recently completed a 1-

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year longitudinal comparison of the relative efficacies of 3 antimalarial regimens in children in Kampala, Uganda [12]. In the present study, we assessed the prevalence of asymptomatic parasitemia detected by use of polymerase chain reaction (PCR) and microscopy, followed all children for the development of symptomatic malaria, and used molecular techniques to differentiate between disease due to enrollment strains and that due to new infections.

## METHODS

**Clinical study and sampling.** Clinical data and blood samples came from a previously published longitudinal study that took place between July 2000 and August 2001 in Kampala, Uganda [12]. In this urban area, malaria is mesoendemic, with perennial peaks during the 2 rainy seasons (Ugandan Ministry of Health, unpublished data). Informed consent was obtained from all children's parents or guardians, and the study was approved by the institutional review boards of the University of California, San Francisco, and Makerere University, Kampala.

In brief, healthy children, aged 6 months to 5 years, were enrolled from the community, by use of convenience sampling, if they fulfilled the following criteria: (1) no history of treatment for malaria during the previous 2 weeks or fever during the previous 48 h, (2) no history of adverse reaction to the study drugs, (3) no history of sickle cell disease, and (4) a hemoglobin level of  $\geq 50$  g/L. A total of 316 children were enrolled and were followed for 1 year, for all of their health-care needs.

Parents were instructed to bring their child to clinic whenever they needed medical attention and to avoid using any drugs not administered or approved by a study physician. Every time a child presented to clinic with a new history of fever (during the previous 48 h) or a tympanic temperature of  $\geq 38.0^\circ\text{C}$ , a thick blood smear was obtained. Symptomatic malaria was diagnosed if any of the following criteria was fulfilled: (1) tympanic temperature of  $\geq 38.0^\circ\text{C}$  and any parasitemia, (2) history of fever (not documented) and  $\geq 500$  asexual parasites/ $\mu\text{L}$ , or (3) severe malaria or danger signs [12] and any parasitemia.

**Detection of malaria parasites by use of microscopy and PCR.** Blood obtained by fingerprick was used for thick and thin smear and for storage on filter paper. Samples were obtained at enrollment, when all children were asymptomatic, and at the time of a child's first diagnosis of malaria. Smears were stained with 2% Giemsa for 30 min and read within 48 h in duplicate by 2 experienced microscopists. All discrepancies were resolved by a third microscopist. Parasite density was calculated by counting the number of asexual parasites per 200 white blood cells and calculating the number of parasites per microliter, assuming a white blood cell count of 8000 cells/ $\mu\text{L}$ . A smear was judged to be negative if no parasites were seen after

review of 100 high-power fields. Thin smears were used to determine the species of parasite.

As an alternate method of parasite detection, speciation by PCR was performed on all enrollment samples. DNA was extracted from filter paper by use of the chelex method [13]. Genus-specific PCR, followed by nested species-specific PCR, of 18S small subunit ribosomal DNA was performed [14]. Reactions were performed in 25  $\mu\text{L}$  containing  $1\times$  buffer, 2.5 mmol/L MgCl<sub>2</sub>, 200  $\mu\text{mol/L}$  dNTP mix, 0.25  $\mu\text{mol/L}$  each primer, and 1 U of *Taq* polymerase, with the following cycling conditions: initial denaturation for 1 min at  $94^\circ\text{C}$ , and 25 cycles (30 cycles for the nested round) of 1 min at  $94^\circ\text{C}$ , 5 min at  $58^\circ\text{C}$ , and 2 min at  $72^\circ\text{C}$ . Species were determined on the basis of product size on 2% agarose gel. Positive and negative controls were used for each round of PCR, and the technician was unaware of the child's microscopy results.

**Molecular genotyping.** Children's samples were selected for comparative genotyping if they were positive for *Plasmodium falciparum* by either microscopy or PCR at enrollment and if the child went on to develop symptomatic *P. falciparum* malaria during the 1-year period of follow-up. Genotyping was performed by use of nested PCR amplification of the block 3 polymorphic region of merozoite surface protein (MSP)-2 on the basis of the results of 2 recent genotyping studies [15, 16]. Initial PCR was performed by use of primers that flanked the IC3D7 and FC27 allelic families [17], and the nested round used family-specific primers [18]. The PCR conditions used have been described elsewhere [16]. Products were run on 2% agarose gels, and digitized gel images were then analyzed by use of GelCompar II software (Applied Maths). Each PCR product identified was considered to be an individual parasite strain. All pairs of asymptomatic parasitemia followed by symptomatic malaria in the same child were run side by side. PCR products were considered to represent the same parasite strain if molecular weights were within a 10-bp range. Complexity of infection (COI) was defined as the mean number of unique strains identified in an individual infection.

To assess the validity of using MSP-2 alleles to distinguish parasite strains, we determined the MSP-2 allelic diversity in our study population. When we compared genotyping results between individual children, there was, on average, only a 5.1% chance at enrollment and a 4.6% chance at the onset of symptomatic malaria that any 2 alleles from independent infections would be classified as being the same. Thus, genetic diversity was high, making it unlikely that a random new infection would be misclassified as the same strain.

**Statistical methods.** Hypothesis testing for categorical variables was performed by use of the  $\chi^2$  or Fisher's exact test. All confidence intervals were set at 95% by use of exact methods. The association between age and asymptomatic parasitemia was assessed by use of the 2-sample *t* test. The risk of developing

symptomatic malaria during follow-up was estimated by use of the Kaplan-Meier product limit formula. Children who did not complete the full year of follow-up before being diagnosed with malaria were censored. Associations between the asymptomatic parasitemia and time to symptomatic malaria were estimated by use of the Cox proportional hazards model. Geometric mean parasite densities (GMPDs) were compared by use of paired and 2-sample *t* tests. COIs were compared by use of the nonparametric Wilcoxon matched pairs signed rank sum test and the Mann-Whitney *U* test.

## RESULTS

**Prevalence of asymptomatic parasitemia at enrollment.** At enrollment, when all children were asymptomatic, the prevalence of parasitemia identified by microscopy was 17% (55/316). The only species identified by microscopy was *P. falciparum*. The range of parasite densities was 16–71,840 parasites/ $\mu$ L, with a GMPD of 2630 parasites/ $\mu$ L. By use of species-specific PCR, the prevalence of asymptomatic malaria was much higher than that identified by microscopy. At enrollment, 148 (47%) of the 316 children had plasmodial infection identified by PCR (36% *P. falciparum* mono-infection, 18% *P. falciparum* mixed infection, 10% *P. ovale*, 7% *P. vivax*, 4% *P. malariae*, and 3% non-*P. falciparum* mixed infection; figure 1). The relative risk of parasite detection by PCR, compared with microscopy, was 2.7 (95% confidence interval [CI], 2.1–3.6;  $P < .001$ ). Two children were microscopy positive but species-specific PCR negative (both had *P. falciparum* DNA detected by MSP-2 genotyping). By use of PCR as the reference standard, the overall sensitivity and specificity of microscopy for detecting asymptomatic *P. falciparum* parasitemia were 44% and 98%, respectively. There was no association, within our limited age range (6 months to 5 years), between age and asymptomatic parasitemia on the basis of detection by PCR ( $P = .4$ ) or microscopy ( $P = .2$ ).

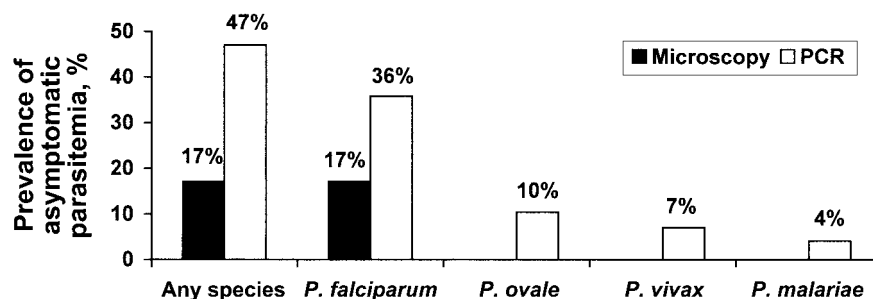
**Natural history of asymptomatic *P. falciparum* parasitemia.** Of the 316 children enrolled in the longitudinal study,

2 had no follow-up and were excluded. The remaining 314 children were classified into 3 groups on the basis of *P. falciparum* detection at enrollment: (1) microscopy negative and PCR negative (198/314 [63%]), (2) PCR positive but microscopy negative (63/314 [20%]), and (3) microscopy positive (53/314 [17%]).

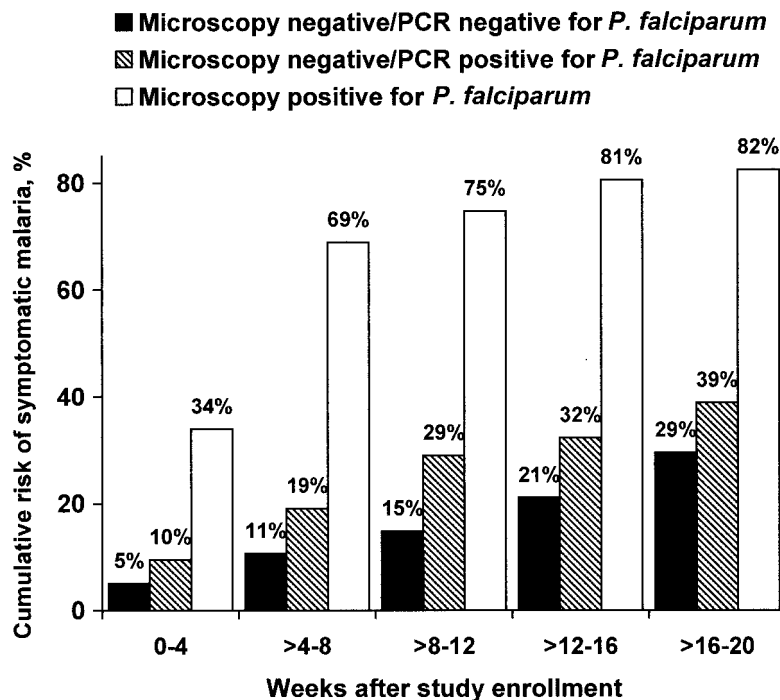
Children were followed until either they developed symptomatic malaria or the 1-year study ended. After 20 weeks of follow-up, the risk of developing symptomatic malaria was 82% in the microscopy-positive group, 39% in the PCR-positive/microscopy-negative group, and 29% in the microscopy- and PCR-negative group (figure 2). Children who were positive by microscopy at enrollment had a much higher rate of developing symptomatic malaria, compared with children who were negative by both microscopy and PCR (hazard ratio [HR], 4.7; 95% CI, 3.3–6.6;  $P < .001$ ). In contrast, children who were positive for *P. falciparum* by PCR only had a similar rate of developing symptomatic malaria, compared with children who were negative by both PCR and microscopy (HR, 1.1; 95% CI, 0.8–1.7;  $P = .54$ ). Children with non-*P. falciparum* infections detected by PCR had a similar rate of developing symptomatic malaria, compared with children who were negative by both microscopy and PCR (data not shown).

Genotyping was performed for all children who were positive for *P. falciparum* by microscopy or PCR at enrollment and went on to develop symptomatic malaria. This included 34 of 63 children in the PCR-positive/microscopy-negative group and 48 of 53 children in the microscopy-positive group. Successful genotyping results were obtained for 88% (30/34) and 98% (47/48) of paired samples, respectively. For these children, we determined the number of strains present at the onset of symptomatic malaria that were also present at the time of enrollment. Symptomatic malaria caused by a non-*P. falciparum* species (2 children) was classified as a new infection.

Among the children who had asymptomatic malaria detected by microscopy and went on to develop symptomatic malaria, 45% (21/47) had identical genotyping patterns at the onset of symptomatic malaria as at enrollment, and 77% (36/47) had



**Figure 1.** Prevalence of asymptomatic parasitemia at enrollment, defined by microscopy or polymerase chain reaction (PCR) using species-specific primers. All microscopy-positive infections were identified morphologically as *Plasmodium falciparum*.



**Figure 2.** Risk of developing symptomatic malaria, based on method of parasite detection at enrollment. Risk was estimated by use of the Kaplan-Meier product limit formula. PCR, polymerase chain reaction.

at least 1 identical strain present on both occasions. When the results were stratified according to the time to development of symptomatic malaria, the proportion of episodes with identical genotyping patterns remained high up to 12 weeks after enrollment (table 1). In the PCR-positive/microscopy-negative group, only 17% (5/30) of children had identical genotyping patterns at the onset of symptomatic malaria as at enrollment, and 47% (14/30) of children had at least 1 identical strain present on both occasions (table 1). In this group, the proportion of episodes with identical genotyping patterns remained high only during the first 4 weeks after enrollment.

**GMPD and COI.** The GMPD and the number of unique MSP-2 alleles COI were compared for the microscopy-positive and PCR-positive/microscopy-negative groups, between enrollment and the onset of symptomatic malaria. Children with asymptomatic parasitemia by microscopy had almost twice the COI as those positive by PCR alone (2.41 vs. 1.28;  $P < .001$ ). At the onset of symptomatic malaria, there was a trend toward a lower GMPD (6139 vs. 15558 parasites/ $\mu$ L;  $P = .08$ ) but a higher COI (2.59 vs. 2.00;  $P = .14$ ), in children who were microscopy positive at enrollment, compared with children who were PCR positive and microscopy negative.

The microscopy-positive and PCR-positive/microscopy-negative children were further stratified into 2 groups: (1) those who harbored the identical strains at the onset of symptomatic malaria as at enrollment and (2) those who developed symptomatic malaria with the detection of a novel strain (table 2).

Those children who were either microscopy or PCR positive and developed symptomatic malaria with the detection of a novel strain had significant increases in GMPD and COI at the onset of symptomatic malaria, compared with enrollment. In contrast, those children who were microscopy or PCR positive and harbored identical strains over time tended to have a similar GMPD and either a lower or unchanged COI at the onset of symptomatic malaria, compared with enrollment. Overall, there was no correlation between age and COI (data not shown).

## DISCUSSION

Asymptomatic parasitemia was assessed in a population-based cohort of children who were prospectively followed for the development of symptomatic malaria. The prevalence of asymptomatic parasitemia was nearly 3-fold higher when PCR was used as the method of detection, compared with microscopy alone. Several studies have previously reported that, for the identification of asymptomatic malaria, PCR is much more sensitive than microscopy [4, 19, 20]. However, the association between these 2 methods of detecting asymptomatic parasitemia and the subsequent risk of symptomatic malaria has not previously been compared, and this association varied greatly in our study population. Children in whom asymptomatic parasitemia was identified by microscopy had a 5-fold greater rate of developing symptomatic malaria than did children without parasites at enrollment. In contrast, children in whom asymp-

**Table 1. Proportion of asymptomatic *Plasmodium falciparum*-infected children who subsequently developed symptomatic malaria due to the same strain that was present at the time of enrollment.**

Time from enrollment to onset of symptomatic malaria, weeks	Microscopy negative/PCR positive			Microscopy positive		
	No. of pairs with successful genotyping	All enrollment strains present, % <sup>a</sup>	Any enrollment strain present, % <sup>a</sup>	No. of pairs with successful genotyping	All enrollment strains present, % <sup>a</sup>	Any enrollment strain present, % <sup>a</sup>
0–4	6	50	67	17	41	88
5–8	6	17	83	18	50	78
9–12	6	17	50	3	67	100
>12	12	0	17	9	33	44

**NOTE.** PCR, polymerase chain reaction.

<sup>a</sup> Comparing genotyping patterns at the onset of symptomatic malaria with genotyping pattern at the time of enrollment.

tomatic parasitemia was detectable only by PCR had no significant increase in the rate of developing symptoms, compared with children who were both PCR and microscopy negative. Thus, the improved sensitivity of PCR was not of added utility in defining those children at risk of developing symptomatic malaria. Furthermore, microscopy-positive children harbored nearly twice as many strains at enrollment, compared with children who were positive by PCR only. These results may be explained by a difference in the level of acquired immunity in these 2 groups of children with asymptomatic parasitemia. Low-level and low-complexity asymptomatic parasitemia, detectable only by PCR, may reflect a fairly effective antimalarial immune response that is relatively likely to prevent clinical illness. In contrast, the higher and more-complex parasitemia detected by microscopy may reflect a less-effective antimalarial

immune response, and, thus, a high risk of subsequent symptomatic malaria.

We initially noted that children with asymptomatic malaria identified by microscopy were at increased risk of clinical malaria [21]. The use of genotyping allowed us to assess whether the increased risk was typically due to persistence of an infecting strain or to an increased risk of new infection. In the majority of children with asymptomatic parasitemia detected by PCR alone and in approximately one-half of the children with asymptomatic parasitemia detected by microscopy, the subsequent development of symptomatic malaria was associated with the emergence of a new parasite strain. These episodes were associated with a significant increase in parasite density and COI. This finding is consistent with those of another study from areas of hyperendemicity, in which small numbers of

**Table 2. Geometric mean parasite density (GMPD) and complexity of infection (COI) in asymptomatic and symptomatic malaria in 314 children in Kampala, Uganda.**

Parameter, test result	No. of isolates <sup>a</sup>	Asymptomatic parasitemia	Symptomatic malaria	<i>P</i> <sup>b</sup>
GMPD, asexual parasites/ $\mu$ L <sup>c</sup>				
Microscopy positive: all strains the same	21	2836	1953	.39 <sup>d</sup>
Microscopy positive: any new strain	26	3050	14578	.03 <sup>d</sup>
PCR positive only: all strains the same	5	0	2349	...
PCR positive only: any new strain	26	0	20494	...
Complexity of infection <sup>c</sup>				
Microscopy positive: all strains the same	21	2.43	1.62	.004 <sup>e</sup>
Microscopy positive: any new strain	26	2.35	3.31	.01 <sup>e</sup>
PCR positive only: all strains the same	5	1.00	1.00	1.00 <sup>e</sup>
PCR positive only: any new strain	25	1.32	2.16	.01 <sup>e</sup>

**NOTE.** PCR, polymerase chain reaction.

<sup>a</sup> Includes all paired samples for which genotyping for *Plasmodium falciparum* was successful at the time of enrollment and at the first episode of symptomatic malaria.

<sup>b</sup> Asymptomatic parasitemia vs. symptomatic malaria.

<sup>c</sup> Comparing genotyping patterns at onset of symptomatic malaria with genotyping pattern at time of asymptomatic parasitemia.

<sup>d</sup> Paired *t* test.

<sup>e</sup> Wilcoxon matched pairs signed rank sum test.

patients with asymptomatic parasitemia were followed prospectively [10, 11]. In these studies, frequent sampling revealed that the development of symptomatic malaria was nearly always associated with the appearance of a novel strains and an abrupt increase in parasitemia. In contrast, in the present study, the onset of symptomatic malaria was associated with the persistence of strains present at enrollment in approximately one-half of the children with microscopically defined asymptomatic parasitemia and in a small number of children with parasitemia detected by PCR alone. In these children, there was no significant change in parasite density at the onset of symptomatic malaria, and the COI was actually lower at the onset of symptomatic malaria, compared with enrollment. The duration of persistent infections before the onset of symptomatic malaria also differed between groups: it was equally distributed over the course of 12 weeks in the microscopy-positive group but mostly occurred within the first 4 weeks in the group that was positive by PCR alone. Thus, children remained susceptible to illness due to persistent strains for many weeks. Similarly, in Sudan, some subjects with asymptomatic parasitemia developed symptomatic malaria due to strains that persisted for months before the onset of illness [22]. In the absence of introduction of a novel strain, it is unclear what factors are responsible for shifting the balance from asymptomatic infection to symptomatic malaria. One possibility, as suggested by others, is the outgrowth of a dominant population that is responsible for the onset of clinical symptoms [10]. This may be due to antigenic switching of erythrocyte surface proteins [23]. Such switching may allow for the immunologic escape of a particular strain. The lack of an increase in parasitemia in these individuals might be explained by the loss of other strains, coincident with an increase in parasitemia of the disease-causing strain.

One possible explanation for the differences in results between the present study and others is the level of transmission intensity. Studies in which symptomatic malaria was always associated with the introduction of novel parasite strains were done in areas of high endemicity, where immunity would be expected to develop more rapidly [10, 11]. In contrast, the present study was conducted in an area of mesoendemicity, where malaria incidence is highly variable and associated with microenvironments [24]. Under these circumstances, immunity is likely to be slower to develop and highly variable, leading to wider variation in the outcome of an asymptomatic infection in a child. This highlights the importance of population-based studies for generalizing results.

Given the high prevalence of asymptomatic parasitemia in sub-Saharan Africa, a better understanding of the epidemiology and natural history of this state is of obvious importance. Our results indicate that the method of parasite detection may have a significant effect on the observed natural history and COI of asymptomatic parasitemia. Of note, the present study benefited

from excellent quality control of microscopy reading, which is essential for accurate classification of asymptomatic parasitemia. Although microscopy alone missed a significant proportion of subpatent and mixed infections in Kampala, the increased sensitivity of parasite detection by PCR did not translate into a higher risk of subsequently developing symptomatic malaria. Furthermore, parasitemia detectable by microscopy appears to be more complex than that detectable only by PCR. Much of this difference might be explained by varying levels of acquired antimalarial immunity in these 2 groups. Future studies attempting to compare the precise immunologic markers in these 2 asymptomatic groups should yield important information regarding the acquired host response to the malaria parasite. Furthermore, because the acquisition of antimalarial immunity is tightly correlated with endemicity and age, future longitudinal studies in varied epidemiological settings will help to shed light on the factors that underlie asymptomatic parasitemia.

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