Epidemiology of multiple *Plasmodium falciparum* infections

4. Age dependence of the multiplicity of *Plasmodium falciparum* infections and of other malariological indices in an area of high endemicity

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Abstract

The relationship between age and various malariological indices in the Kilombero valley of Tanzania were examined by compiling data from 6 different community studies carried out between 1989 and 1996. The rate of acquisition of *Plasmodium falciparum* infection was highest in children 1–5 years of age, while recovery rates were lowest between the first birthday and early adolescence. As a result, peak prevalence was reached in 3–5 years old children. However, the prevalence of clinical malaria (estimated from the excess risk of axillary temperatures ≥37·5°C attributable to parasitaemia) was highest in children under one year of age. The peak in multiplicity of infection (identified by polymerase chain reaction–restriction fragment length polymorphism of the *msp2* locus) occurred in 3–7 years old children. There was a significant correlation between parasite density and multiplicity of infection in infants and young children (1–2 years of age) but not in older individuals.

Keywords: malaria, *Plasmodium falciparum*, multiple infection, prevalence, *msp2* gene, Tanzania

Introduction

The age distribution of infection is a key indicator of endemicity and the acquisition of natural immunity to an infectious agent. With *Plasmodium falciparum* malaria in endemic areas, not only is the risk of being infected age-dependent, so are the parasite densities, the likely duration of an infection, and the risks of different clinical outcomes.

The analysis of parasite genotypes provides new measures which can be used to characterize the malariological picture in human populations, and to extend our understanding of the epidemiological effects of natural immunity to *P. falciparum*. Recent studies have indicated that the same individual can simultaneously be parasitized by many genetically distinct infections. The number, or multiplicity, of such infections in any one infected host may be related to the endemicity of malaria (KONATÉ *et al.*, 1998) and to the degree of immunity against *P. falciparum*, and the risk of clinical malaria (AL-YAMAN *et al.*, 1997; BECK *et al.*, 1997).

We have now combined results from several studies using genotyping of the *msp2* locus of *P. falciparum* in blood samples from villages in a highly endemic area in southern Tanzania. We used these data to describe how the multiplicity of infections varies by age in this population. We analysed how the age trend in multiplicity of infection is related to those of other parasitological measures used to characterize transmission intensity and malaria endemicity. We also considered the extent to which the apparent age trends might be affected by variations in the sensitivities of the parasitological techniques.

Methods

Study population

The flood plain of the Kilombero river in the Morogoro Region of south-eastern Tanzania is low-lying with a population mainly dependent on subsistence farming of rice, maize and cassava, and some fishing (TANNER *et al.*, 1991). Houses have walls built from bamboo and earth with grass thatch.

P. falciparum malaria is the leading cause of morbidity and mortality in the area (TANNER *et al.*, 1991) and the most frequent single diagnosis at the Saint Francis Designated District Hospital (SFDDH) in the main town in the valley, Ifakara. Severe malaria presents there mainly as malarial anaemia in children less than one year of age (SNOW *et al.*, 1994). Prevalence and density of *P. falciparum* parasitaemia show little seasonality. *P. malariae* also occurs but at a low and unstable frequency (SMITH *et al.*, 1993).

In the present paper we review data from 4 villages within the valley, all lying along the northern edge of the flood plain of the Kilombero river. Namawala, Idete and Michenga lie to the west of Ifakara, whilst Kiberege lies to its north-east (Fig. 1). In contrast to the situation

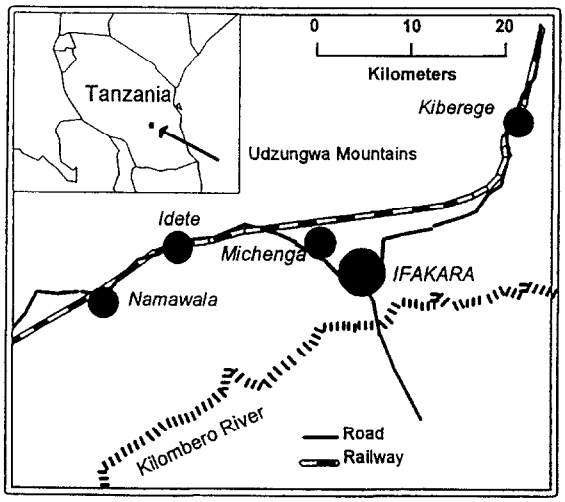

Fig. 1. Map of Kilombero valley in Tanzania, indicating villages studied.

in Ifakara town (MENENDEZ *et al.*, 1997), the rural parts of the valley exhibit similar high levels of infection, and we thus consider it reasonable to combine data from different villages in our description of age distributions. The average entomological inoculation rate in adults in these villages has been estimated to be above one inoculation per person per night (Namawala village: SMITH *et al.*, 1993; Idete village: CHARLWOOD *et al.*, 1998; Michenga village: BABIKER *et al.*, 1997).

We have combined results of malaria epidemiology studies carried out in Idete during 1993–1994 (ALONSO

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Table. Studies providing data for the present analyses

Reference	Age (years)	Group analysed	Village	Time	No. analysed[a]	Interval between surveys[b]	Malariological indices From published source	Malariological indices Newly determined
SMITH *et al.* (1993, 1995)	0–82	Age ≥1 year	Namwala & Michenga	1989–1991	1076(7·3)	2 months	Prevalence, density, prevalence of malaria fever	Transition rates (h,r)
SMITH *et al.* (1993)	0–57	Age-stratified sample[c]	Namawala	1991	61(1)	NA	–	Multiplicity of infection. Correlation of multiplicity with parasite density
KITUA *et al.* (1997)	0–1	All <1 year	Idete	1993–1994	304(4·5)	2 weeks	Prevalence, density, prevalence of malaria fever, transition rates (h,r)	–
FELGER *et al.* (1999b)	0–1	Same children as KITUA *et al.* (1997)	Idete	1993–1994	100(2)	4 weeks	Multiplicity of infection, correlation of multiplicity with parasite density	–
FRASER-HURT *et al.* (1999)	0·5–2·5	Non-ITN[e] users	Kiberege	1996	60(5·8)[d]	1 month	Multiplicity of infection	Correlation of multiplicity with parasite density
BECK *et al.* (1997)	2–7	Placebo group[f]	Idete	1993-1994	76(1)	NA	Multiplicity of infection	Correlation of multiplicity with parasite density

[a]Figures in parentheses are the mean numbers of samples analysed per individual.
[b]The intervals given are those intended in the study design; the actual interval varied somewhat. NA: not applicable.
[c]Age-stratified sample from the survey carried out in April 1991.
[d]Excluding baseline survey samples.
[e]Insecticide-treated bed net.
[f]In trial of SPf66 vaccine (ALONSO *et al.*, 1994).

et al., 1994; KITUA *et al.*, 1997; BECK *et al.*, 1997), in Namawala and Michenga during 1989–1991 (SMITH *et al.*, 1993), and in Kiberege during 1996 (FRASER-HURT *et al.*, 1999) (Table). These data were all derived from either repeated malariological surveys carried out as baseline studies for subsequent interventions (SMITH *et al.*, 1993; KITUA *et al.*, 1997; FELGER *et al.*, 1999b) or the control arms of randomized controlled trials of insecticide-treated bed nets (ITNs) (FRASER-HURT *et al.*, 1999) or of the SPf66 malaria vaccine (BECK *et al.*, 1997).

Although entomological measures of transmission show seasonal variation in the Kilombero valley, there is substantial transmission all year round and little seasonality in parasite prevalence or density (SMITH *et al.*, 1993). The major studies included in our analyses spanned several seasons or entire annual cycles and we do not further consider seasonality in the analyses.

In addition, we have used data from a randomized controlled trial of the new antimalarial drug CGP 56697 (co-artemether) (HATZ *et al.*, 1998; IRION *et al.*, 1998) to estimate the sensitivity of the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) technique for detecting individual infections of *P. falciparum*. This trial was carried out among paediatric outpatients at SFDDH during 1996.

Laboratory techniques

Studies of the prevalence and density of malaria parasitaemia used standard Giemsa staining methods and light microscopy (Wild–Heerbrug, Switzerland, with a ×100 oil immersion lens and ×10 eyepiece). As applied in our studies, these methods, details of which have been described elsewhere (SMITH *et al.*, 1993; ALONSO *et al.*, 1994), have a detection limit of approximately 40 parasites/μL. Infections with malaria species other than *P. falciparum* were not considered in the present analyses.

The same PCR–RFLP technique for the typing of the *msp2* locus of *P. falciparum* was used throughout (FELGER *et al.*, 1999a). Mean multiplicity was defined as the mean number of distinct genotypes detected in infected samples (i.e., aparasitaemic samples were excluded from the analyses of multiplicity).

Data analysis

Prevalence of malaria-attributable fever. Estimates of the prevalence of malaria-attributable fever in the community in children over one year of age and in adults used the data of surveys repeated every 2 months in Namawala and Michenga villages during 1989–1991 (SMITH *et al.*, 1993). A total of 5132 observations of parasitological status and contemporaneous axillary temperatures in individuals from Namawala and Michenga was analysed. For children under one year of age, 1385 observations from community surveys every 2 weeks of axillary temperatures and parasitaemia were available from the study of KITUA *et al.* (1997). For these analyses, fever was defined as an axillary temperature ≥37·5°C. The excess risk of fever attributable to parasitaemia, and hence the percentage of fevers attributable to malaria, were then estimated using models of the relative risk of malaria morbidity as a function of parasite density (SMITH *et al.*, 1994).

Estimation of transition rates for patent infections. The instantaneous attack rate (h) (force of infection) for microscopically patent *P. falciparum* infection and the recovery rate from infection (r) were obtained from data for individuals who participated in the repeated malariological surveys. Estimates of h and r for infants in Idete were taken from KITUA *et al.* (1997). For older individuals estimates were made using the data of surveys repeated every 2 months in Namawala and Michenga villages during 1989–1991 (SMITH *et al.*, 1993).

The records for each individual were considered as a set of time intervals, corresponding to the intervals between successive survey attendances. Each interval was assigned to an age class depending on the age of the individual at the midpoint of the interval. Reversible catalytic models (BEKESSY *et al.*, 1976; KITUA *et al.*, 1997) were fitted separately for each age class to estimate h and r by maximum likelihood, assuming binomial errors and using the SAS non-linear regression procedure (SAS, 1989).

Estimates of sensitivity of PCR–RFLP. The sensitivity (S) of the PCR–RFLP technique for detecting individual genotypes can be estimated from determinations on repeated samples from the same individuals. Analysis of triplets of samples provides one approach (SMITH *et al.*, 1999). We here present another technique using the data collected during the trial of CGP 56697 (IRION *et al.*, 1998), in which blood samples were collected on presentation from patients with uncomplicated malaria, and again 72 h later, and the *msp2* locus of *P. falciparum* was genotyped in both samples. The proportion of those infections detected in the 72 h sample which were also found in the baseline sample provides an estimate of S. This estimate assumes (i) that a negligible number of new infections appeared between baseline and 72 h and (ii) that the probability that an infection present at both times was detected at 72 h is independent of whether it was detected at baseline.

Results

The compilation of optical microscopy data from in-

fants in Idete village and from older individuals in Namawala and Michenga indicated that the incidence rate of infection, determined from microscopy data, was highest in children aged 2–5 years (Fig. 2). However, the incidence estimate for children 1–2 years of age was very imprecise (due to the availability of only limited data) and we could not exclude the possibility that the maximum was in this age group. This maximum incidence was approximately one infection event per 20 d.

The corresponding recovery rate estimates were at a minimum in children older than on year and adolescents (Fig. 2). The prevalence of malaria infection detected by microscopy (Fig. 3) consequently reached a peak around age 3–5 years, when the incidence of infection was high and the recovery rate was at this minimum.

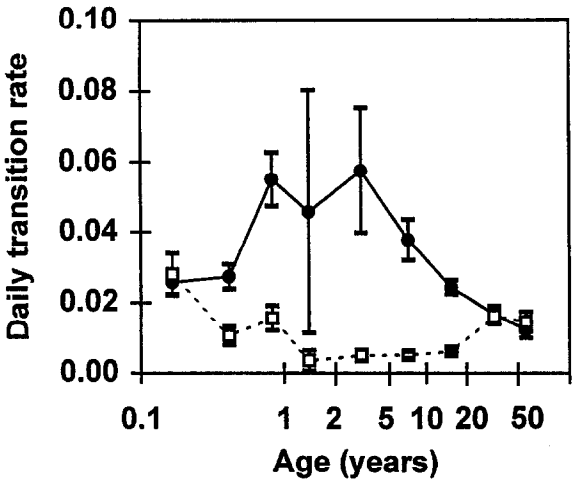

Fig. 2. Transition rates by age. Force of infection, h (●) and recovery rate, r (□). Rates for children under 1 year of age from Idete (KITUA *et al.*, 1997); those for adults and children over one year of age calcualted from Namawala and Michenga data (SMITH *et al.*, 1993). Error bars indicate ±1 standard error.

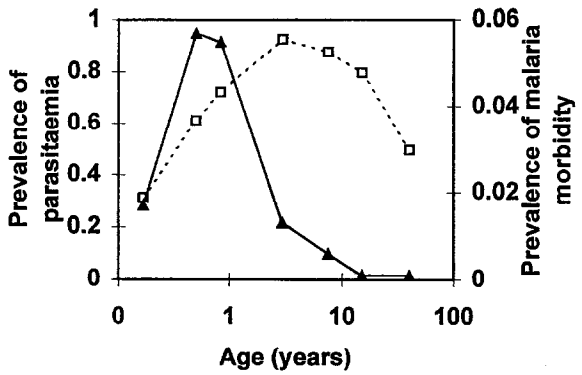

Fig. 3. Prevalence by age of asexual stage *Plasmodium falciparum* parasitaemia (□) and malaria-attributable morbidity (▲). Data for children under 1 year of age from Idete (KITUA *et al.*, 1997); those for adults and children over 1 year of age calculated from Namawala and Michenga data (SMITH *et al.*, 1995).

Not all malaria infections result in acute morbidity, and the age distribution of clinical malaria episodes was very different from that of infection. In contrast to the peak in the infection prevalence in 3–5 years old children, the highest prevalence (and indeed incidence, data not shown) of malaria fevers was in children under one year of age (Fig. 3). However, even the very high risk of malaria fevers in these children was a very conservative measure of the prevalence of malaria morbidity among them. In such young children, malaria illness often does not present as fever (SMITH *et al.*, 1995). When malaria morbidity was defined as any current illness (not only fever) associated with peripheral parasite densities higher than those in asymptomatic control individuals, a prevalence of 9·8% of malaria morbidity was estimated for children under one year of age in Namawala and Michenga (SMITH *et al.*, 1995).

The age trends in mean multiplicity of infection (Fig. 4), determined by PCR–RFLP broadly corresponded to

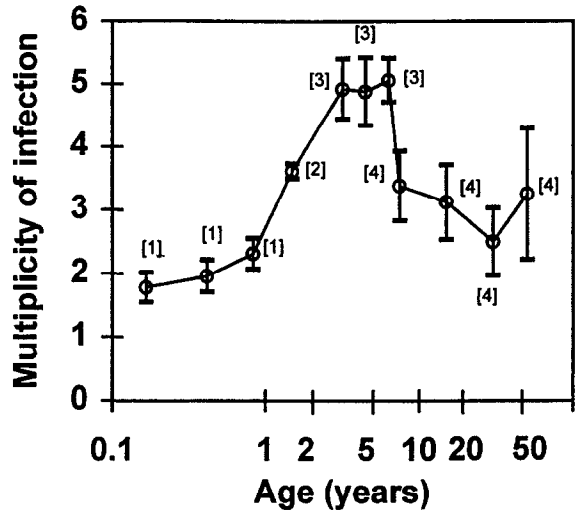

Fig. 4. Multiplicity of *Plasmodium falciparum* infection by age. Sources of data: [1] FELGER *et al.* (1999b); [2] FRASER-HURT *et al.* (1999); [3] BECK *et al.* (1997); [4] samples from Namawala (see Table 1). PCR-negative samples were excluded from the analysis; error bars indicate ±1 standard error.

the trend in prevalence by microscopy, with peak multiplicity and peak prevalence both occurring in 3–7 years old children. Since aparasitaemic samples were excluded from the calculation of mean multiplicity, this does not merely reflect the trend in prevalence. There was a statistically significant negative correlation between age and multiplicity in the series of samples from Namawala (Spearman's ρ=–0·28, P=0·03). The ratio of the number of infections belonging to the FC27-like *msp2* gene family to those of the 3D7 family was 0·8 with no substantial variation between the different villages or age groups.

The Spearman correlation between parasite density and multiplicity of infection was calculated separately for each study (Fig. 5). In each case the correlation was based on data for microscopically positive samples only. In the infants and control group of the ITN study, there were substantial correlations (infants: n=94, ρ=0·35,

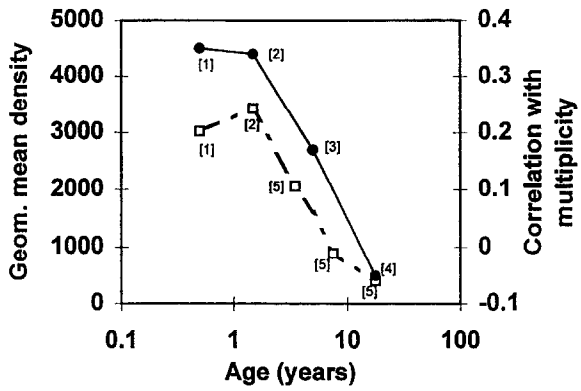

Fig. 5. *Plasmodium falciparum* parasite geometric mean density (per μL) by optical microscopy (□) (aparasitaemic samples excluded from the analysis) and Spearman correlation between density and multiplicity (●). Sources of data: [1] FELGER *et al.* (1999b); [2] FRASER-HURT *et al.* (1999); [3] BECK *et al.* (1997); [4] samples from Namawala; [5] SMITH *et al.* (1993).

P=0·0005; ITN study: n=266, ρ=0·34, P=0·0001). In older individuals the correlation was lower and not statistically significant (placebo recipients in the SPf66 trial, aged 2–7 years, n=76 ρ=0·17, P=0·14; individuals from Namawala village, aged 1–10 years: n=32, ρ=0·35, P=0·048; Namawala inhabitants over 10 years old, n=29, ρ=–0·05, P=0·8).

Discussion

When overall patterns are deduced from a composite of different field studies there is always a danger that important causes of variation will be overlooked, but the present overview nevertheless presents a coherent picture of malaria in the rural Kilombero valley. That picture still corresponds closely to that described for holoendemic areas of Africa before the widespread use of chloroquine and is little different from that in the Kilombero valley when it was described first in 1909–1910 (CLYDE 1967) and later by the malaria service in 1953 (CLYDE, 1967). However, molecular analyses of parasite genotypes now provide us with additional malariometric indices not available in the classical descriptions of holoendemic malaria.

Malaria infection in Kilombero shows little seasonality, although there is substantial seasonality in both the entomological inoculation rate (SMITH *et al.*, 1993) and the incidence of clinical malaria in the youngest children (SMITH *et al.*, 1998), but not in the older ones (SMITH *et al.*, 1994). Data on seasonality of multiplicity of infections in the Kilombero valley are not yet available.

The high prevalence of *P. falciparum*, in comparison with other intensively studied sites, is a consequence of more frequent acquisition of infections, rather than their longer duration. Both the average recovery rate and its age distribution were very similar to those recorded using similar methods of analysis in the Garki study in northern Nigeria (BEKESSY *et al.*, 1976) and also in the Wosera area of Papua New Guinea (PNG) (GENTON *et al.*, 1995). The daily incidence of infection (conversion rate) in the Kilombero valley was about 3 times higher than that in Garki and roughly 5 times that in Wosera. All 3 studies nevertheless showed similar age dependence in the incidence of infection (possibly with a slight shift to an older age group in peak incidence of infection in the PNG study), despite the very different overall rates. These differences were consistent with the entomological inoculation rates estimated for the various sites (MOLINEAUX & GRAMICCIA, 1980 for Garki; J. Hii, unpublished data for Wosera; SMITH *et al.*, 1993, and CHARLWOOD *et al.*, 1998, for Kilombero) and suggested that variations in entomological inoculation rate are reflected, as might be expected, in the incidence of infection but not in the recovery rate (MOLINEAUX & GRAMICCIA, 1980; CHARLWOOD *et al.*, 1997).

Variations between sites in the entomological inoculation rate also seem to be reflected in the average multiplicity of infection (ARNOT, 1998). In view of the variation in multiplicity with age in both our study and that by KONATÉ *et al.* (1999), it is important in such comparisons to ensure that individuals of the same age are compared between sites. It seems likely that effects of transmission intensity will be more evident in the young, and both optical microscopy and PCR-based genotyping are probably also more reliable indicators of the true picture in young children than in adults.

Since aparasitaemic samples were excluded from the calculation of multiplicity, the age trend in mean multiplicity is not merely a structural effect of the trend in prevalence, but the interpretation of the age trends is also potentially complicated by age differences in the sensitivity of microscopy in detecting low density infections. In the study of infants (FELGER *et al.*, 1999b), 79·2% (87/109) of PCR-positive samples from community surveys were shown by microscopy to contain asexual stages of *P. falciparum*. Using the same microscopy procedures, the sensitivity of microscopy was 72·5% in the study of effects of ITNs (FRASER-HURT *et al.*, 1999). Our other studies did not provide estimates of this sensitivity because no attempt was made in them to amplify parasite deoxyribonucleic acid from microscopically negative samples. However, since the average parasite density decreases strongly with age (Fig. 5), there are many more samples with low densities close to the detection limit of optical microscopy among older than younger individuals, and hence one would expect the sensitivity of the microscopy procedures to fall with age. Other studies have also found that, in endemic areas, the sensitivity of optical microscopy compared with PCR for detecting infections decreases with age of the host (FELGER *et al.*, 1995). Adults are better able to control parasitaemia, because a long period of time is required for the accumulation of the large repertoire of immune responses involved (MCGREGOR & WILSON, 1988), or possibly because of innate factors (i.e., a more mature immune system) (BAIRD *et al.*, 1991).

A consequence of the low parasite densities in older individuals is that estimates of infection and recovery rates based on optical microscopy data from adults are probably unreliable. Even PCR-based parasite detection underestimates true prevalence if infected erythrocytes are so sparse that there is a substantial chance of there being no template in the volume of blood tested. Further studies are needed to determine conclusively whether the apparent decrease in prevalence over the older age range is indeed an artefact resulting from the more reliable detection of infection in younger individuals.

The age trend in multiplicity is also affected by age differences in the proportion, S, of genetically distinct *P. falciparum* infections within a host which are detected by the PCR–RFLP technique. S can be estimated only from longitudinal studies, and we have conducted such studies only in small children. In our study of the effects of impregnated bed nets we estimated that a greater proportion of the genotypes infecting an individual were detected in younger than in older children (61% in children 6–18 months old and 41% in children 18–24 months old) (SMITH *et al.*, 1999). If this trend continued in older individuals, it could easily account for the decrease in apparent multiplicity in older individuals (which has also been recorded in Senegal by NTOUMI *et al.*, 1995) even if the true multiplicity continued to increase with age. A very similar estimate of S was obtained using the data from the CGP 56697 trial; 146 of the 241 infections (61%) detected on day 3 by PCR–RFLP had also been detected at day 0. The median age of these patients was 2 years.

The state of chronic malaria infection experienced by adults in the Kilombero valley is characterized not only by low parasite densities but also by a lack of correlation between multiplicity of infection and parasite density. This lack of correlation could be merely a consequence of the uncertainties in determination of the parasitological status of older individuals. However, another explanation could be that, in adults, the immune system regulates the overall level of parasitaemia rather than that of individual infections, resulting in apparent competition between genotypes. Such apparent competition has been observed in mixed infections of *P. chabaudi* in mice (TAYLOR *et al.*, 1997). A similar explanation has been proposed for the lack of seasonality in parasite densities in the Kilombero valley (SMITH *et al.*, 1993).

Chronic infections with *P. falciparum* are not well understood. It has been suggested that they are maintained as a result of clonal antigenic variation (REEDER & BROWN, 1996). An indirect, monocyte-dependent mechanism could also be involved (DRUILHE & PÉRIGNON, 1997). It is important to understand not only how and when chronic infections arise, but also what are their consequences, in terms both of chronic morbidity and protective immunity, which they both elicit and maintain. Elsewhere, we show how some of these

questions can be addressed using PCR–RFLP typing of the *msp2* locus.

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