The epidemiology of multiple *Plasmodium falciparum* infections

7. Dynamics of multiple *Plasmodium falciparum* infections in infants in a highly endemic area of Tanzania

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Abstract

The force of infection and recovery rate for malaria in infants in a highly endemic area of Tanzania were analysed using polymerase chain reaction–restriction fragment length polymorphism genotyping of the *Plasmodium falciparum msp2* locus in 99 paired blood samples. Overall, new genotypes were acquired at a rate of 0·064 per day, and the average duration of infections was estimated to be 23 d. The highest recovery rates were in children under 4 months of age. The higher susceptibility of infants to clinical malaria in comparison with older children, in areas of very high transmission, may be largely a consequence of the short duration of infections which precludes the establishment of concomitant immunity. The high turnover of infections also implies that infection prevalence and multiplicity approach an equilibrium even in very young children, and calls into question the use of infant conversion rates as a measure of transmission intensity.

Keywords: malaria, *Plasmodium falciparum*, multiple infection, genotypes, *msp2* gene, force of infection, recovery rate, children, Tanzania

Introduction

Changes in prevalence of infectious agents with host age are widely used to measure the rate at which individuals acquire infections (ANDERSON & MAY, 1992). A rapid increase in prevalence with age is generally indicative of a high infection rate. Conversely, a low rate of infection results in a slow increase in prevalence with age. MACDONALD (1950) consequently suggested that the increase with age in the prevalence of asexual stages of malaria during the first year of life could be used to estimate the force of infection. This approach has more recently been revived by SNOW *et al.* (1996) as a means of evaluating effects on transmission of insecticide-impregnated bed nets.

Since *Plasmodium falciparum* infections are not lifelong, the prevalence depends on the duration of infections as well as the force of infection (λ). We recently carried out a longitudinal study of *P. falciparum* malaria in infants in a highly endemic area of Tanzania (KITUA *et al.*, 1996, 1997; CHARLWOOD *et al.*, 1998; SMITH *et al.*, 1998). By fitting reversible catalytic models (MUENCH, 1959; BEKESSY *et al.*, 1976) to data from microscopical examination of paired blood samples, we found that for the first 4 months of life the infections were of only very short duration, with much lower recovery rates (μ) in older infants; λ showed a small increase with age. In such populations, the increase in prevalence of malaria with age is more a consequence of changes in the equilibrium between infection and recovery than of gradual accumulation of infections in perfectly susceptible individuals as modelled by MACDONALD (1950).

Estimates of λ from such studies depend on the sensitivity of the method for detecting parasites, and polymerase chain reaction (PCR) techniques therefore potentially provide better estimates than does light microscopy. The PCR–RFLP (restriction fragment length polymorphism) technique used in our laboratory also offers the possibility of enumerating and genotyping multiple clones in superinfected individuals. Such techniques can therefore be used to estimate λ from the numbers and persistence of individual infecting genotypes rather than from overall presence–absence data.

Using PCR–RFLP genotyping to analyse the diversity of the *msp2* gene of *P. falciparum* infections in the children studied by KITUA *et al.* (1996), we recently found that the multiplicity of infections (i.e., multiplicity of *P. falciparum* genotypes) in infected infants was much lower than in older children (FELGER *et al.*, 1999b). We have now carried out further analyses of these data to determine whether this is a consequence of slow accumulation or of rapid clearance of infections. We consider the implications of our results for the hypothesis that clinical immunity in highly endemic areas depends on cross-protection by concomitant infections.

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Materials and Methods

Study area

The study was carried out in the village of Idete (8° 5′ S; 36° 30′ E), in Kilombero District, Morogoro Region, southern Tanzania (ALONSO *et al.*, 1994). The general features of the Kilombero area have been described elsewhere (TANNER *et al.*, 1991). Malaria (predominantly due to *P. falciparum*) is the leading cause of morbidity and mortality (TANNER *et al.*, 1991). Patterns of malaria infection and morbidity in infants in Idete have been described by KITUA *et al.* (1996, 1997), and the average entomological inoculation rate in adults in the village is about one inoculation per person per night (CHARLWOOD *et al.*, 1998). In keeping with Tanzanian Ministry of Health guidelines, malaria was treated presumptively at the village dispensary. Chloroquine was the first-line antimalarial drug at the time of the study and was dispensed free of charge.

Finger-prick blood samples were collected from an age-stratified random sample of infants (identified on a population database) at intervals of one month, using a rolling cohort design described in detail by KITUA *et al.* (1996). The present report analyses results of parasite genotyping carried out on samples collected between November 1993 and July 1994 (FELGER *et al.*, 1999b). These comprised pairs of samples collected at intervals of approximately one month from a random subsample of 99 children; in this paper only one pair of samples from each child was analysed.

Laboratory methods

Deoxyribonucleic acid (DNA) preparation and genotyping of *msp2* by PCR–RFLP were carried out as described elsewhere (FELGER *et al.*, 1999a).

Restriction endonuclease *Hinf*1 digests from paired samples from the same individuals were run alongside each other, to ensure that persisting 3D7-like genotypes were correctly identified as such (FELGER *et al.*, 1999a).

Data analysis

Consider an infant from whom parasites from 2 blood samples have been typed, with an interval of t days between them. Let $n_{0,t}$ be the expected number of distinct genotypes identified in the initial sample which are still present at time t, and $n_{+,t}$ be the expected number of genotypes not present in the initial sample but which can be identified at time t. Furthermore, assume that new infections arise at a rate λ per day, and that existing infections are cleared at a rate μ being independent of genotype. We assume that reinfection with the same genotype which has just been lost during the same interval is a very unlikely event which can be ignored. The acquisition of new genotypes can then be described by the following differential equation:

$$\frac{dn_{+,t}}{dt} = \lambda - \mu n_{+,t} \quad (1)$$

and the loss of pre-existing genotypes by:

$$\frac{dn_{0,t}}{dt} = -\mu n_{0,t} \quad (2)$$

Solving these equations for the initial condition $n_{+,0}=0$, we obtain:

$$n_{0,t} = n_0 e^{-\mu t} \quad (3)$$

and

$$n_{+,t} = \frac{\lambda}{\mu}(1-e^{-\mu t}) \quad (4)$$

To estimate the rate constants λ and μ, equations (3) and (4) were simultaneously fitted by maximum likelihood to the observed numbers of genotypes present at time t. The fitting procedure assumed both the numbers of new infections and of persistent infections to be Poisson-distributed about their expectations, and used the observed number of infections present at time 0 to estimate n_0.

A dynamic system such as this approaches an equilibrium over time, in which infections are lost at the same rate as they are gained. To determine whether the actual multiplicity at each age was more a function of the equilibrium multiplicity (λ/μ), or whether the available time since birth was a more important determinant of multiplicity, plots of λ/μ by age were compared with those of observed multiplicity (using the data of FELGER *et al.*, 1999b).

Results

Eighty-nine infections were detected in the 99 initial (baseline) samples. Fifty-one samples were uninfected, 24 contained one infection only, and the remaining 24 contained up to 5 genotypes each. Corresponding to each of these samples, a second sample, collected after a mean period of 29·5 d (SD=5·5), was tested. Twenty-eight of the original 89 infections were retained in the follow-up samples, and another 104 infections had been acquired. These data correspond to maximum likelihood estimates of λ=0·064 per day (SE=0·008) and μ=0·044 per day (SE=0·007), corresponding to an average duration of an infection (estimated as $1/\mu$) of 23 d.

Table 1. Numbers of children and numbers of *Plasmodium falciparum* infections in the different age groups

Age (months)	No. of children	No. of infections: Initial Σn_0	Retained $\Sigma n_{0,t}$	Acquired $\Sigma n_{+,t}$
0–1	19	5	1	11
2–3	30	25	5	28
4–5	16	10	5	21
6–7	10	8	3	10
8–9	15	25	7	25
10–11	9	16	7	9

The children were classified by average age into age groups of 2 months each (Table 1). Younger children were deliberately over-represented in the original sampling strategy (KITUA *et al.*, 1996).

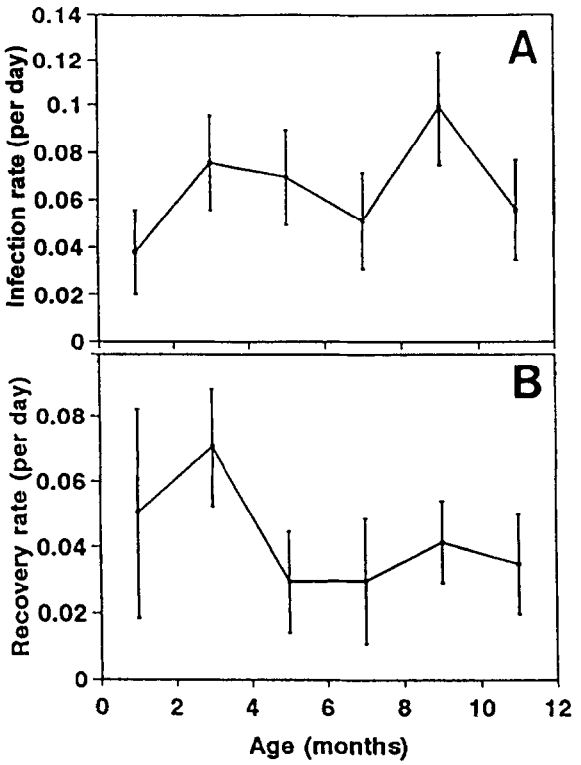

Fig. 1. *Plasmodium falciparum* infection: in children aged 1–11 months. A, Force of infection, λ; B, Recovery rate, μ. Error bars represent standard errors.

The estimated average duration of intervals between samples was similar in each age group. The maximum likelihood estimate of the force of infection (λ) was very low in the youngest age group, but showed little increase with age after the first few months of life (Fig. 1, A). The age heterogeneity was not statistically significant [likelihood ratio (LR) χ_5^2=4·4, P=0·5]. However, there was significant heterogeneity between ages in the recovery rate (μ), which was highest in children under 4 months of age (LR χ_5^2=11·3, P=0·047) (Fig. 1, B).

Consequently, the equilibrium multiplicity, estimated as λ/μ, was lowest in the youngest children (Fig. 2). This equilibrium multiplicity was in general somewhat higher than the observed mean multiplicity, but the difference was small.

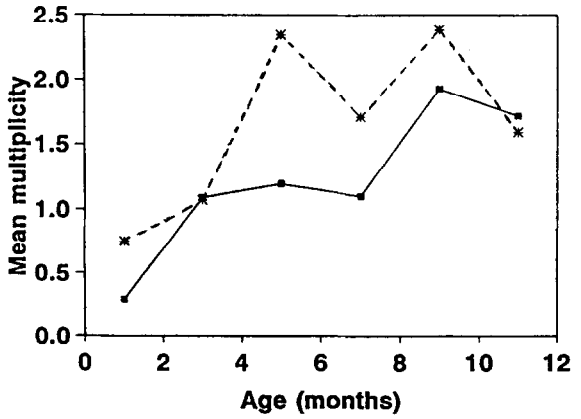

Fig. 2. Mean numbers of *Plasmodium falciparum* genotypes per sample (including negative samples) from children aged 1–11 months, showing the equilibrium numbers (λ/μ; broken line) and the observed numbers (solid line).

To test whether existing infections affect either (i) the rate at which new genotypes become established or (ii) the rate of elimination, the analysis was repeated separately for subgroups defined by the number of genotypes present in the baseline sample (n_0) (Table 2).

Table 2. *Plasmodium falciparum* infections in children: transition rates according to multiplicity of baseline infections

No. of genotypes at baseline n_0	Initial Σn_0	No. of infections Retained $\Sigma n_{0,t}$	Acquired $\Sigma n_{0+,t}$	Force of infection (λ)[a]	Recovery rate (μ)[a]
0	0	0	41	0·049(0·016)	0·044(–)[b]
1	24	9	19	0·042(0·011)	0·033(0·011)
≥2	65	19	44	0·115(0·021)	0·045(0·008)

[a]Maximum likelihood estimates (standard error in parentheses).
[b]λ and μ are not simultaneously identifiable when there is no baseline infection; the estimate of μ from the overall model was assumed in order to estimate λ.

When multiple genotypes were present at baseline, the force of infection during the interval was much higher than when a single or no genotype was present. There was little difference between the recovery rates for children with multiple infections at baseline and with single infections at baseline. (Note that these are recovery rates per genotype per day.) Hence the actual number of genotypes lost during the interval between samples was much greater when there were many genotypes present initially.

A further analysis considered whether there was a difference in estimated turnover rates depending on whether an interval lasted an even or odd number of days. The tendency was for intervals with even duration to be associated with higher turnover of parasites [λ=0·082 per day (SE=0·015), μ=0·058 per day (SE=0·011)] than those lasting an odd number of days [λ=0·051 per day (SE=0·009), μ=0·031 per day (SE=0·009)], though the difference was not statistically significant (LR χ_2^2=4·6, P=0·10).

A further factor modifying the turnover rate of infections is the use of antimalarial drugs. Chloroquine was prescribed at the dispensary for the child during 39 of the 99 intervals between paired samples (Table 3) (LR χ_2^2=14·1, P=0·0009), including 18 intervals (38%) in children less than 4 months old, and 21 in older children (42%).

The main difference between children for whom chloroquine was prescribed and those for whom it was not was in the force of infection: antimalarial prescription was associated with increased acquisition of new genotypes. The rate of clearance of infections (μ) was only a little higher among treated infants than among those with no treatment.

Alleles of *msp2* can be classified into 2 families, FC27-like and 3D7-like (SMYTHE *et al.*, 1988). Further analyses considered whether either λ and/or μ differed depending on the allelic family. Estimates of both λ and μ were nearly identical in the 2 allelic families (Table 4).

Table 3. *Plasmodium falciparum* infections in children: transition rates according to chloroquine prescription

Chloroquine	Initial Σn_0	No. of infections Retained $\Sigma n_{0,t}$	Acquired $\Sigma n_{+,t}$	Force of infection (λ)[a]	Recovery rate (μ)[a]
Yes	32	8	58	0·107(0·021)	0·057(0·013)
No	57	20	46	0·043(0·008)	0·037(0·008)

[a]Maximum likelihood estimates (standard error in parentheses).

Table 4. *Plasmodium falciparum* infections in children: transition rates according to *msp2* allelic family

Allelic family	Initial Σn_0	No. of infections Retained $\Sigma n_{0,t}$	Acquired $\Sigma n_{+,t}$	Force of infection (λ)[a]	Recovery rate (μ)[a]
3D7	37	11	49	0·031(0·006)	0·045(0·011)
FC27	52	17	53	0·032(0·006)	0·043(0·009)

[a]Maximum likelihood estimates (standard error in parentheses).

Discussion

The trends with age in both prevalence and multiplicity of *P. falciparum* during the first year of life in Idete village resulted mainly from changes in the duration of infections rather than from slow accumulation of persisting genotypes. The age-specific infection and recovery rates define age-specific equilibria in the prevalence and numbers of infections. These were only a little higher than the observed levels of infection, implying that this dynamic system is close to equilibrium and that changes in immune status account for most of the age trends in levels of infection. Since human populations vary in their responses to malaria exposure (see, e.g., MODIANO *et al.*, 1995), comparisons of infant conversion rates between populations may therefore not be a reliable indicator of transmission pressure.

Our previous analyses, based on light microscopy (KITUA *et al.*, 1996), suggested that the low prevalence of *P. falciparum* infection during the first 4 months of life is a consequence of short duration of infections rather than a low infection rate. However, because parasite densities of infected children under 4 months of age are usually very low, in the present study we considered the possibility that the high recovery rate estimated for these children from the light microscopy data was an artefact of the low sensitivity of the conventional diagnostic technique. The analyses confirmed the original conclusion that the main difference between the youngest infants and older children is in the rate of clearance of

parasites, possibly as a result of maternal antibody or the presence of fetal haemoglobin.

Failure to detect low density infections is only one of several potential sources of bias in estimates of infection and recovery rates from light microsopy data. Malaria transmission in highly endemic areas occurs disproportionately in a small proportion of the host population (WOOLHOUSE *et al.*, 1997). Such heterogeneities in transmission rates can bias estimates of the parameters of transmission models such as the catalytic model which we have used with microscopy data (BEKESSY *et al.*, 1976; KITUA *et al.*, 1996; CHARLWOOD *et al.*, 1998). These models assume homogeneous infection rate and ignore superinfections. When only parasite positivity is considered, a single continuously infected individual contributes neither to the pool of new infections nor to that of infections which have been cleared. Therefore, if all the infections are concentrated in a small number of such individuals, the number of infection events is considerably underestimated. In contrast, when genotype-specific transition data are available it may become apparent that such individuals are experiencing repeated superinfection and clearance of specific genotypes.

The overall estimate of the force of infection (infection rate), of 0·064 (SE=0·008) per day based on the PCR–RFLP data, was a little over twice that of 0·029 per day estimated from the catalytic model fitted to the light microscopy results (KITUA *et al.*, 1996). Much of this difference is probably a consequence of simultaneous inoculation of multiple genotypes. Most infected mosquitoes in the Kilombero area harbour genetically heterogenous parasite populations (BABIKER *et al.*, 1995) and hence individual infection events (which are what the attack rate estimated from the conventional catalytic model measures) involve inoculation with multiple genotypes.

Recovery rates estimated using the genotyping data are also higher than those estimated from the microscopy results. However, this may partly be an artefact caused by parasite densities temporarily falling below the limit of detection. Studies of day to day changes in parasite populations within multiply infected individuals have found frequent appearance and disappearance of genotypes (DAUBERSIES *et al.*, 1996; FÄRNERT *et al.*, 1997). The main effect of this on our models is overestimation of recovery rates derived from genotyping data. Sequestration of synchronized infections is one way in which temporary disappearance of genotypes could arise. Since the schizogonic periodicity is 48 h in *P. falciparum* (see GILLES, 1993), there should in particular be overestimation of the clearance rates if the duration of the interval between paired samples is an odd number of days. In the present study, however, the tendency was for intervals which lasted an even number of days to be associated with higher turnover of parasites. Synchronization over long intervals therefore does not seem to be an important consideration.

The multiplicity of infections in infants was much less than that found in older children both at our own study site (BECK *et al.*, 1997) and at a comparable study site in Senegal (NTOUMI *et al.*, 1995). This implies that either the duration of infections must be greater in older children, or incidence must be much higher.

The lower multiplicity in infants cannot be a consequence of greater use of antimalarial drugs. Indeed, among infants the duration of infections was longest in the 4–7 months age groups which are those with the highest incidence of morbidity and hence of drug treatment. Antimalarial prescription was concentrated in children who acquired new parasite genotypes and these are probably the ones who did indeed suffer from clinical malaria, but the chloroquine dispensed was quite ineffective in clearing these infections. This is consistent with recent findings of very high levels of chloroquine resistance *in vivo* in Idete (MSHINDA *et al.*, 1996; HATZ *et al.*, 1998).

While the youngest children are protected from clinical attacks via mechanisms unrelated to the multiplicity of infection, the vulnerability of infants 4–12 months of age in Idete to clinical malaria may well be related to their low multiplicity of infection. There is evidence that a high number of different co-infecting genotypes in older semi-immune individuals protects against clinical malaria (AL-YAMAN *et al.*, 1997; BECK *et al.*, 1997), presumably via a mechanism of partial cross-protection. Such protection does not appear to operate in infants in Idete (FELGER *et al.*, 1999b). One explanation may be that in infants non-specific responses clear infections before such more specific mechanisms become established. If this is the case, variation in μ could easily be an important determinant of the age distribution of clinical malaria in highly endemic areas, since the duration of an infection affects the type of host response which it provokes. Further studies of the age dependence of the dynamics of individual infections will be needed to evaluate this hypothesis.

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