The epidemiology of multiple *Plasmodium falciparum* infections

10. Effect of insecticide-treated bed nets on the dynamics of multiple *Plasmodium falciparum* infections

T. Smith[1]*, I. Felger[1], N. Fraser-Hurt[2] and H.-P. Beck[1] *[1]Swiss Tropical Institute, Socinstrasse 57, 4002, Basel, Switzerland; [2]Ifakara Health Research and Development Centre, P.O. Box 53, Ifakara, Tanzania*

Abstract

The rates of acquisition and loss of individual genotypes belonging to the FC27 family of the *Plasmodium falciparum* merozoite surface protein 2 (*msp2*) gene were studied in 120 children aged 5 months to 2·5 years, in a randomized controlled trial of insecticide-treated bed nets (ITNs) in Kiberege village, Tanzania. Analysis of longitudinal changes in positivity for individual alleles in samples collected at intervals of one month indicated that the average duration of infections, allowing for undetected parasite genotypes, was 73 d in those aged <18 months and 160 d in children aged ≥18 months, consistent with a shift from acute to chronic infection with age. Overall, 51% of genotypes infecting the host were estimated to be detected by polymerase chain reaction–restriction fragment length polymorphism analysis in any one sample of 0·5 μL of packed peripheral blood cells. In children less than 18 months old this sensitivity was 61% (SE=6%) compared with 41% (SE=6%) in older children. Conversely, the rate of appearance of new parasite genotypes was higher in children <18 months of age than in older children, but this partly reflected the difference in sensitivity. The overall incidence of new infections was estimated to be reduced by 17% in ITN users. There was no statistically significant difference between users and non-users in observed infection multiplicity, sensitivity, recovery rate, or estimated infection rates for individual alleles. This suggests that, in areas of high *P. falciparum* endemicity, ITNs have little effect on the establishment of chronic malaria infection.

Keywords: malaria, *Plasmodium falciparum*, multiple infection, infection dynamics, insecticide-treated bed nets, children, Tanzania

Introduction

Several molecular epidemiology studies have indicated that, in children in areas highly endemic for malaria, the number of concurrent infections with *Plasmodium falciparum* might be an important indicator of the degree of acquired immunity against malaria. In the Kilombero valley, an area of high endemicity in Tanzania, we found that multiplicity increases with age over the first few years of life in parallel with the increase in clinical immunity (SMITH *et al.*, 1999a). A high multiplicity of asymptomatic infections may indeed protect against clinical malaria (AL-YAMAN *et al.*, 1997; BECK *et al.*, 1997).

A series of recent trials has demonstrated the effectiveness of insecticide-treated bed nets (ITNs) in reducing child mortality in areas endemic for malaria (D'ALESSANDRO *et al.*, 1995; BINKA *et al.*, 1996; NEVILL *et al.*, 1996). However, it has also been suggested that long-term use of ITNs might be less beneficial because reduction in exposure to infective stages of the malaria parasite could interfere with the development of natural immunity (SNOW & MARSH, 1995; TRAPE & ROGIER, 1996; SNOW *et al.*, 1997). There is evidence that a high multiplicity of infection can provide clinical protection against super-infection (AL-YAMAN *et al.*, 1997; BECK *et al.*, 1997). One possible way in which ITNs could interfere with natural immunity might therefore be via reduction in the multiplicity of infection.

In a randomized controlled trial of ITNs in the Kilombero valley of Tanzania we found that multiplicity of infection [assessed by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) typing of the *msp2* locus of *P. falciparum*] was little affected by provision of ITNs, although the prevalences of both microscopically detectable infection and anaemia were decreased (FRASER-HURT *et al.*, 1999).

In children under one year of age from an area similar to that of the ITN trial we found high rates of both infection and clearance of infections, giving a picture of rapid turnover of the genotypes of infecting parasites (SMITH *et al.*, 1999b). One possible explanation for the similar multiplicities of infection in the ITN and control groups could therefore be that in the control group there was also a rapid turnover, with a higher rate of loss of infections than in ITN users compensating for the higher incidence of infection. This might have important implications for the development of concomitant immunity.

In the study of children under one year of age (FELGER *et al.*, 1999b; SMITH *et al.*, 1999b), we assumed that the PCR detection method was 100% sensitive. This cannot be taken for granted. In hosts of all ages, malaria parasite densities can fall below the limit of detection even by PCR, without infections being cleared, and an alternative explanation for the lack of difference between the ITN group and controls in measured infection multiplicity might be that the sensitivity of detection of individual genotypes differed between the 2 groups.

The availability of multiple (up to 6) follow-up samples from each child in the ITN trial meant that the longitudinal patterns of infection could be analysed to provide simultaneous estimates of the sensitivity of the PCR detection method, infection rates and recovery rates. We now present such estimates and consider how they vary depending on ITN use and on the age of the child. In the context of our findings we discuss how ITNs are likely to affect the acquisition of clinical immunity.

Methods

Sampling and laboratory methods

The trial was carried out in Kiberege village in the Kilombero valley of Tanzania, an area highly endemic for *P. falciparum* malaria (TANNER *et al.*, 1991; SMITH *et al.*, 1993). The design has been described in detail by FRASER-HURT *et al.* (1999). The present analyses involved repeated finger-prick blood samples collected at intervals of one month over a period of 6 months from 60 children initially aged 5 months–2 years and sleeping under ITNs and from 60 children of the same age not sleeping under ITNs. The ITNs were installed at the same time as collection of a baseline blood sample in

*Author for correspondence; phone +41 61 284 8273, fax +41 61 271 7951, e-mail <smith@ubaclu. unibas.ch>

May 1996. In the present analyses we considered only the follow-up samples collected from June to November 1996.

The *msp2* genotypes of *P. falciparum* parasites were determined by PCR–RFLP analysis (FELGER *et al.*, 1999a). The present analyses concern only parasites with *msp2* genotypes belonging to the FC27 family. 3D7-like alleles were not included because persisting infections belonging to this family could not be reliably identified since *Hin*f1 digests from samples from the same individuals were not run alongside each other in this study (FELGER *et al.*, 1999a). Data for a total of 30 different FC27 alleles were analysed (Table 1). Three sequences of observations could thus be defined, one corresponding to each allele, and each consisting of up to 6 sequential determinations of the presence or absence of the allele. In addition, we assume that an infection is detected with a probability equal to S, the sensitivity.

Estimation of the probability of recovery, M, and the sensitivity of detection, S

M and S were estimated by maximum likelihood using follow-up data for samples containing *msp2* FC27 parasites. For each sample at $t=t_0$ and positive for allele i, either the next 2 samples were considered (if the sample at $t=t_0+1$ were negative) or the next one sample only (if the sample at $t=t_0+1$ were positive, or if no sample from $t=t_0+2$ were available). The likelihood for each sub-sequence defined in this way (L_1, L_2, L_3, L_4) was determined by simple probability calculations (see Table 2). These likelihoods assume that M and S are both

Table 1. *Plasmodium falciparum msp2* genotypes of the FC27 family identified in the study children

	Carriage prevalence of genotype p_i	No. of intervals At risk N_{0i}	No. of intervals New infections $n_{1,i}$	Incidence of infection Unadjusted[a] Λ'_i	Incidence of infection Adjusted Λ_i
Allele					
D10	0·016	557	11	0·020 (0·006)	0·016
Ifa1	0·038	548	17	0·031 (0·007)	0·030
Ifa10	0·003	567	2	0·004 (0·002)	0·003
Ifa11	0·010	561	4	0·007 (0·004)	0·007
Ifa14	0·001	567	1	0·002 (0·002)	0·001
Ifa15	0·003	566	1	0·002 (0·002)	0·002
Ifa16	0·009	563	5	0·009 (0·004)	0·008
Ifa18	0·004	565	2	0·004 (0·002)	0·003
Ifa2	0·001	567	1	0·002 (0·002)	0·001
Ifa21	0·001	567	1	0·002 (0·002)	0·001
Ifa28	0·019	559	8	0·014 (0·005)	0·014
Ifa31	0·019	555	5	0·009 (0·004)	0·012
Ifa32	0·006	567	4	0·007 (0·004)	0·006
Ifa33	0·006	564	4	0·007 (0·004)	0·006
Ifa34	0·006	564	3	0·005 (0·003)	0·005
Ifa36	0·006	566	4	0·007 (0·004)	0·006
Ifa4	0·006	564	4	0·007 (0·004)	0·006
Ifa40	0·009	564	4	0·007 (0·004)	0·007
Ifa44	0·003	566	2	0·004 (0·002)	0·003
Ifa46	0·019	558	11	0·020 (0·006)	0·017
Ifa49	0·001	567	1	0·002 (0·002)	0·001
Ifa50	0·007	565	5	0·009 (0·004)	0·007
Ifa51	0·001	568	1	0·002 (0·002)	0·001
Iaf52	0·001	568	1	0·002 (0·002)	0·001
K1	0·142	490	50	0·102 (0·013)	0·104
Wos10	0·022	557	8	0·014 (0·005)	0·015
Wos12	0·274	407	65	0·160 (0·017)	0·190
Wos3	0·104	507	42	0·083 (0·012)	0·080
Wos6	0·099	510	33	0·065 (0·011)	0·070
Wos7	0·132	494	44	0·089 (0·012)	0·094
Sum[b]	0·968	16488	344	0·695 (0·004)	0·718

[a]Standard error in parentheses.
[b]Σp_i is the observed multiplicity of FC27 alleles. $\Sigma\Lambda'_i=\Lambda'$, the expected number of incident alleles, without allowance for imperfect sensitivity. $\Sigma\Lambda_i=\Lambda$, the expected number of incident alleles, with allowance for imperfect sensitivity. See text for further explanation of the symbols and see FELGER *et al.* (1999b) for the definition of each allele.

FC27 alleles (Ifa13, Ifa30, Ifa41) occurred only once each at baseline, and a fourth (Ifa43) occurred twice, but only in baseline samples. These 4 alleles were excluded because their incidence rates could not be estimated.

Definitions

Each blood sample was classified according to presence or absence of each of 30 different FC27 family alleles of *msp2* numbered $i=1, 2...30$. The processes of infection and recovery from infection were then approximated with a simple transition model (Figure) in which Λ_i is the allele-specific risk of infection (i.e., the probability of a transition from being negative for allele i at survey t_0 to being positive at survey t_0+1) and M is the probability of recovery (i.e., the probability of a transition from being positive for allele i at survey t_0 to being negative at survey t_0+1). For each child in the trial, 30

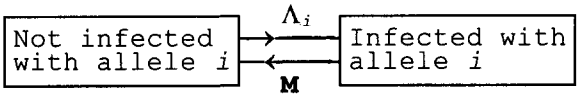

Figure. Compartment model for allele i of the *Plasmodium falciparum* gene *msp2*. The boxes indicate states and Λ_i is the allele-specific risk of infection (i.e., the probability of a transition from being negative for allele i at survey t_0 to being positive at survey t_0+1), and M is the probability of recovery (i.e., the probability of a transition from being positive for allele i at survey t_0 to being negative at survey t_0+1).

independent of the parasite genotype, and the sequence (+–+) is indicative of undetected infection at $t=t_0+1$. Hence the relative frequencies of (+–+) and (+++) sequences provide information about S. The overall likelihood as a function of M and S was calculated as the product over all sub-sequences and the maximum likelihood solution determined by a derivative-free maximization algorithm (GILL & MURRAY, 1976), using the NAG software library.

Estimation of the incidence of new infections, Λ

Unadjusted estimates of the allele-specific risks of infection, Λ_i, were calculated as $n_{1,i}/N_{0,i}$ where $n_{1,i}$ is the number of samples found negative for allele i at survey t_0 which were positive by PCR–RFLP at survey t_0+1 (see Table 1), and $N_{0,i}$ is the total number of samples found negative for allele i at survey t_0 when the child had also been sampled at t_0+1. This estimate (Λ'_i) is slightly biased due to undetected infections at both t_0 and t_0+1. An estimate corrected for this bias is

$$\Lambda_i=[\Lambda'_i(1-p_i)+(1-S)(1-M)p_i]/(S-p_i)$$

where p_i is the prevalence of genotype i.

Estimates of Λ_i corrected for bias were made by substituting the maximum likelihood estimates of M and S into this equation. The expected number of incident alleles, Λ, was then $\Sigma\Lambda_i$, summing over all the genotypes. Standard errors for Λ'_i and Λ′ were computed assuming independence of infection events. Interval estimation for Λ is not straightforward and was not carried out. For comparison with other studies, the estimates of M and Λ were converted to transition rates per day, as described in the Appendix.

Results

The frequencies of different patterns of subsequent detection of the same FC27 genotype following a positive sample are shown in Table 2. Overall, 38% of genotypes detected were also detected at the next survey. In 11% of the follow-ups, the first follow-up sample was negative for the genotype but the second was positive; 38% of follow-ups consisted of 2 negative samples, and for the remaining 13% one negative sample was available, but no second follow-up sample.

The follow-up patterns observed for ITN users were very similar to those for non-users. There were, however, substantial differences according to age. The disappearance and subsequent reappearance of the same allele was observed more frequently in older children (Table 2). This pattern indicates that the genotype probably persisted throughout, although it had not been observed in the middle sample.

Of the 16488 transitions analysed for incident infections (considering each sampling interval once for each allele), 344 (2·1%) ended with an infection of the relevant allele (Table 1). Among children <18 months of age at the time of the second sample, 190/7282 (2·6%) intervals ended with a sample positive for the allele compared with 154/9206 (1·6%) in children >18 months old (Pearsons's $\chi_1^2=17{\cdot}5$, $P<0{\cdot}0001$). Among ITN users, 167/8475 transitions (2·0%) ended with positive samples compared with 177/8013 transitions (2·1%) among non-users (Pearson's $\chi_1^2=1{\cdot}15$, $P=0{\cdot}3$). Further analyses, allowing for possible variation between alleles, gave similar test statistics.

The transition models confirmed the impression that incidence, recovery rates and sensitivities were all age-dependent but were similar in both users and non-users of ITNs (Table 3).

Likelihood ratio tests, comparing statistical models with only single parameters for either M or S with models where both M and S varied depending on whether ITNs were used, indicated that neither the difference in M between ITN users and non-users ($\chi_1^2=0{\cdot}05$, $P=0{\cdot}8$) nor that between values of S ($\chi_1^2=0{\cdot}3$, $P=0{\cdot}6$) was statistically significant. The estimated effect of the ITNs on incidence was also modest. ITNs were associated with a 13% reduction in the crude incidence summed over all alleles, Λ′ (Table 3). When adjustment was carried out for the bias caused by imperfect sensitivity, the ITNs appeared to have reduced incidence by 17% (note that the numbers in the Tables are rounded). Because Λ could be estimated much more precisely than M or S, this modest reduction in incidence associated with ITN use was clearly statistically significant.

Table 2. Patterns of *Plasmodium falciparum* infection detected by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) in samples collected following the detection of the same *msp2* (family FC27) allele in an initial sample

Pattern of infection Allele *i*[a]			No. of positive examinations followed up					
			Insecticide-treated bed nets		Age (months)			Likelihood
t_0	t_0+1	t_0+2	Non-users	Users	<18	≥18	Overall	(L)
+	+		110(37%)	101(40%)	120(41%)	91(35%)	211(38%)	$L_1=(1-M)S$
+	–	+	36(12%)	25(10%)	26 (9%)	35(14%)	61(11%)	$L_2=(1-M)^2(1-S)S$
+	–	–	113(38%)	96(38%)	118(40%)	91(35%)	209(38%)	$L_3=1-L_2-L_1$
+	–	?	38(13%)	33(13%)	31(11%)	40(16%)	71(13%)	L_4[b]$=1-L_1$
Total			297(100%)	255(100%)	295(100%)	257(100%)	552(100%)	–

[a]Presence (+) or absence (–) of allele i in 2 or 3 successive samples.The probability of reinfection with allele i at t_0+2 (given no infection with allele i at t_0+1) was assumed to be negligible.The pattern +–+ is thus assumed to be indicative of undetected infection at t_0+1.

[b]The likelihood if only one (negative) follow-up sample were available.

Table 3. Rates of recovery, detection sensitivity and incidence of infection with *Plasmodium falciparum*[a]

	Recovery probability M	Sensitivity of detection S	Incidence of infection Unadjusted Λ′	Adjusted Λ
Insecticide-treated bed nets				
Non-users	0·22(0·06)	0·47(0·06)	0·75(0·005)	0·79
Users	0·29(0·06)	0·56(0·07)	0·65(0·005)	0·65
Age (months)				
<18	0·33(0·05)	0·61(0·06)	0·89(0·005)	0·83
≥18	0·14(0·08)	0·41(0·06)	0·55(0·004)	0·67
Overall	0·25(0·04)	0·51(0·05)	0·70(0·004)	0·72

[a]See text for explanation of symbols; standard errors are indicated in parentheses.

The mean multiplicity of FC27 infections observed was 1·04 in children without ITNs and 0·90 in those with ITNs, a reduction of 14%, similar in magnitude to the reduction in crude incidence summed over all alleles. Allowing for the sensitivity, S (0·47 in non-users, and 0·56 in users, see Table 3), the true mean FC27 multiplicity in non-users of ITNs was estimated to be 2·2, and that in users to be 1·6, a decrease of 27%.

Corresponding analyses of age effects indicated that M was significantly lower in older children than in those less than 18 months old (χ_1^2=3·86, P=0·050), as was S (χ_1^2=4·18, P=0·041). Daily rates (see Appendix) corresponding to the transition probabilities in Table 3 are shown in Table 4.

Table 4. Estimated daily recovery and infection rates, and duration of individual infections, for *Plasmodium falciparum* (FC27 clones)

Age (months)	Recovery rate (μ/d)	Infection rate ($\Sigma\lambda_i$/d)	Mean duration (1/μ) (d)
<18	0·014	0·037	73
≥18	0·006	0·026	160
Overall	0·010	0·029	101

Discussion

In areas highly endemic for *P. falciparum*, the incidence of infection estimated using conventional microscopy results from longitudinal sampling increases with age in young children (MOLINEAUX & GRAMMICIA, 1980; GENTON *et al.*, 1995). This trend has been thought to be due to the increase in body surface area as children grow, making them attract more mosquitoes (PORT *et al.*, 1980). No entomological exposure data were available in the present study, but the age trend in infection rate was the reverse: the rate was lower in older children.

Much of this age variation in force of infection appeared, however, to be an artefact of decreasing sensitivity as the child ages, this decrease being presumably related to parasite densities. When infected with *P. falciparum*, infants in highly endemic areas (with the exception of those in the first few months of life) have higher parasite densities than older children (MOLINEAUX & GRAMMICIA, 1980; SMITH *et al*,. 1993, 1998a; KITUA *et al.*, 1996) and parasites usually cannot be detected by PCR in blood films found to be aparasitaemic by microscopy (FELGER *et al.*, 1999b). As children grow older, parasite densities fall and the prevalence of microscopically sub-patent parasitaemia increases (FELGER *et al.*, 1995). Our present results also indicate that the proportion of infecting parasite genotypes which can be detected even by PCR also decreases as the host grows older. This is probably because the densities of parasites with the individual genotypes in peripheral blood are so low that they fall below even the threshold of detection of PCR techniques (about 2 per μL, using our techniques).

In younger children, the effect on the analysis of undetected infections is consequently relatively small. Thus, the estimated infection rate of 0·029 FC27 infections per day was very similar to that of 0·032 per day estimated for infants (SMITH *et al.*, 1999b) assuming a sensitivity of 100%. Similarly, although the estimated average duration of infections in the present study was considerably higher (101 d) than that obtained in our study of infants (23 d), the steep age trend confirms that the youngest children have infections with very short duration. Although there is, no doubt, some bias caused by undetected infections in the estimate of duration in infants (SMITH *et al.*, 1999b), this is probably insufficient to affect the conclusion that infections in the youngest children are not very persistent. The lack of persistence of infections in infants was also not a consequence of chloroquine use (SMITH *et al.*, 1999b).

It is in older children that the problem of incomplete ascertainment of infections is likely to be more important. This is especially the case for estimates of the recovery rate in older children, made using microscopy data. Although the estimated recovery rate decreased with age in the Garki study in northern Nigeria (MOLINEAUX & GRAMMICIA, 1980), it showed little age-dependence in children in the Wosera area of Papua New Guinea (GENTON *et al.*, 1995). Our results indicate that the recovery rate for individual genotypes decreases markedly over the 5 months' to 2 years' age range. Thus, despite the very low densities of many infections in older children, these infections persist chronically for longer periods.

We conjecture that the decreased recovery rate as children age is indicative of not only a shift away from acute towards chronic infection, but also of the development of clinical immunity. Children under one year old form the age group most susceptible to clinical malaria in Kilombero (KITUA *et al.*, 1996). In these children with the highest recovery rates, acute episodes of clinical malaria are positively associated with multiplicity of infection (FELGER *et al.*, 1999a). Elsewhere we have hypothesized (SMITH *et al.*, 1999c) that, in older children and adults, low density chronic infections have a protective effect against clinical malaria and that this could explain negative relationships between multiplicity and clinical attacks (AL-YAMAN *et al.*, 1997; BECK *et al.*, 1997; FÄRNERT *et al.*, 1997).

In contrast to the pronounced effects of age, infection rates, duration of infection and sensitivity were all surprisingly unaffected by the use of ITNs. ITNs are clearly effective against anaemia and parasitaemia as assessed by microscopy (FRASER-HURT *et al.*, 1999), and this effect can only be a consequence of a reduced infection rate. Yet the estimated reduction in infection rate was modest. The effect of the ITNs on sensitivity was not statistically significant. This could be because there really was little effect on either incidence or sensitivity. Another explanation could be that, although the power of the study was inadequate to establish it, the sensitivity of the PCR–RFLP technique may indeed be lower in non-users of ITNs. There are plausible mechanisms for this. It is known that fever can synchronize malaria parasites (KWIATKOWSKI, 1989) and, if the parasites carrying a given allele are synchronized, then they may all be sequestered at the time of sampling. The higher risk of clinical malaria in children without ITNs might thus lead to a reduced sensitivity in detecting infection.

An alternative explanation is that those infections prevented by the ITNs were disproportionately those accounting for morbidity. It may be that the age dependence of multiplicity results from inherent host factors, so that the number of infections which become established depends mainly on age, and little on the inoculation rate. If this is the case, then a reduction in the reinfection rate resulting from ITN use would be compensated for by an increase in duration of the infections. In our study we did not observe such an increase in duration and the tendency was for the estimated duration of infection to be lower in ITN users. Nevertheless, such a model is supported by seasonal patterns in turnover rates of *P. falciparum* infections in Senegal (MERCEREAU-PUIJALON, 1996). There, transmission reduction during the dry season resulted in persistence of infections, in contrast to the rapid turnover during the rainy season.

If this is the case, then the effect of the ITNs would be predominantly to cut out short-term, acute, infections. Such transient infections might well be those which account for the highest parasite densities, and hence for most of both the anaemia and clinical attacks. We hypothesize that it is likely to be longer-term, chronic infections which stimulate specific immune responses and which are, in particular, involved in concomitant immunity.

Acknowledgements

We thank the people of Kiberege for their co-operation which made the study possible, and the field and laboratory staff of the Ifakara Health Research and Development Centre for their assistance. Research clearance was granted by the Medical Research Co-ordinating Committee of the Tanzanian National Institute of Medical Research through the Tanzanian Commission of Science and Technology (UTAFITI), reference no. NSR/RCA 90. The laboratory investigations were supported by the Friedrich Miescher Institute, Basel and Swiss National Science Foundation grant no. 3200-045616.95/1.

Appendix

The transition probabilities, M and Λ_i, quantify recovery from infection and acquisition of infection over time periods of one month. Corresponding to M and Λ_i, directly comparable with rates estimated in other studies are the recovery rate (per day), μ, and the allele-specific infection rates (per day), λ_i. Estimates of μ and λ_i were made from M and Λ_i by solving the following set of simultaneous equations derived from the continuous time version of the model shown in the Figure:

$$\Lambda_i = \frac{\lambda_i}{\lambda_i + \mu}[1 - \exp(-(\lambda_i + \mu)t)]$$

$$M = \frac{\mu}{\lambda_i + \mu}[1 - \exp(-(\lambda_i + \mu)t)]$$

Note that t is the average duration of an inter-survey interval in days. This gave very slightly different values of μ for the different alleles. A simple mean of these allele-specific values is presented in the results section. The average duration of an infection is then estimated by $1/\mu$.

References

Al-Yaman, F., Genton, B., Reeder, J., Anders, R. F., Smith, T. & Alpers, M. P. (1997). Reduced risk of clinical malaria in children infected with multiple clones of *Plasmodium falciparum* in a highly endemic area: a prospective community study. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **91**, 602–605.

Beck, H.-P., Felger, I., Huber, W., Steiger, S., Smith, T., Weiss, N., Alonso, P. L. & Tanner, W. (1997). Analysis of multiple *Plasmodium falciparum* infections in Tanzanian children during the trial of the malaria vaccine SPf66. *Journal of Infectious Diseases*, **175**, 921–926.

Binka, F. N., Kubaje, A., Adjuik, M., Williams, L. A., Lengeler, C., Maude, G. H., Armah, G. E., Kajihara, B., Adiamah, J. H. & Smith, P. G. (1996). Impact of permethrin-impregnated bednets on child mortality in Kassena–Nankana District, Ghana: a randomized controlled trial. *Tropical Medicine and International Health*, **1**, 147–154.

D'Alessandro, U., Olaleye, B. O., McGuire, W., Langerock, P., Bennett, S., Aikins, M. K., Thomson, M. C., Cham, M. K., Cham, B. A. & Greenwood, B. (1995). Mortality and morbidity from malaria in Gambian children after introduction of an impregnated bednet programme. *Lancet*, **345**, 479–483.

Färnert, A., Snounou, G., Rooth, I. & Björkman, A. (1997). Daily dynamics of *Plasmodium falciparum* subpopulations in asymptomatic children in a holoendemic area. *American Journal of Tropical Medicine and Hygiene*, **56**, 538–547.

Felger, I., Tavul, L., Narara, A., Genton, B., Alpers, M. & Beck, H.-P. (1995). The use of the polymerase chain reaction for more sensitive detection of *Plasmodium falciparum*. *Papua New Guinea Medical Journal*, **38**, 52–56.

Felger, I., Irion, A., Steiger, S. & Beck, H.-P. (1999a). The epidemiology of multiple *Plasmodium falciparum* infections. 2. Genotypes of merozoite surface protein 2 of *Plasmodium falciparum* in Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93**, supplement 1, S1/3–S1/9.

Felger, I., Smith, T., Edoh, D., Kitua, A., Alonso, P. L., Tanner, M. & Beck, H.-P. (1999b). The epidemiology of multiple *Plasmodium falciparum* infections 6. Multiple *Plasmodium falciparum* infections in Tanzanian infants. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93**, supplement 1, S1/29–S1/34.

Fraser-Hurt, N., Felger, I., Edoh, D., Steiger, S., Mashaka, M., Masanja, H., Smith, T., Mbena, F. & Beck, H.-P. (1999). The epidemiology of multiple *Plasmodium falciparum* infections 9. Effect of insecticide-treated bed nets on haemoglobin values, prevalence and multiplicity of infection with *Plasmodium falciparum* in a randomized controlled trial in Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93**, supplement 1, S1/47–S1/51.

Genton, B., Al-Yaman, F., Beck, H.-P., Hii, J., Mellor, S., Narara, A., Gibson, N., Smith, T. & Alpers, M. P. (1995). the epidemiology of malaria in the Wosera area, East Sepik Province of Papua New Guinea, in preparation for vaccine trials. I. Malariometric indices and immunity. *Annals of Tropical Medicine and Parasitology*, **89**, 359–376.

Gill, P. E. & Murray, W. (1976). *Minimization subject to bounds on the variables*. National Physical Laboratory, UK, Report no. NAG 72.

Kitua, A. Y., Smith, T., Alonso, P. L., Masanja, H., Menendez, C., Urassa, H., Kimario, J. & Tanner, M. (1996). *Plasmodium falciparum* malaria in the first year of life in an area of intense and perennial transmisson. *Tropical Medicine and International Health*, **1**, 475–484.

Kwiatkowski, D. (1989). Febrile temperatures can synchronize the growth of *Plasmodium falciparum in vitro*. *Journal of Experimental Medicine*, **169**, 357–361.

Mercereau-Puijalon, O. (1996). Revisiting host/parasite interactions: molecular analysis of parasites collected during longitudinal and cross-sectional surveys in humans. *Parasite Immunology*, **18**, 173–180.

Molineaux, L. & Gramiccia, G. (1980). *The Garki Project*. Geneva: World Health Organization.

Nevill, C. G., Some, E. S., Mung'ala, V. O., Mutemi, W., New, L., Marsh, K., Lengeler, C. & Snow, R. W. (1996). Insecticide-treated bednets reduce mortality and severe morbidity from malaria among children on the Kenyan coast. *Tropical Medicine and International Health*, **1**, 139–146.

Port, G. R., Boreham, P. F. L. & Bryan, J. H. (1980). The relationship of host size to feeding by mosquitoes of the *Anopheles gambiae* Giles complex (Diptera: Culicidae). *Bulletin of Entomological Research*, **70**, 133–144.

Smith, T., Charlwood, J. D., Kihonda, J., Mwankusye, S., Billingsley, P., Meuwissen, J., Lyimo, Takken, W., Teuscher, T. & Tanner, M. (1993). Absence of seasonal variation in malaria parasitaemia in an area of intense seasonal transmission. *Acta Tropica*, **54**, 55–72.

Smith, T., Beck, H.-P., Kitua, A., Mwankusye, S., Felger, I., Fraser-Hurt, N., Irion, A., Alonso, P., Teuscher, T. & Tanner, M. (1999a). The epidemiology of multiple *Plasmodium falciparum* infections 4. Age-dependence of multiplicity of infection with *Plasmodium falciparum* and other malariological indices in an area of high endemicity. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93**, supplement 1, S1/15–S1/20.

Smith, T., Felger, I., Kitua, A., Tanner, M. & Beck, H.-P. (1999b). The epidemiology of multiple *Plasmodium falciparum* infections 7. Dynamics of multiple infections with *Plasmodium falciparum* in infants in a highly endemic area of Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93**, supplement 1, S1/35–S1/39.

Smith, T., Felger, I., Tanner, M. & Beck, H.-P. (1999c). The epidemiology of multiple *Plasmodium falciparum* infections 11. Premunition in *Plasmodium falciparum* infections: insights from studies of multiple infections. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93**, supplement 1, S1/59–S1/64.

Snow, R. W. & Marsh, K. (1995). Will reducing *Plasmodium falciparum* transmission alter malaria mortality among African children? *Parasitology Today*, **11**, 188–190.

Snow, R. W., Omumbo, J. A., Lowe, B., Molyneux, C. S., Obiero, J. O., Palmer, A., Weber, M. W., Pinder, M., Nahlen, B., Obonyo, C., Newbold, C., Gupta, S. & Marsh, K. (1997). Relation between severe malaria morbidity in children and level of *Plasmodium falciparum* transmission in Africa. *Lancet*, **349**, 1650–1654.

Tanner, M., De Savigny, D., Mayombana, C., Hatz, C., Burnier, E., Tayari, S. & Degrémont, A. (1991). Morbidity and mortality at Kilombero, Tanzania, 1982–88. In: *Disease and Mortality in Sub-Saharan Africa*, Feachem, R. G. & Jamison, D. T. (editors). Oxford: Oxford University Press, pp. 286–305.

Trape, J. F. & Rogier, C. (1996). Combating malaria morbidity and mortality by reducing transmission. *Parasitology Today*, **12**, 236–240.