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Novel *Plasmodium falciparum* clones and rising clone multiplicities are associated with the increase in malaria morbidity in Ghanaian children during the transition into the high transmission season

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SUMMARY

A survey of *Plasmodium falciparum* infection and clone multiplicity in Ghanaian children was carried out to study the effect of the onset of the malaria transmission season on disease incidence. Fortnightly blood samples were collected from 40 children living in the rural town of Dodowa, between February and August 1998. *P. falciparum* parasite densities were calculated and PCR genotyping was carried out using the polymorphic MSP-1 and MSP-2 genes as target loci for the estimation of the number of parasite clones in each sample. The average clone number was estimated using maximum likelihood techniques and the minimum number of clones per patient was analysed for the effects of age, sex, season, minimum number of clones per child, level of parasitaemia and parasite genotype. The statistical analysis indicated that the more clones a child carried, the more likely they were to have a clinical malaria episode. This was true after adjusting for age and season effects and for the measured circulating parasitaemia. The probability of clinical disease also increased if the MSP-1 MAD 20 and the MSP-2 FC 27 alleles were present. This longitudinal analysis thus indicates that the probability of a Ghanaian child having a symptomatic malaria episode is positively associated with both increasing numbers and novel types of *P. falciparum* clones.

Key words: malaria, *Plasmodium falciparum*, clone multiplicity, virulence, seasonal transmission, Ghana.

INTRODUCTION

In areas of holo-endemic malaria transmission in West Africa, although infections are present throughout the year, there is a marked seasonal effect on disease incidence. Morbidity and mortality are concentrated in the periods of maximum mosquito population size and parasite inoculation rate, during and just after the rainy seasons (Greenwood *et al.* 1987; Lines & Armstrong, 1992). However, how the 'transmission season effect' – the observed increase in the virulence of malaria infections induced by increased parasite inoculation rates, can be accounted for in terms of super-infection, clone multiplicity and immunity remains unclear. In an attempt to clarify this relationship between clone multiplicity, clinical disease and the malaria transmission season we have carried out a longitudinal study in which blood samples were collected from a cohort of Ghanaian children during the dry season–rainy season transition when malaria transmission intensifies. These samples were typed for allelic polymorphisms at 2 loci in order to obtain an estimate of the number of parasite genotypes per patient. This permitted analysis of the effects of genetic complexity of *P. falciparum* infections on the probability of suffering a clinical malaria episode, while taking into account the effects of age, sex, parasitaemia and the seasonal transmission cycle.

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MATERIALS AND METHODS

Study area

The study was conducted in Dodowa, the main town of the Dangbe West district of Ghana, 50 km to the north east of the Ghanaian capital, Accra. Dodowa is a predominantly farming community with a population of 6600 (Appawu *et al.* 1997). Dodowa is 15 km from the Atlantic Ocean and was once surrounded by coastal forest, lying between the coastal savannah and secondary forest areas to the north although little of the virgin forest remains. Mean annual temperature is around 26 °C and rainfall is seasonal with 2 peaks occurring in June

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and September. *P. malariae* infections are occasionally seen but most malaria cases are caused by *P. falciparum*, transmitted mainly by *Anopheles gambiae sensu strictu*.

Study population and design

Surveillance and treatment of clinical malaria cases in Dodowa was carried out by community health worker-based case detection supplemented with fortnightly cross-sectional surveys conducted between February and August 1998. Prior to the start of the work it was established that none of the study cohort of 110 children carried the sickle cell allele of the alpha haemoglobin gene. Ethical clearance for the study was obtained from the Review Board of the Ghanaian Ministry of Health. Parental consent for recruitment of the children was carried out at a local malaria clinic operated by the Noguchi Institute and the children were monitored for clinical symptoms by daily visits from 3 resident field workers. Treatment was initiated immediately after microscopical confirmation of diagnosis. Fingerprick blood samples were also collected at diagnosis and further blood films were made on Days 3 and 7 after the initiation of treatment to monitor clearance of parasitaemia. Filter paper samples were kept in individual plastic bags and stored at –20 °C. At the end of the study, 19 children had at least 1 clinical episode of malaria. At this point, samples from 21 children were randomly selected from the remaining cohort to act as a control group. This group had approximately the same age and sex distribution as those in the clinically ill group.

DNA extraction and PCR analysis

The bi-monthly blood samples from the 40 children in this study were PCR genotyped to estimate the number of parasite clones present in each sample. Laboratory-cultured *P. falciparum* strains (HB3 and 3D7) were used as positive controls. Parasite DNA for PCR was extracted using published protocols for liquid and filter paper-bound blood (Foley, Ranford-Cartwright & Babiker, 1992; Wooden, Keyes & Sibley, 1993). Submicroscopically patent *P. falciparum* parasitaemias were detected by nested amplification of the rRNA gene (Snounou *et al*. 1993). Infections were typed for MSP-1 and MSP-2 gene alleles as published (Roper *et al*. 1996, 1998).

Statistical analysis

The data were analysed with 2 major questions in mind. First, what factors, both permanent and transient, were associated with a clinical episode? Second, how did the complexity of infection (number of clones per host) change through the transmission season and with what consequences to illness?

Individual children were classified into 2 groups – those who remained asymptomatic throughout the study period (called 'asymptomatics'), and those who experienced at least one clinical episode during the study (called 'symptomatics'). Before analysis, records from the symptomatic children after their first clinical malaria episode were discarded because drug treatment was expected to affect the results. The remaining records were then classified into those taken at the malaria attack (termed 'clinical') and those taken prior to this episode (termed 'pre-symptomatic').

The probability of having a clinical episode was analysed in SAS (SAS, 1997) as a binomial variable using the PROC GENMOD procedure with the REPEATED option. The PROC GENMOD procedure performs linear regression analysis on the logit-transformed scale in order to map the binomial zero-one scale to the real scale i.e. the $-\infty$, range. The REPEATED option makes allowance for the fact that several observations were taken on the same child, thus effectively reducing the number of independent data points in the analysis below that of the actual number of data points. This allowance is achieved by incorporating a correlation matrix into the model which reflects the similarity between successive records on the same child. The stronger these correlations, the lower the information content of each individual record, and hence the greater the reduction in the effective residual degrees of freedom and increase in the residual error. Under these models, significance levels are determined by chi-squared tests with appropriate adjustments for the correlation structure of the data, and for any over- or under-dispersion of the binomial data.

Using this method, first a basic model with fixed effects for age, sex and month was fitted to test for the influence of these factors. Following this, to investigate the influence of additional factors on the probability of illness, this model was extended to include an additional fixed effect for the following factors: (i) the minimum number of clones in the infection (m), defined as the maximum number of detectable alleles at either of the 2 loci, (ii) the log parasitaemia, fitted as a linear continuous covariate, and (iii) the presence versus absence of each of the 3 MSP-1 and 2 MSP-2 alleles. Each of these effects was fitted separately, but a further model was fitted which included both the number of clones and log parasitaemia. Records where parasites were detectable by PCR but not by microscopy were excluded from the latter analyses because symptomatic children were classified as such partly on the basis that they were slide positive, whereas asymptomatic children were defined by being either PCR or slide positive. Thus the effects of parasitaemia on the

probability of becoming ill would be biased if these data had not been excluded.

The improvement in fit of the model to data was assessed by likelihood ratio tests assumed to be distributed as a chi-squared with degrees of freedom equal to the difference in the number of parameters estimated by the model. Significance tests for individual effects were based on Wald chi-squared statistics after adjusting for the correlations between repeated records on the same child, and over-dispersion in the binomial distribution as estimated during the model-fitting procedure.

For analyses of the factors affecting the complexity of infection, two measures of the number of clones per host were analysed – the actual number of clones (μ) as estimated by maximum likelihood (Hill & Babiker, 1995), and the minimum number of clones detected (m), as defined above. The maximum likelihood method takes account of the fact that the typing system used does not detect >3 clones but uses information on population allele frequencies to obtain estimates of the true number of clones. A conditional (i.e. excluding zeroes) Poisson distribution for the number of clones was assumed. Separate analyses were performed on all records from the asymptomatic group, on records from the symptomatic group at the times when the child was pre-symptomatic, and records from the symptomatic group when the child was ill. Mean clone number and allele frequencies were also estimated separately for each month in the asymptomatic and symptomatic children. The 95% confidence intervals around μ over all months were constructed based on a drop in the likelihood ratio (Hill *et al*. 1995). However, as samples were not independent across the months, these represent underestimates of the sampling error.

A drawback of the maximum likelihood method for comparing mean clone numbers between the groups is that confounding factors, such as age or sex, cannot be taken into account. Estimates of mean clone number and allele frequencies may therefore be affected by any systematic influences that these factors may have. Therefore, the second measure of clone number – the minimum number of clones detectable in the blood (m) – was analysed by linear regression fitting fixed effects for age, sex and month, with a random effect for child to allow for correlations between repeated records on the same individual. An additional effect for log parasitaemia was incorporated into the model to determine its relationship with clone number. These analyses were carried out assuming a Poisson distribution (log transformation) using SAS, as described above.

RESULTS

Parasite point prevalence in dry and wet seasons

The parasite rate in the cohort ranged between 61 and 79% at each fortnightly survey, a normal situation in this area of holo-endemic transmission. Nineteen children had an episode of clinical malaria during the study, as defined by observed fevers $>37{\cdot}5$ °C together with symptoms suggestive of malaria such as headaches, vomiting or diarrhoea and a concurrent *P. falciparum*-positive slide. Three of the 19 children had 2 distinct disease episodes while the other 16 had 1 clinical episode. Twenty-one continuously asymptomatically infected individuals were randomly selected to constitute a control group for the 19 affected children. Fig. 1 summarizes the average monthly parasite point prevalence from February to August 1998 starting in the dry season, continuing through the start of the rainy season in May and ending after the peak of the rainy season in August. PCR assays confirmed the microscopical diagnosis and also revealed additional infections (between 10 and 20%, depending on the month) that were not detected microscopically. There was a small decline in the number of infected individuals from March to May and then a rise in June and July before falling again in August as drug treatment of symptomatic cases reached its maximum. The mean age of those continuously asymptomatic was 7·5 years compared to an average age of 5·4 years in those who had symptomatic malaria during the study.

Individual infection profiles during study period

Fig. 2 shows the asexual parasitaemias observed in each of the 40 children from February to August 1998. During this period a total of 22 symptomatic episodes of malaria were recorded in 19 cohort members. Estimated parasitaemia at malaria diagnosis ranged widely from 100 to $>200\,000$ infected red blood cells/μl of blood. (Parasitaemia at the survey points in asymptomatic children, ranged from around 100 to 43 000 parasites/μl of blood). Most clinical cases (14/22) cleared their parasites 3 days after starting treatment with chloroquine. Five cases cleared by Day 7 after the start of treatment. Three children were clinically cured but remained microscopically positive on Day 7 after treatment. They were considered to have chloroquine-resistant parasites and were treated with a course of sulphadoxine/pyrimethamine. However, only 7/22 had actually cleared all parasites by the 7th day when assayed by PCR.

Factors affecting the probability of becoming clinically ill

Table 1 shows the effects of age, month and sex on the probability of becoming ill. Correlations between successive records on the same individual were close to zero so that the effective number of records approached that of the actual number of records,

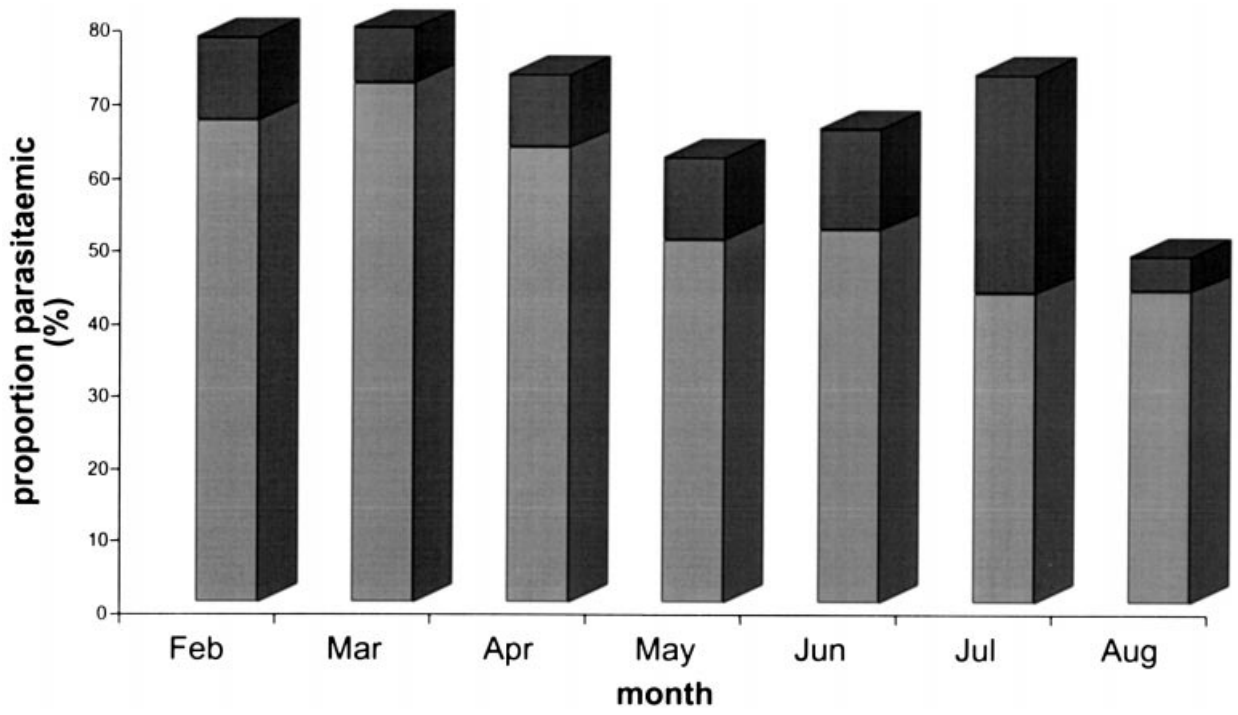

Fig. 1. The prevalence of *Plasmodium falciparum* infections in a cohort of 40 children in Dodowa during the months between February and August 1998. Infections were detected by blood film examination and confirmed in nested PCR assays amplifying the *P. falciparum* rRNA genes (light shaded bar). Additional infections detectable only in the PCR assays are shown in darker shading.

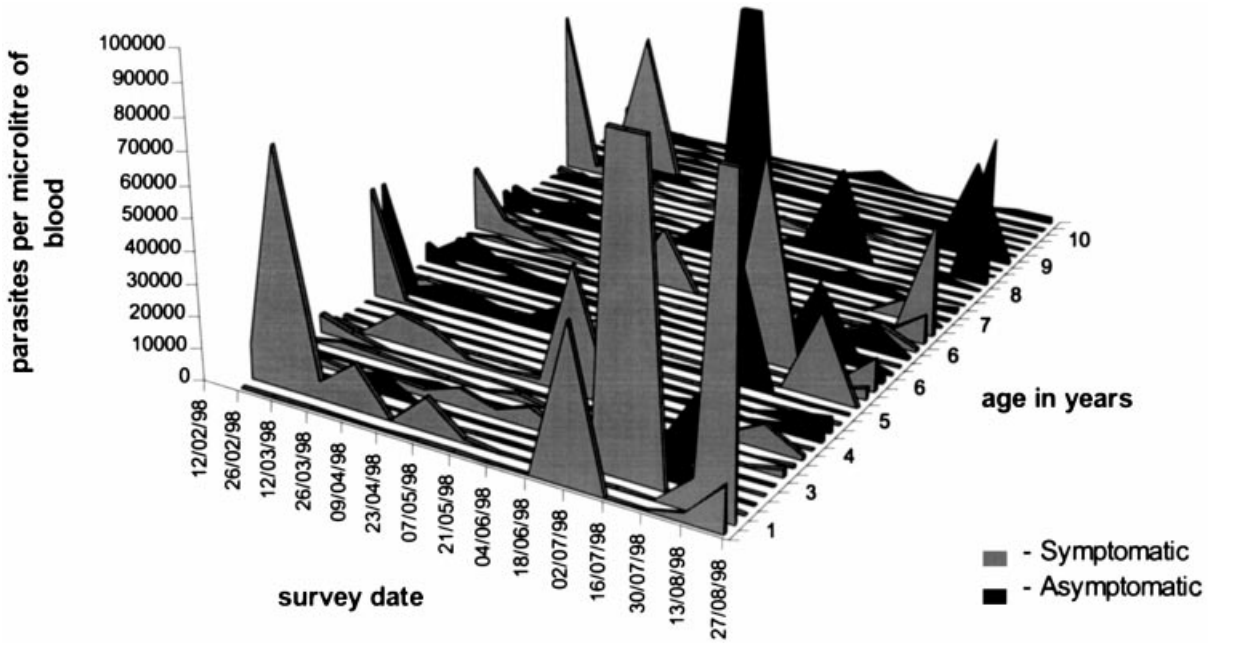

Fig. 2. Fluctuations in asexual parasitaemia in the 40 cohort members during the survey period. Parasitaemias measured in children who remained asymptomatic throughout the study are shaded black. Parasitaemia measured in the group of children who had a malaria attack during the study period are shaded grey. The truncations observed in the peaks on 02/07/98 and 13/08/98 are due to a parasitaemia in excess of 100000/μl of blood.

giving considerable sample sizes for each class, hence justifying the use of chi-squared statistics for significance testing. In comparison to 10-year-olds, the younger age classes, especially 1-year-olds, had significantly higher risks of becoming clinically ill with malaria. This risk generally declined with age although the pattern was variable, probably partly due to the small numbers of children per age class. The risk of illness increased significantly between February and June, the period covering the start of the transmission season, after which it declined. There was also a significant difference, $P < 0{\cdot}01$,

Table 1. Effects of age, month and sex on the probability of having a clinical malaria attack in Ghanaian children

Factor	No. of records	No. of children	Relative probability (odds ratio)†
Age (years)			
1	7	1	92·1***
2	325	3	33·8***
3	27	3	15·4***
4	32	3	12·1***
5	67	5	6·8**
6	89	8	11·8***
7	31	3	4·0
8	54	4	N.E.
9	26	3	52·3***
10	82	7	1
Month			
February	75	—	1
March	76	—	4·1*
April	69	—	N.E.
May	64	—	12·0***
June	62	—	27·4***
July	71	—	18·8***
August	23	—	N.E.
Sex			
Male	203	19	1
Female	237	21	3·1**

† Estimates are expressed as odds ratios relative to the reference class indicated with a 1. They were obtained by logistic regression analysis fitting all factors simultaneously, allowing for repeated observations on each child.
N.E., Not estimable due to there being zero records of clinical attacks in this class.
* Significant differences from the reference class are indicated by: * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$. The overall model fit was highly significant ($P < 0{\cdot}001$) by a likelihood ratio test.

Table 2. Effects of parasitaemia, number of clones per host and the presence of specific MSP-1 and MSP-2 alleles on the probability of having a clinical malaria attack after adjustment for age, sex and month effects

Factor		No. of records	Relative probability (odds ratio)†
Log parasitaemia‡§		286	7·64*** (5·85***)
No. of clones¶‖			
1		42	Small***
2		142	1
3		102	8·70*** (4·78***)
MSP-1 allele¶			
RO33	No	103	1
	Yes	263	1·43
MAD20	No	176	1
	Yes	190	Large***
K1	No	83	1
	Yes	283	2·37
MSP-2 allele¶			
FC27	No	112	1
	Yes	254	11·2***
IC1	No	54	1
	Yes	312	0·25***

† Estimates were obtained by fitting a logistic regression model fitting age, sex, month, and only one of the above factors, while allowing for repeated records on the same child. Figures in parentheses show estimates when both log parasitaemia and number of clones per host were included in the model. Small and Large indicate where the odds ratio was not estimable because 0 or 100% of the observations, respectively, came from children with a clinical attack. Significance tests in these cases came from Fisher's exact test. Significance levels are indicated by *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.
‡ Data include only slide-positive records.
§ Fitted as a linear covariate.
‖ Minimum clone number (m).
¶ Data include only PCR-positive records.

between the sexes (females more likely than males) in the probability of becoming ill.

Table 2 shows the effects of parasitaemia, parasite clone multiplicity and the presence of particular alleles on the probability of having a slide-positive clinical malaria attack, after accounting for age, sex and month effects. Among slide-positive patients, there was an increase in the frequency of illness from 0% when a minimum of 1 clone was present ($m = 1$) to 2·8% when $m = 2$, and a further significant ($P < 0{\cdot}001$) increase to 13·7% when $m = 3$ ($P < 0{\cdot}001$, Table 2). Among these same patients, there was a significant positive relationship between parasitaemia and the probability of becoming ill ($P < 0{\cdot}001$). Since there was also a significant positive relationship between the number of clones and parasitaemia ($P < 0{\cdot}001$), both of these factors were included in the analysis of clinical illness to see whether parasitaemia could fully explain the effect of clone multiplicity. The outcome was a small reduction in the magnitude of both effects, but the significance levels for each remained high ($P < 0{\cdot}001$). Thus the presence of more clones and more parasites was positively associated with clinical malaria episodes, and despite the positive association with each other, each had an independent effect on the probability of becoming ill. Note that this effect did not arise due to the fact that having a parasite-positive slide was required for definition as clinically ill because slide-negative data were excluded from these analyses.

It was also found that the presence of the MAD 20 allele of MSP-1, and the FC 27 allele of MSP-2 were positively associated with illness, while the presence of the IC1 allele of MSP-2 was negatively associated with illness (Table 2). In fact, 19/19 of the infections sampled at the point of illness contained parasite clones with the MSP-1 MAD 20 allele compared with only 9/19 (47%) containing this allele when these same children were asymptomatic ($P < 0{\cdot}001$,

Table 3. Estimates of mean clone number (μ), mean clone minimum clone number (m) and allele frequencies for each month and over all months for asymptomatic and symptomatic patients

Month	No. of records	μ*	m†	Allele frequency* MSP-1 RO33	MSP-1 MAD20	MSP-1 K1	MSP-2 FC27	MSP-2 IC1
Asymptomatic patients								
2	34	3·34	2·02	0·45	0·13	0·32	0·44	0·56
3	31	3·02	1·89	0·31	0·14	0·55	0·43	0·57
4	32	3·11	1·96	0·33	0·14	0·53	0·30	0·70
5	25	2·68	1·94	0·40	0·14	0·46	0·35	0·65
6	30	3·47	2·01	0·30	0·16	0·54	0·23	0·77
7	39	2·77	1·88	0·45	0·10	0·45	0·42	0·58
8	16	2·47	1·99	0·11	0·31	0·58	0·47	0·53
Overall	207	2·94	1·89	0·35	0·14	0·51	0·37	0·63
Symptomatic patients								
2	36	4·12	2·51	0·32	0·34	0·34	0·37	0·63
3	35	3·98	2·53	0·37	0·34	0·29	0·42	0·58
4	27	3·40	2·33	0·39	0·27	0·66	0·38	0·62
5	23	3·82	2·38	0·40	0·28	0·32	0·57	0·43
6	21	4·55	2·77	0·31	0·29	0·50	0·54	0·46
7	13	3·96	2·33	0·37	0·27	0·36	0·38	0·62
8	0	—	—	—	—	—	—	—
Overall	155	3·89	2·53	0·35	0·32	0·33	0·44	0·56
Pre-symptomatic‡	136	3·77	2·35	0·37	0·30	0·33	0·40	0·60
Clinic§	19	6·46	2·73	0·26	0·42	0·32	0·79	0·21

* Maximum likelihood estimates.
† Estimated by regression analysis, adjusted for age and sex.
‡ Estimates are based on data during the period before the child became symptomatic.
§ Based on data when the child was symptomatic.

using Fisher's exact test). For the FC 27 allele, the equivalent figures were 18/19 (95%) versus 68% ($P < 0·05$), and for the IC1 allele 13/18 (68%) versus 86% ($P < 0·05$). Thus there appeared to be a very marked influence of parasite genotype on the probability that the infection would develop into symptomatic malaria. Interestingly, the MSP-1 allele frequency distributions in Dodowa infections are around 1/3 : 1/3 : 1/3, a feature also noted in other studies of MSP-1 allele frequency distributions and indicative of perhaps selection in maintaining these polymorphisms in the parasite population (Conway *et al*. 2000).

Mean clone numbers and allele frequencies over time

Table 3 shows the maximum likelihood estimates of mean clone number (μ) and allele frequencies for the asymptomatic and symptomatic children for each month. Means for minimum clone number (m), adjusted for age and sex, are also shown for comparison. These underestimated μ quite severely, especially as m approached 3 when the detection limit in the 3-allele typing system was reached. Strikingly, the clone number was, on average, higher in the symptomatic group than in the asymptomatic group ($P < 0·001$), and higher still when the children in the symptomatic group were clinically ill ($P < 0·01$). Because of the inability to account for repeated records in the maximum likelihood analysis, these statistical tests are based on the analysis of m and were the same whether the trait was analysed as a Poisson variable or a normal variable. These significance levels are also consistent with those based on the confidence limits around μ (not shown) although the latter were expected to be underestimates. This is because the repeatability (correlation between repeated records) of m was quite low (0·3).

The frequency of the MSP-1 MAD 20 allele was higher in the symptomatic group than the asymptomatic group, and even higher when those children were clinically ill. The frequency of the MSP-1 K1 allele was lower in the symptomatic group than in the asymptomatic group regardless of whether they were clinically ill at the time of sampling. The frequency of FC-27 was similar in the 2 groups, but was higher when children in the symptomatic group became ill compared with when they were asymptomatic. Significance tests of differences in maximum likelihood estimates of allele frequencies were not performed because of the strong correlation between repeated records on the same individual for the presence or absence of each allele. The tests would therefore exaggerate the importance of the differences. Furthermore, allele frequencies could

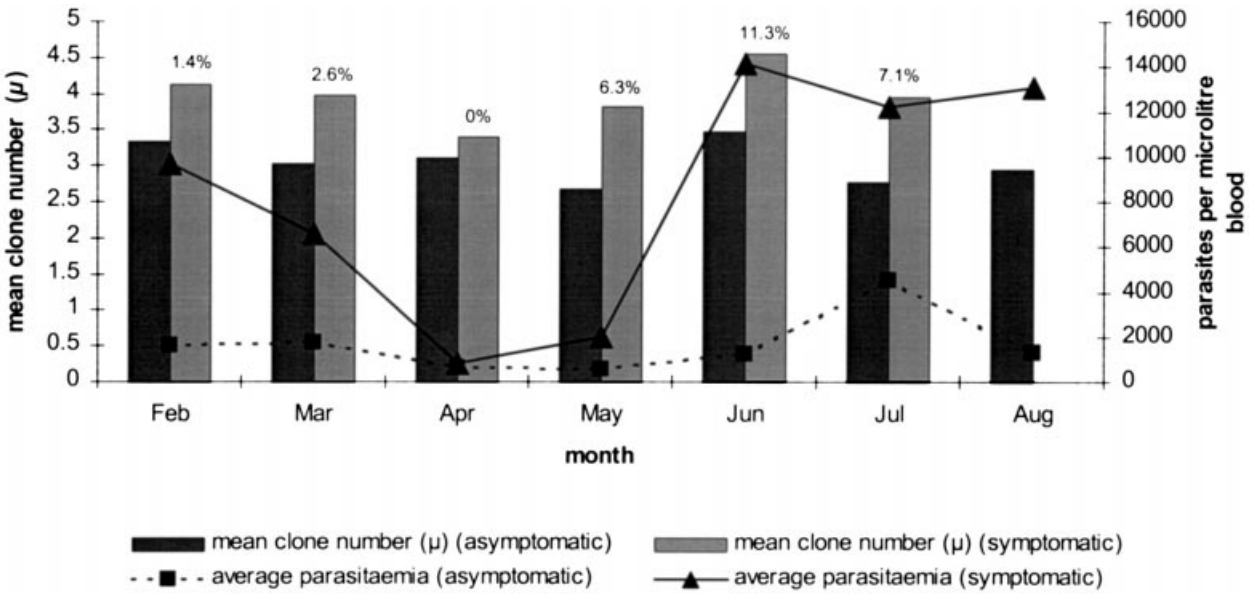

Fig. 3. Average monthly allelic frequencies and the maximum likelihood derived clone number estimations in the group of children who had a malaria-attack during the study period and in those who remained continuously asymptomatic throughout the survey in Dodowa. The average monthly allelic frequency records in the continuously asymptomatic individuals and in the group who had clinical malaria during the study are shown in broken and continuous lines respectively. The maximum likelihood derived clone number estimations for the two groups are shown as black and grey bars respectively.

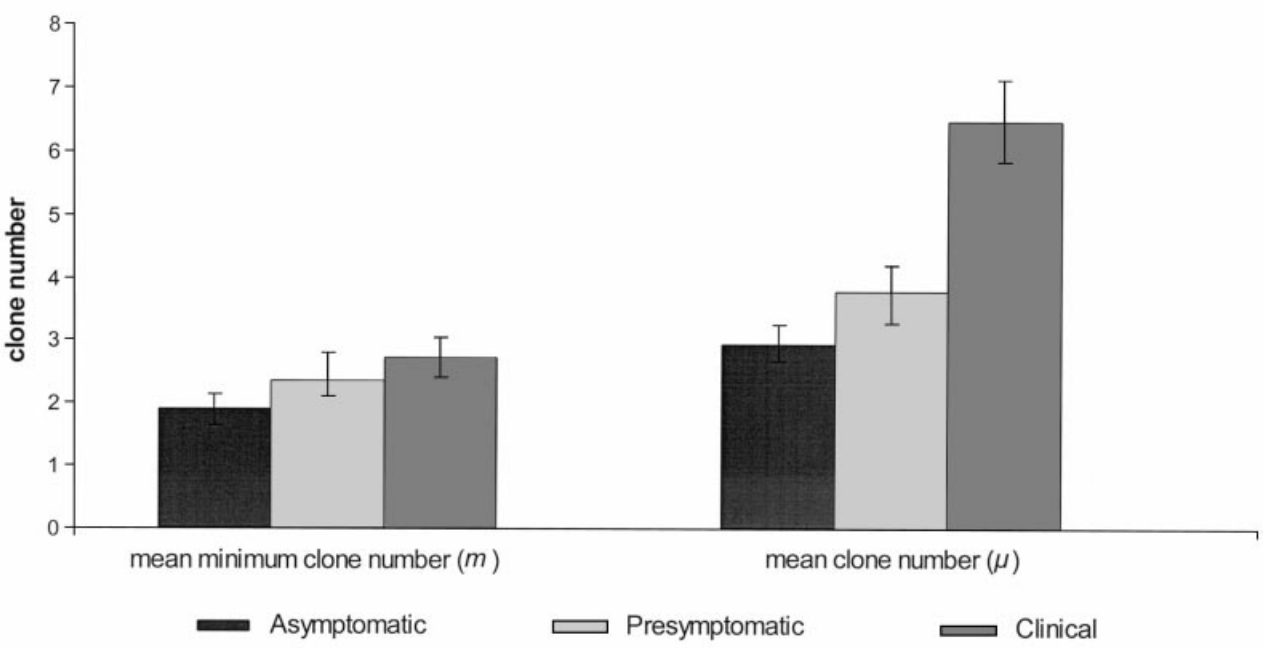

Fig. 4. Maximum likelihood-derived clone number estimations and mean minimum clone numbers of the continuously asymptomatic individuals (black bars), the pre-symptomatic samples of individuals who had a malaria attack (grey bars) and pre-treatment samples collected during clinical attacks (dotted bars).

not be analysed accounting for repeated records because of the occurrence of multiple alleles per host.

Figs 3 and 4 summarize all the observations on the relationship between clone number and patient clinical status, whether calculated as a maximum likelihood derived mean clone number or as a mean minimum clone number. The percentages given above the bars in Fig. 3 indicate the proportion of the cohort experiencing clinical illness in that particular month of the survey. In summary, these analyses showed that symptomatic children carried more clones than their asymptomatic counterparts (3·9 versus 2·9) and notably more when they were clinically ill (average $\mu = 6{\cdot}5$) and that MAD 20 and FC-27 alleles were associated with this illness. Both of these results are consistent with the earlier analysis of the probability of becoming ill (Table 2).

DISCUSSION

This study has revealed that the following factors significantly affected the probability of a child in Dodowa suffering clinical malaria during the 1998 transmission season. (i) The risks of suffering clinical malaria declined with age. (ii) Children with higher levels of asymptomatic parasitaemia had a higher risk of subsequently suffering clinical malaria. (iii) Children carrying higher numbers of parasite clones had a higher risk of clinical attacks. Mean clone number (μ) was always higher in the malaria-affected group than in the asymptomatic group, particularly when children were actually clinically ill. (iv) Based on assays of the clinical infection sample, the presence of clones carrying particular alleles of merozoite surface protein genes (MSP-1 MAD 20 and MSP-2 FC-27) was strongly associated with disease episodes although overall allele frequencies in the study population changed little over the course of the study.

The acquisition of natural immunity to malaria is not well understood and measurable correlates of protection and immunity have been difficult to identify. As it has become clear that human malaria infections are frequently composed of genetically diverse populations of parasites, the relationship between the number of different parasite clones in infection samples (multiplicity) and immunity and pathology has come under scrutiny. A number of possible relationships between multiplicity and virulence are conceivable and 4 distinct types of models have been considered in recent years.

The simplest multiplicity/virulence relationship is that there is a direct diversity effect (represented by polymorphic genes) such that more complex infections presenting more antigenic variants are less easily controlled and more pathogenic. Such a model is certainly consistent with controlled laboratory experiments showing that induced infections with 2 distinct clones of rodent malaria parasites are more virulent (measured by parasitaemia, anaemia and weight loss in the host) than single-clone infections (Taylor, Mackinnon & Read, 1998).

The second class of model derives from theories of kin and group selection, specifically aimed at optimizing tradeoffs between high parasite asexual growth rates and sustainable transmission (Hamilton, 1972; Frank, 1992, 1996). These models predict that where mixed-genotype parasitism occurs, natural selection may favour levels of virulence that are higher than those optimal for single genotype infections (Bremermann & Pickering, 1983; Nowak & May, 1994; May & Nowak, 1995). Thus, if a parasite has the capacity to adopt a facultative strategy of increasing its growth (and thus increasing the rate of host exploitation) when in the presence of competing unrelated clones, then more virulence will result from multiple than single-clone infections. Direct evidence for facultative adoption of less prudent host exploitation strategies (which require that new parasites can detect existing infections) has been difficult to obtain. However, such models imply that *P. falciparum* infections, irrespective of clone multiplicity, may have evolved relatively high levels of intrinsic virulence because immune selection for antigenic diversity in the parasite population necessarily leads to inter-clone competition. Increases in clone multiplicity might therefore be associated with increased virulence in human infections (Read *et al*. 1998).

The third class of model proposes that the multiplicity/virulence relationship may change as the immune status of the host develops (Smith *et al*. 1999). It proposes that chronic multi-clonal infections actually provide protection against super-infection, a development of the concept of 'premunition' by continuous infection, first proposed by Sergent & Parrot (1935). This could lead to higher clone numbers in individuals who have established clinical immunity than in those who have not. However, whether established clones give protection against disease to the host by 'preventing super-infecting clones from becoming established or via tolerance of the new infections' is uncertain (Smith *et al*. 1999). Thus whether 'clonal premunition' leads to chronic infections composed of very many clones, or relatively few, is unclear. Experimental resolution of this question awaits application of more quantitative PCR measurements of clone numbers (Arnot, 1998).

The clonal premunition theory is based on analysis of a number of field studies of clone multiplicity under different conditions of age, morbidity and transmission. During clinical malaria episodes, older children generally have been observed to have lower measured clone multiplicities than equivalent groups of asymptomatically infected children (Engelbrecht *et al*. 1995; Robert *et al*. 1996; Farnert *et al*. 1997). Higher multiplicity in older children has also been correlated with reduction in risk of contracting clinical malaria (Al Yaman *et al*. 1997). Higher average multiplicities were also found in relatively malaria-protected sickle cell heterozygotic as opposed to normal (HbAA) Gabonese children (Ntoumi *et al*. 1997) and in SPf66 immunized children as opposed to children given another vaccine (Beck *et al*. 1997; Haywood *et al*. 1999).

However, higher clone numbers were not associated with protection in Tanzanian infants (1-year-olds), where those experiencing a febrile episode actually had significantly higher mean multiplicities than asymptomatic neonates (Felger *et al*. 1999). Nor were higher clone numbers associated with protection in areas of very low endemicity such as Sudan (Roper *et al*. 1998). Further, as age (and presumably immunity) increased in a Senegalese study, a decrease in clone multiplicity was observed

(Ntoumi *et al*. 1995). To explain the apparent contradiction between these studies, the clonal premunition theory proposes that higher multiplicity in older Tanzanian children is due to longer clone survival periods after age and transmission intensity-dependent transitions in the mechanism of control of parasitaemia have occurred. After this transition, infections cease to be controlled by non-specific, morbidity-inducing mechanisms such as fever and cytokine production but by less sterilizing, less pathogenic, clone and antigen-specific responses (Smith *et al*. 1999). Such a mechanism could, therefore, explain the positive relationship between clone number and disease in those with incomplete immunity and the negative relationship measured in older and more immune individuals.

A fourth possible hypothesis to explain the marked increase in morbidity as the inoculation rate rises makes no predictions about the relationship between clone multiplicity and virulence but merely proposes that increased rates of infection by novel clones cause morbidity to rise. Such clones may have higher virulence (Gupta & Day, 1994), or they may simply have hitherto unencountered antigenic specificities (Bull *et al*. 1999; Giha *et al*. 2000). With one exception, we observed sharp increases in average parasitaemias and multiplicity only in the group of children who actually suffered malaria. Interestingly, these clinical malaria episodes were significantly associated with the presence of the MSP-1 MAD 20 allele and the MSP-2 FC27 allele. The transmission season increase in malaria morbidity in some Dodowa children thus appears linked to the appearance of 'new' clones carrying a higher frequency of certain alleles than was present in the parasite population infecting these children earlier in the year. Children who did not get malaria either avoided new inoculations or were more immune to the 'new' clones. This study, therefore, provides novel and direct experimental evidence for the importance of super-infection with genetically different clones in causing increases in malaria morbidity during the transmission season.

Malaria is holoendemic in Dodowa with entomological inoculation rate estimates of around 30 per annum (Appawu *et al*. 1997). Transmission is more intense in the Kilombero valley-based Tanzanian studies where inoculation rates are in the 4–600 infective bites per annum range and a large degree of clinical immunity is acquired by age 2 or 3 years (Smith *et al*. 1993). Both Kilombero infants and Dodowa children show increasing morbidity and parasite density with increasing number of infecting genotypes (this positive relationship between parasitaemia and multiplicity was also found in the Senegalese study of Daubersies *et al*. 1996). A possible explanation for the similarity between Kilombero infants and the older Dodowa children is, therefore, that the positive relationship between clone multiplicity and clinical malaria is associated with the early stages in the development of immunity and relatively short clone survival times. The Dodowa data can thus be interpreted in terms of the clonal premunition theory but do not provide any new evidence for the theory. In fact, none of the 4 hypothetical relationships between multiplicity and virulence considered here are excluded by this (or any other) data set. A similar conclusion has also been reached in a recent Sudanese study, with the additional proviso that long clone survival periods were observed in a population with low levels of acquired immunity (Hamad *et al*. 2000).

Experimental testing of the relationship between multiplicity and virulence is proving difficult in both field and laboratory (experimental rodent malaria studies have been informative but multiple super-infections are difficult to establish). Furthermore, if the acknowledged underestimation of infection complexity is more severe at higher infection complexities (Arnot, 1998), then difficult and reagent-intensive PCR methods need to be used. For example, limiting dilution to clone out single templates may need to be carried out on each blood sample to obtain a true estimate of complexity. Combining this analytical approach with carefully designed longitudinal analyses of large, age-stratified study populations growing up under varying intensities of transmission may offer the best chance of resolving this question. Alternatively, similar improvements to provide more accurate and quantitative measurement in controlled experiments of mosquito-transmitted inoculations of the natural *Thamnomys* rodent host could be considered.

Taken as a whole, the simplest interpretation of these data is that the positive relationship observed between clone number and morbidity is a consequence of additive super-infection with novel clones. Thus we propose that the evocative title of an earlier analysis of the transmission season effect on disease incidence (Lines & Armstrong 1992) – 'for a few parasites more', be changed to 'for some new parasites more'?

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