

Genetic diversity and dynamics of *Plasmodium falciparum* and *P. vivax* populations in multiply infected children with asymptomatic malaria infections in Papua New Guinea

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(Received 9 November 1999; revised 16 March 2000; accepted 16 March 2000)

SUMMARY

We describe the dynamics of co-infections of *Plasmodium falciparum* and *P. vivax* in 28 asymptomatic children by genotyping these species using the polymorphic loci Msp2 and Msp3 α , respectively. The total number of *Plasmodium* spp. infections detected using 3 day sampling over 61 days varied between 1 and 14 (mean 6.6). The dynamics of *P. falciparum* and *P. vivax* genotypes varied greatly both within and amongst children. Periodicity in the detection of *P. falciparum* infections is consistent with the synchronous replication of individual genotypes. Replication synchrony of multiple co-infecting genotypes was not detected. In 4-year-old children *P. falciparum* genotype complexity was reduced and episodes lasted significantly longer (median duration > 60 days) when compared to children aged 5–14 years (median duration 9 days). *P. vivax* genotype complexity was not correlated with age but the episode duration was also longer for this species in 4-year-olds than in older children but was not as long as *P. falciparum* episodes. Recurrence of *P. falciparum* and *P. vivax* genotypes over weeks was observed. We interpret these major fluctuations in the density of genotypes over time as the result of the mechanism of antigenic variation thought to be present in these *Plasmodium* species.

Key words: *Plasmodium falciparum*, *Plasmodium vivax*, dynamics, asymptomatic infection, genotyping.

INTRODUCTION

Malaria parasite populations are made up of multiple genetic variants. The turnover in *Plasmodium* populations over short intervals of time within single human hosts has been shown to be high in regions of high endemicity, both for *P. falciparum* (Daubersies *et al.* 1996; Färnert *et al.* 1997) and *P. vivax* (Bruce *et al.* 1999). The rate of turnover depends upon the rate of acquisition of new infections and the rate of clearance of existing infections. Clearance rates depend on immunity that is species- and most probably genotype-specific (Ciuca, Ballif & Chelarescu-Vieru, 1934; Taliaferro, 1939; Brown & Brown, 1965). Evidence is accumulating that important targets of naturally acquired immunity that

regulates parasite clearance are molecules on the surface of infected erythrocytes which undergo antigenic variation (Brown & Brown, 1965; Mendis, Ihalamulla & David, 1988; Baruch *et al.* 1995; Reeder & Brown, 1996; Al-Khedery, Barnwell & Galinski, 1999). Polymorphic molecules on the surface of merozoites may also play a role in protective immunity (Riley *et al.* 1992; Taylor *et al.* 1998) but the relative importance of the response to each type of molecule is not known.

We have previously shown that most individuals living in a highly malaria-endemic region of Papua New Guinea carry multiple species infections that fluctuate over time (Bruce *et al.* 2000*a*). Both *P. falciparum* and *P. vivax* infections in this region are known to consist of multiple genotypes (Paul *et al.* 1995; Kolakovich *et al.* 1996; Bruce *et al.* 1999, 2000*a*).

However, the nature of the factors that determine the fluctuations in parasitaemia of such multiple infections are not fully understood. It has been

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suggested that innate immune responses play an important role in regulating parasite density in asymptomatic children (Kwiatkowski & Nowak, 1991; Kwiatkowski, 1995). Non-specific regulatory mechanisms are thought to act in concert with specific responses against diverse *Plasmodium* antigens resulting in an interaction between co-infecting species that can alter the pattern of infection of each species (Bruce *et al.* 2000a).

To study the dynamics of *Plasmodium* infections frequent sampling from patient's blood is required at intervals close to that of the parasite replication cycle, i.e. 48 h for *P. falciparum* and *P. vivax*. In previous studies, dynamics of multiple clones of *P. falciparum* in asymptomatic hosts have been followed by daily sampling over short periods (Daubersies *et al.* 1996; Färnert *et al.* 1997). In this study, we have examined samples of parasitized blood from patients in Papua New Guinea every 3 days over a 61 day period. By plotting the genotype data of both *P. falciparum* and *P. vivax* with species density data we have built up an integrated picture of the dynamics of co-infections over time. For the first time, this study has allowed re-interpretation of the nature of multi-species infections normally identified only by microscopy.

MATERIALS AND METHODS

Study area, population and sampling protocol

These are described in an accompanying paper (Bruce *et al.* 2000b).

Sample collection and DNA extraction

Samples for genetic analysis were collected at the same time as blood smears (see Bruce *et al.* 2000a, b). Approximately 500 μ l of blood were collected into anti-coagulant tubes containing Na₂EDTA (Sarstedt). Genotyping was carried out only on samples from children (aged 4–14 years), due to the greater prevalence of infection in children compared to adults. Children with the highest prevalence of infection were selected. *P. falciparum* genotyping was carried out on all samples (including those negative by microscopy) from children with 2 or more *P. falciparum* smear-positive samples. *P. vivax* genotyping was carried out on samples from children with 4 or more *P. vivax* smear-positive samples. Samples from 1 child aged 4 years with only 2 *P. vivax* smear positive samples were also analysed, to allow better comparison across age groups. Samples were stored at -70°C before transfer of 20 μ l of blood to filter paper (Whatman no.3). DNA extraction was carried out using a Chelex boiling method (Kyes *et al.* 1993) and the final extract volume was 200 μ l.

P. falciparum genotyping

Genetic analysis of *P. falciparum* populations was carried out for detection of allelic diversity at the *P. falciparum* merozoite surface protein 2 (Msp2) locus (Fenton *et al.* 1991; Smythe *et al.* 1991). Nested polymerase chain reaction (PCR) amplification of alleles was carried out using 2 μ l of DNA extract in the primary reaction and 2–0.02 μ l of the primary reaction as target in the nested reaction. PCR reaction volumes were 20 μ l. Deoxynucleotide triphosphates were at 0.75 mM, oligonucleotide primers at 0.1 μ M and 0.5 units of Taq polymerase (Promega) were used per reaction. Reaction buffer was as supplied, with the addition of 2.5 mM MgCl₂. Primer sequences and cycling temperatures were as previously described (Ranford-Cartwright *et al.* 1993). Amplification products were run on 1.8% agarose gels stained with ethidium bromide. Alleles were discriminated by both product size and sequence dimorphism (Smythe *et al.* 1991). Size was determined to within 20 bp using a standard curve drawn from DNA size standards (Boehringer–Mannheim). Sequence type was determined by Southern blotting of products to nylon membrane and hybridization with IC1-type and FC27-type sequence-specific probes, followed by chemiluminescent detection (Amersham) (Babiker *et al.* 1994).

P. vivax genotyping

P. vivax genotypes were distinguished using PCR/restriction fragment length polymorphism (RFLP) analysis (Bruce *et al.* 1999) of alleles at the merozoite surface protein 3 alpha (Msp3 α) locus (Galinski *et al.* 1999). The combination of RFLPs from *Hha*I and *Alu*I digests were used to differentiate genotypes (Bruce *et al.* 1999). Multiple *P. vivax* populations present in a single sample were easily distinguished as they resulted in multiple PCR products or RFLP patterns in which fragment sizes sum to greater than that of the uncut product. A consensus approach was taken in assigning genotypes in samples from individual children containing mixed parasite populations. Where a mixed pattern contained fragment sizes identical to those observed in one or more other samples from the same child these patterns were assigned. Only when the observed fragment sizes could not be a combination of other patterns observed within samples from a child was a new genotype assumed.

Genotyping replicates, controls and sensitivity

One negative control was run per 10 samples in all primary and nested reactions. Samples in which multiple products were observed, were repeated

with 1/100th the amount of primary reaction transferred to the nested to ensure that these were not a result of carry-over. Where replicate results varied due to stochasticity in the amplification of low density populations, genotypes observed in both amplifications were used. Samples from individual children were run in chronological order on gels. Genotype identity between children was ensured by running representative samples containing each genotype from each child on the same gel. Replicates were carried out for 65% of smear-positive and 77% of smear-negative *P. falciparum* samples genotyped. Eighty-five per cent of *P. vivax* smear-positive samples were assayed in replicate and due to the lower sensitivity of *P. vivax* genotyping of a selection of 20% of smear-negative samples covering all children, was duplicated.

The lower limit of the sensitivity of detection of *P. falciparum* genotypes was tested using mock field samples containing 1000–0.5 parasites/ μ l in 40% haematocrit blood. *In vitro*-cultured parasite clones of different allelic types (3D7 and Ro33, ICI-type and HB3, FC27-type) were combined to assess sensitivity of detection and allelic amplification bias in mixed infections with varying ratios. The lower sensitivity limit of *P. vivax* genotyping was previously determined as 100 parasites/ μ l (Bruce *et al.* 1999).

Statistical analysis

To test for periodicity in the detection of individual *P. falciparum* and *P. vivax* genotypes a paired sample analysis was carried out to calculate the conditional probability of infection with the same genotype at 3–60 day intervals following detection in the first of the pair of samples. This was carried out as for blood smear data (Bruce *et al.* 2000*b*).

Duration of episodes of infection with individual genotypes of *P. falciparum* and *P. vivax* was measured according to the same rules as used for microscopy data (Bruce *et al.* 2000*b*). Episodes were defined as consecutive genotype positive samples for *P. vivax*. For *P. falciparum*, genotype episodes were taken as consecutive or 6 day periodic positive time points to allow for observed periodicity in detection of genotypes of this species (see Results section). Episodes defined using consecutive or 6 day periodic samples are called moderate estimates. A maximum measure of duration was also taken. This was the time between the first and the last positive sample for each genotype within each child. Survival analysis of genotype duration estimates was carried out. Episodes estimated using the moderate method were considered censored if they bounded either end of the study period. Maximum estimates were censored if they fell within 2 days of the beginning or end of the study period.

RESULTS

The genetic identity of *P. falciparum* and *P. vivax* infections were determined from longitudinal samples from 28 children aged 4–14 years, living in a malaria endemic region of Papua New Guinea. *P. falciparum* genotypes were determined in samples from 25/28 children, *P. vivax* genotypes were determined for 16/28 children and 13/28 children were analysed for both species (Table 1). Genotype data for all children can be obtained at <http://www.ceid.ox.ac.uk/download/>. For both species, single loci were used for genetic typing and we refer to this as the parasite 'genotype' throughout. Multi-locus typing was precluded due to the frequent presence of multiple infections. Alleles at the *Msp2* locus of *P. falciparum* and those at the *Msp3 α* locus of *P. vivax* were used to distinguish individual infections of these species.

Genetic diversity at the *Msp2* locus of *P. falciparum*

A total of 395 samples were available from 25 children selected for *P. falciparum* genotyping (Table 1). Genotypes could be assigned in 227/395 samples (57.5%). *P. falciparum* parasites were detected by microscopy in only 62.1% of samples positive by PCR (Table 1), illustrating the higher sensitivity of PCR detection. A total of 305/395 (77.2%) samples were assayed in duplicate. Replicates of 150 samples were consistently negative (87.7% of these were *P. falciparum* smear-negative) and in 41 samples amplification occurred in only 1 replicate. In 114 samples amplification was observed in both replicates and in 72.8% of these samples, results were identical. In the remainder, a difference in the number and/or size of products was observed. Such differences have been commonly observed by other workers (Molecular Epidemiology in Malaria Collaborative Research Network (Björkman *et al.* 1998, personal communication) and are likely to be a result of random amplification of populations present at densities close to the detection threshold, estimated to be approximately 10 parasites/ μ l of blood in this study. A total of 27 distinct *Msp2* alleles were found in 227 PCR-positive samples (Fig. 1A and Table 2). Eight null alleles did not react with either repeat-specific probe.

Genetic diversity at the *Msp3 α* locus of *P. vivax*

A total of 258 samples were available for PCR analysis from 16 children selected for *P. vivax* genotyping (Table 1). PCR amplification at the *Msp3 α* locus was successful in 95/258 (36.8%) samples, of which 57 were smear-positive. Of these, 108/258 samples (41.9%) were assayed in duplicate. Replicates of 64 samples were consistently negative

Table 1. Number of samples used in PCR genotyping and genotypes detected per child for *Plasmodium falciparum* and *P. vivax*

(Child identifiers are the same as those given in Bruce *et al.* (2000*a, b*) Age is in years. Samples are the total number of samples per child available for genotyping. PCR-positive or negative refers to a successful amplification with species specific PCR genotyping method. Smear-positive or negative refers to detection of each species of parasite by microscopy. Allele numbers for *P. falciparum* at the Msp2 locus and *P. vivax* at the Msp3 α locus refer to those given in Tables 2 and 3, respectively. Numbers in parentheses for *P. vivax* alleles are the number of undefined alleles per child. The minimum number of infections is the total of *Plasmodium* genotypes present (i.e. the sum of *P. falciparum* and *P. vivax* genotypes plus additional species not genotyped but detected by microscopy (see Bruce *et al.* (2000*a, b*) counted as single genotypes.)

Child	Sex	Age	Samples	PCR	PCR	PCR	Geno- types/ child	Msp2 alleles detected	Samples	PCR	PCR	PCR	Geno- types/ child	Msp3 α alleles detected	Minimum number of infections
				+ve smear	+ve smear	-ve smear				+ve smear	+ve smear	+ve smear			
1	m	4	14	7	6	0	1	9	14	7	0	1	4	1, 18 (2)	5
2	m	4	18	8	5	1	1	16	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1
4	f	4	12	6	2	1	1	27	12	2	1	0	1	15	2
5	m	4	16	13	2	1	2	3, 25	16	7	4	3	7	1, 7, 9 (4)	9
6	m	7	11	7	4	0	6	3, 4, 5, 10, 14, 17	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	8
7	m	7	17	4	4	1	3	6, 9, 16	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	5
8	f	7	19	2	4	0	3	1, 3, 9	19	2	1	3	1	17	4
10	m	8	16	3	6	0	5	9, 12, 14, 18, 24	16	3	4	2	3	7, 16 (1)	8
11	f	9	13	4	2	0	5	6, 7, 9, 10, 14	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	6
12	m	8	17	8	3	2	5	3, 4, 9, 13, 15	17	1	0	2	1	1	7
14	f	6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	19	8	1	3	3	16, 22 (1)	4
15	f	9	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	19	4	7	2	4	1, 10 (2)	6
16	f	7	15	7	2	1	6	4, 8, 15, 18, 19, 21	14	7	1	1	6	1, 6, 11 (3)	14
17	m	6	18	6	1	1	4	4, 13, 15, 24	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	6
18	f	5	13	4	7	1	4	3, 9, 11, 14	13	1	2	2	3	6, 5, 23	7
19	m	14	17	7	3	0	5	3, 5, 6, 11, 15	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	7
20	m	14	15	3	6	2	6	4, 7, 11, 12, 15, 16	14	2	0	3	3	3 (2)	10
21	f	11	17	4	6	0	2	5, 10	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	2
22	f	14	18	6	3	3	4	4, 14, 16, 18	18	2	3	2	3	24 (2)	8
24	m	14	17	0	1	3	1	5	17	3	4	5	8	4, 6, 12, 13, 21 (3)	10
25	m	12	13	4	3	0	5	3, 14, 17, 25, 26	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	7
26	m	12	17	4	4	0	4	9, 11, 12, 23	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	6
27	f	10	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	19	2	5	3	4	1, 9, 19, 20	5
28	f	11	18	1	0	1	1	16	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	2
31	m	10	16	11	3	1	9	3, 4, 9, 10, 11, 12, 13, 14, 15	16	1	3	3	4	2, 14 (2)	13
32	m	13	19	10	5	2	5	2, 5, 15, 16, 22	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	6
33	f	10	15	5	3	0	3	3, 4, 5	15	5	2	5	7	2, 8 (5)	10
34	m	14	14	7	1	2	5	2, 4, 13, 15, 20	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	7
Total			395	141	86	23			258	57	38	40			

N.D., Not determined.

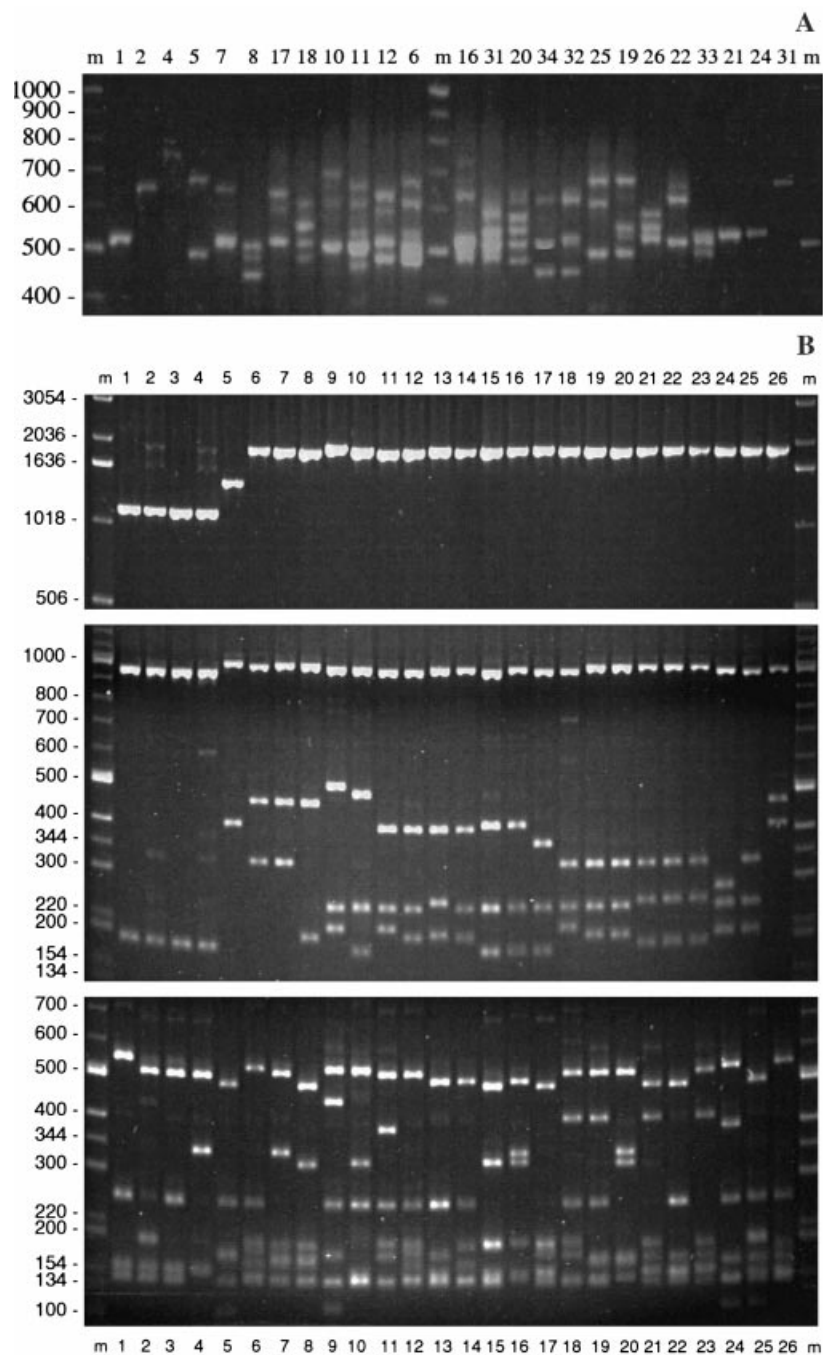


Fig. 1. Allelic polymorphism at (A) the *Plasmodium falciparum* Msp2 locus and (B) the *P. vivax* Msp3 α locus. Msp2 alleles from multiple time-points during the 61 day study period have been combined to show the size polymorphism present within each child during the study. Lanes in (A) are numbered with the child identifiers as in Table 1. Due to the higher background when samples are pooled some alleles are detectable only as faint bands, and some, which are close in size, appear merged. Msp2 allele 13 in child 34 is not shown. Msp3 α alleles are distinguished by size (top panel) and RFLP patterns after digestion with restriction enzymes *Hha*I (middle panel) and *Alu*I (bottom panel). Lanes 1–24 are labelled with the allele number as referred to in Table 1 and 3. Lanes 25 and 26 contain products from *P. vivax* laboratory strains Belem and Sal 1, respectively. Sizes of markers, Lanes m, are shown in base pairs.

(37.5% of these samples were *P. vivax* smear-negative) and in 18 samples amplification was observed in only 1 replicate. In 26 samples, amplification was observed in both replicates and in 16/26 identical results were obtained.

Three sizes of PCR amplification products (1100, 1400 and 1900 bp) were observed at the Msp3 α locus

(Fig. 1B, top panel and Table 3). Two of these (1100 and 1900 bp) had been previously described in a set of samples from Gonoa village, which contained some overlap with those presented here (Bruce *et al.* 1999). An increased sensitivity in distinguishing different *P. vivax* infections using a combination of *Hha*I and *Alu*I has been previously shown (Bruce *et*

Table 2. Size and sequence types of alleles detected at the *Plasmodium falciparum* Msp2 locus

(Numbers of samples and numbers of children do not add to the total numbers assayed as detection of multiple alleles per child and per sample was common. Numbers are given for the 3 sequence categories without reference to allele size.)

Allele	Size (bp)	Sequence type	Number of samples in which allele detected	Number of children in which allele detected
1	440–459	FC27	3	1
2	460–479	FC27	7	2
3	480–499	FC27	29	9
4	500–519	FC27	40	9
5	520–539	FC27	21	7
6	660–679	IC1	10	2
7	460–479	IC1	2	2
8	480–499	IC1	1	1
9	500–519	IC1	35	9
10	520–539	IC1	16	4
11	540–559	IC1	12	5
12	560–579	IC1	9	4
13	580–599	IC1	14	4
14	600–619	IC1	24	7
15	620–639	IC1	26	8
16	640–659	IC1	27	6
17	660–679	IC1	5	2
18	680–699	IC1	6	3
19	720–739	IC1	2	1
20	240–259	Null	1	1
21	500–519	Null	3	1
22	520–539	Null	1	1
23	560–579	Null	1	1
24	620–639	Null	4	2
25	660–679	Null	17	2
26	700–719	Null	1	1
27	740–759	Null	8	1
	Any	FC27	100	19
	Any	IC1	144	21
	Any	Null	36	9

al. 1999). The combination of these RFLP patterns allowed 24 Msp3 α alleles to be distinguished (Fig. 1B, and Table 3).

Some RFLP patterns were impossible to define as they were observed only in samples with mixed populations. Undefined genotypes were detected in 29 samples from 11 children. Despite not being able to assign restriction fragments for such genotypes these samples were still useful. Undefined genotypes could be distinguished from those present at other time-points within the same child if fragment sizes did not match. Undefined allele results were used in genotype dynamics and duration analyses as they rely only on comparison within a child and not between children.

Multiplicity of P. falciparum and P. vivax infections per sample

The mean multiplicity of *P. falciparum* infections across PCR-positive samples was 1.4 genotypes per sample (range 1–4). The number of *P. falciparum* genotypes per sample was not correlated with *P. falciparum* parasite density ($r = 0.07$, $P = 0.287$).

Mean multiplicity of *P. vivax* infection was 1.4 genotypes per sample (range 1–6) and there was also a lack of correlation with *P. vivax* density ($r = 0.18$, $P = 0.08$).

Multiplicity of Plasmodium spp. infections per child

Most children were infected with multiple genotypes of each species over the course of the study (Table 1). Single genotypes were found in only 5/25 children assayed for *P. falciparum* and 3/16 children assayed for *P. vivax*. Up to 9 *P. falciparum* genotypes (mean = 3.8) and 8 *P. vivax* genotypes (mean = 3.9) were distinguished per child. These results have been in part reported and discussed elsewhere (Bruce *et al.* 2000a).

Genotypes for each species (*P. falciparum* and *P. vivax*) were considered together with microscopy data for species not genotyped to calculate the minimum number of *Plasmodium* infections for each child during the study (Table 1). Only 1 child had a single *Plasmodium* infection throughout days 0–60 of the study. All others had between 2 and 14 different infections (mean = 6.6). The number of *P.*

Table 3. Size and RFLP patterns of alleles detected at the *Plasmodium vivax* Msp3 α locus

(The number of samples and number of children do not add to the total number assayed as detection of multiple alleles per child and per sample was common. Data for individual alleles 1–24 comes only from samples in which defined alleles were detected. Numbers are given for the 3 size categories without reference to RFLP patterns. These data are from all samples in which PCR amplification was successful. *AluI* RFLP pattern a10 is not used to define any allele as it was only detected in samples shown to be mixed infections using *HhaI* digestion. *HhaI* patterns h1, h3, h8, h9, h12, h13 and *AluI* pattern a11 are used, in combination with other patterns to define multiple alleles.)

Allele	Size (bp)	<i>HhaI</i> RFLP pattern	<i>AluI</i> RFLP pattern	Number of samples in which allele detected	Number of children in which allele detected
1	1100	h1	a1	20	6
2	1100	h1	a2	2	2
3	1100	h1	a3	2	1
4	1100	h1	a4	1	1
5	1400	h2	a6	1	1
6	1900	h3	a11	5	3
7	1900	h3	a18	6	2
8	1900	h4	a19	3	1
9	1900	h5	a12	8	2
10	1900	h6	a21	4	1
11	1900	h7	a17	3	1
12	1900	h8	a11	1	1
13	1900	h8	a8	1	1
14	1900	h8	a9	1	1
15	1900	h9	a20	3	1
16	1900	h9	a22	10	2
17	1900	h10	a5	3	1
18	1900	h11	a14	1	1
19	1900	h12	a13	1	1
20	1900	h12	a23	2	1
21	1900	h13	a16	1	1
22	1900	h13	a7	2	1
23	1900	h13	a24	1	1
24	1900	h14	a15	5	1
	1100			24	10
	1400			7	3
	1900			74	15

falciparum genotypes was not correlated with that for *P. vivax* amongst the 13 children whose samples were genotyped for both species ($r = -0.163$, $P = 0.594$). The number of genotypes present per child was not correlated with age for either *P. falciparum* or *P. vivax* when all children were analysed together (*P. falciparum*: $r = 0.316$, $P = 0.312$; *P. vivax*: $r = 0.219$, $P = 0.413$) nor was the total number of all *Plasmodium* infections, when all 4 species were considered together (Table 1) ($r = 0.262$, $P = 0.178$). Despite the lack of correlation with age there was a notable reduction in the complexity of *P. falciparum* infections in 4-year-old children compared to older children. Three out of 4 of these children have a single *P. falciparum* infection during the whole study period and the other had only 2 infections.

A correlation between the proportion of time-points microscopy positive for *P. vivax* and the number of *P. vivax* genotypes has been previously reported (Bruce *et al.* 2000a). This relationship also holds for *P. falciparum* in children aged 5–14 years but not *P. falciparum* for 4-year-olds (Bruce *et al.* 2000a). In addition, the total number of *Plasmodium*

infections (Table 1) shows a significant association with the number of smear-positive samples of any species in 5 to 14-year-olds ($r = 0.453$, $P = 0.026$) but when 4-year-old children were also included significance was abolished ($r = 0.330$, $P = 0.086$).

Dynamics of PCR-detectable *P. falciparum* genotypes

The dynamics of PCR-detectable *P. falciparum* infections differed greatly both within and between individual children. The simplest dynamics were observed in *P. falciparum* infections in children aged 4 years (Fig. 2, child 1). In all of the 4-year-old children a single *P. falciparum* infection lasted for almost the total duration of the study.

Dynamics of *P. falciparum* infections were significantly different in children greater than 4 years of age. The pattern of infection was more complex in 5 to 14-year-olds (Fig. 2, children 22, 6 and 31). The dynamics of individual genotypes were characterized by shorter periods of infection and some were seen on only a single occasion per child. Some *P.*

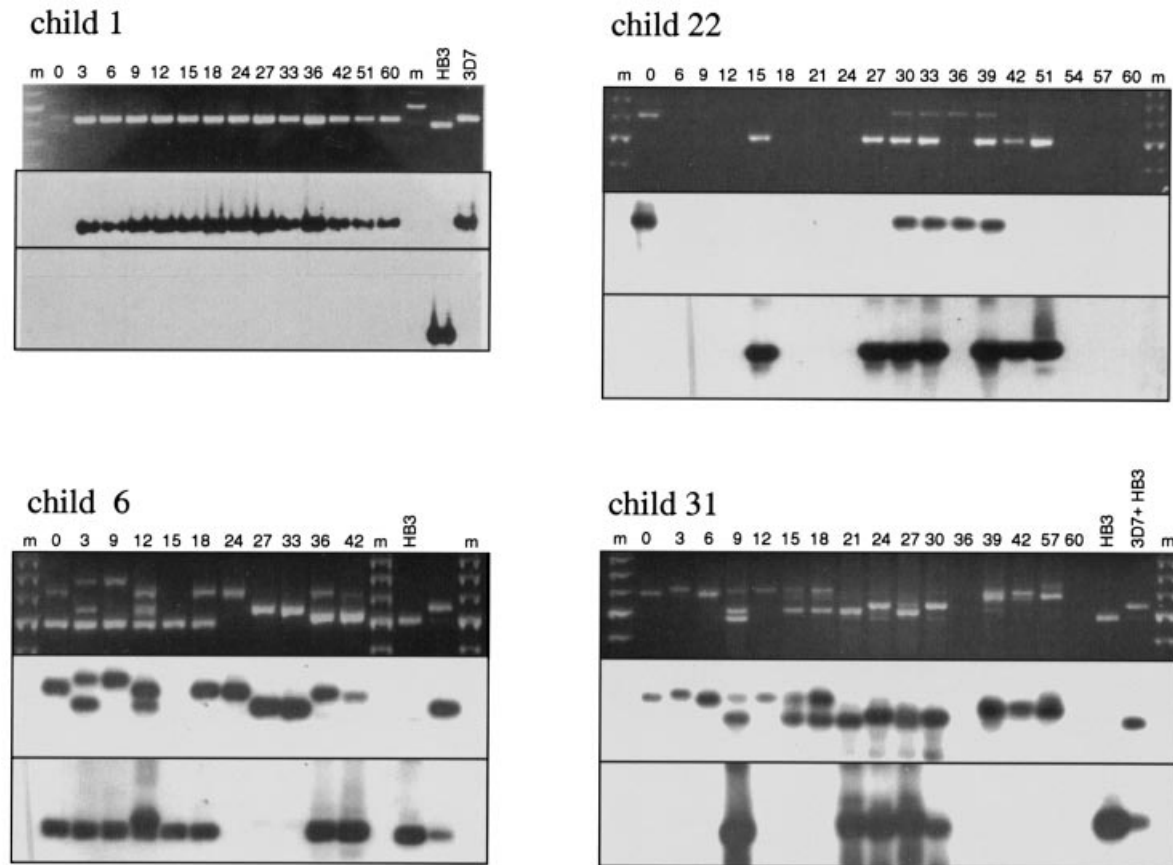


Fig. 2. Intra-host dynamics of *Plasmodium falciparum* genotypes in 4 children detected by allelic variation at the *Msp2* locus. Data available from time-points throughout the study period, including those negative for PCR amplification products, are shown. The child identity number (Table 1) is shown at the top of each set of samples. The top panels show size polymorphism of PCR products. Lower panels show sequence dimorphism of products detected by hybridization of products with sequence-specific oligonucleotide probes: IC1 type (middle panels), FC27 (lower panels). DNA size marker fragment with double intensity in Lanes m, is 500 bp. Fragments above and below have increments or decrements of 100 bp. Products amplified from laboratory lines 3D7 and HB3 shown in some panels are hybridization controls for IC1 and FC27-type probes, respectively.

falciparum infections were seen at consecutive time-points (e.g. Fig. 2, child 22 and child 6) but others were characterized by periodic detection at 6 day intervals. This periodicity was investigated statistically (see below). Recurrence of genotypes also occurred at longer intervals e.g. in child 22 (Fig. 2), the FC27 genotype was detected on day 15 but not again until days 27–33. The appearance and clearance of individual genotypes was independent of other co-infecting *P. falciparum* genotypes.

Dynamics of PCR-detectable *P. vivax* genotypes

The dynamics of *P. vivax* infections were also variable within and between individuals (Fig. 3). The same genotype was detected at sequential time-points in some children (e.g. Fig. 3, child 4) and in others detection was more sporadic. Some genotypes were only detected at a single time-point (e.g. Fig. 3, child 18). In contrast to *P. falciparum*, there was no difference in the dynamics of *P. vivax* infections in children aged 4 years compared to those

in older children. There was apparent replacement of one genotype with another in 1 child (Fig. 3, child 10) but in most children turnover of each genotype appeared independent of co-infecting *P. vivax* infections.

Periodicity in the detection of *P. falciparum* and *P. vivax* genotypes

Periodicity in the detection of *P. falciparum* parasites by blood smear had been previously noted in these children (Bruce *et al.* 2000 *b*). To test if there was a corresponding periodicity in the detection of individual genotypes of *P. falciparum*, a paired analysis similar to that carried out on blood smear data was done. The conditional probability of infection with each distinct genotype after its detection in the first pair of samples from individual children was calculated for intervals of 3–60 days. A marked periodicity in the probability of infection was seen for *P. falciparum* (Fig. 4A). There was a statistically significant lower probability of infection at 3, 9, 15,

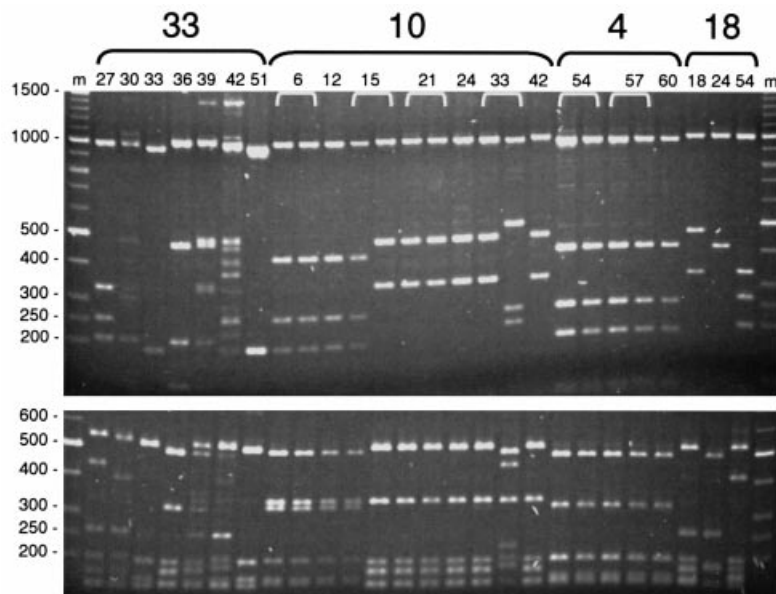


Fig. 3. Intra-host *Plasmodium vivax* genotype dynamics in 4 children detected by PCR amplification and RFLP analysis of allelic variation at the *Msp3 α* locus. PCR products were digested with restriction enzyme *Hha*I (top panel) or *Alu*I (lower panel). The child identity number (Table 1) is shown above each set of samples bracketed in black. Samples for each child are ordered chronologically and are labelled with the time-point of collection during the study. Only samples that gave PCR products are shown. Replicate results from a single sample are shown in white brackets. Sizes of markers, Lanes m, are shown in bp.

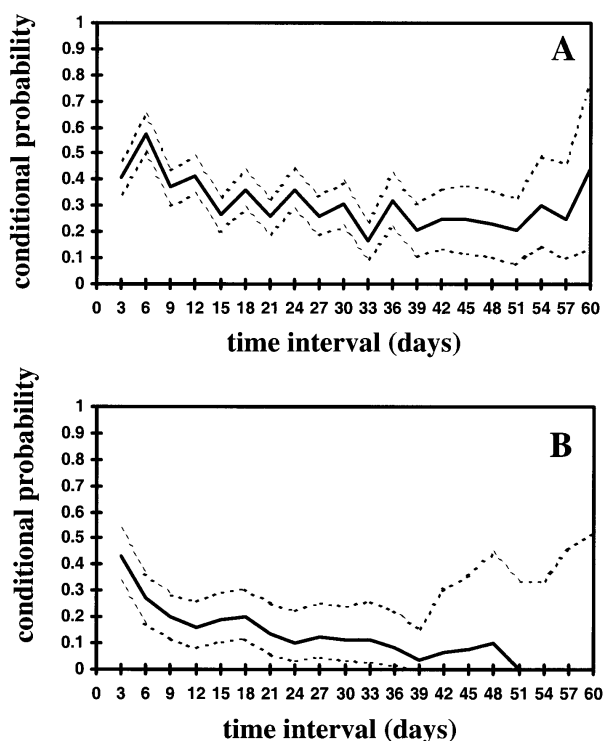


Fig. 4. Conditional probability of detection of the same genotype in pairs of samples from each individual at 3–60 day intervals for (A) *Plasmodium falciparum* and (B) *P. vivax* genotypes from children aged 4–14 years. Probability is conditional on detection of an identical genotype in the chronologically first sample in the pair.

21 and 27 day intervals than at 6, 12, 18 and 24 days ($P < 0.05$). Examination of the pattern of multiple *P. falciparum* genotypes in each child revealed no evidence for synchrony of different co-infecting genotypes. Conditional probabilities were also calculated for *P. vivax* genotypes for comparison but periodicity was absent from these data (Fig. 4B).

Duration of genotype episodes of infection

Estimates of the duration of genotype infections of *P. falciparum* and *P. vivax* were made using 2 different methods. Moderate estimates used the duration of consecutive positive samples for each *P. vivax* genotype. Due to periodicity in the detection of *P. falciparum* genotypes, consecutive or 6 day periodic genotype samples were used for this species. A maximum estimate was also used which measures the total detected duration of each genotype. Data for genotypes of each species from each individual were pooled by age group. The distribution of duration estimates was not significantly different between age groups 5–9 and 10–14 years for either species or method and so were combined (*P. falciparum* moderate, $\chi^2 = 0.7$, 1 D.F., $P = 0.399$; *P. falciparum* maximum, $\chi^2 = 0.1$, 1 D.F., $P = 0.770$; *P. vivax* moderate, $\chi^2 = 1.2$, 1 D.F., $P = 0.557$; *P. vivax* maximum, $\chi^2 = 1.6$, 1 D.F., $P = 0.209$). Estimates for *P. falciparum* and *P. vivax* for children aged 4 years

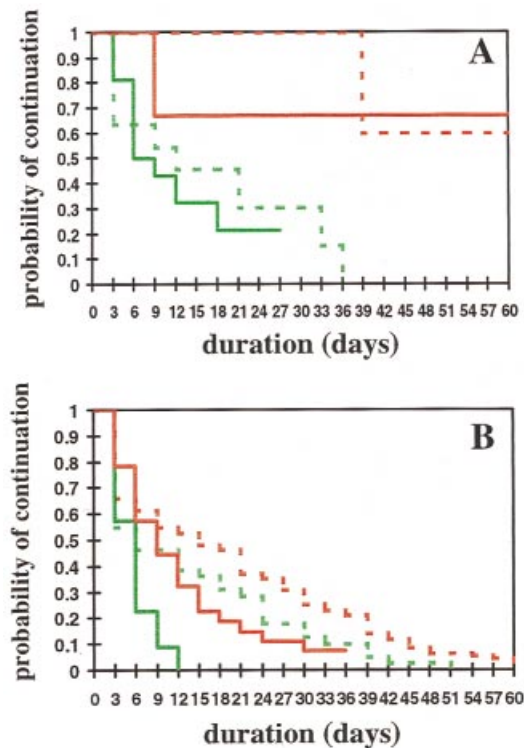


Fig. 5. Kaplan-Meier plots of duration of infection for children aged (A) 4 years and (B) 5–14 years with genotypes of *Plasmodium falciparum* (red) and *P. vivax* (green). Moderate estimates are solid lines and maximum estimates are broken lines.

and 5–14 years are shown as Kaplan-Meier survival plots (Fig. 5) and the median duration of infection for each estimate is shown in Table 4.

The duration of *P. falciparum* genotypes in 4-year-olds was significantly longer than those of the 5 to 14-year-olds when either estimate was used (moderate, $\chi^2 = 8.7$, 2 D.F., $P = 0.012$; maximum, $\chi^2 = 9.5$, 2 D.F., $P = 0.009$). Median duration in 4-year-olds exceeded 60 days using both methods (Fig. 5A and Table 4). In 5 to 14-year-olds *P. falciparum* maximum estimates were significantly longer than the moderate ones ($\chi^2 = 6.9$, 1 D.F., $P = 0.009$).

Moderate *P. vivax* duration estimates from the 4-year-old age group were also significantly longer than those of 5 to 14-year-olds ($\chi^2 = 10.5$, 2 D.F., $P = 0.015$). Significance was abolished when the maximal estimates were tested ($\chi^2 = 1.6$, 2 D.F., $P = 0.446$). The duration of *P. falciparum* and *P. vivax* genotypes cannot be directly compared due to the 10-fold difference in the sensitivity level of the 2 genotyping techniques. Comparison of estimates of episode duration using blood smear data and the moderate genotype method revealed no significant differences for the same age groups for either *P. falciparum* or *P. vivax* (*P. falciparum*: 4 years, $\chi^2 = 0.3$, 1 D.F., $P = 0.579$; 5–14 years, $\chi^2 = 3.3$, 1 D.F., $P = 0.071$; *P. vivax*: 4 years, $\chi^2 = 0.6$, 1 D.F., $P = 0.455$; 5–14 years, $\chi^2 = 0.4$, 1 D.F., $P = 0.551$).

Relating multi-species genotype detection and parasite density data

Plotting the dynamics of genotypes of both *P. falciparum* and *P. vivax* in individual children gave a fuller picture of the co-infections within multiply infected children. By also plotting total and species-specific parasite density obtained from microscopy and relating this to the genotypes of *P. falciparum* and *P. vivax* present at each point, detailed pictures of how the dynamics of multiple infections were related within each child was built up. If multiple genotypes are present in a sample where the species density is known the density of individual genotypes cannot be determined due to the non-quantitative nature of the genotyping methods. The greater sensitivity of the genotyping methods often allowed determination of genotype dynamics below the microscopy detection level. The dynamics of parasite density together with detection data of individual genotypes of *P. falciparum* and *P. vivax* are shown for 6 representative children (Fig. 6).

The long duration of *P. falciparum* genotypes observed in 4-year-old children is illustrated in Fig. 6A and B. *P. falciparum* has the greatest density over the longest time in child 5 (Fig. 6A) whilst *P. vivax* was seen as short, intermittent episodes of much lower density. Despite this the complexity of *P. vivax* infection was much greater than of *P. falciparum*.

A greater number of *P. vivax* compared with *P. falciparum* genotypes was also observed in another 4-year-old, child 1 (Fig. 6B). This child harboured a single *P. falciparum* infection continuing over 57 days but the parasite density of this genotype changes by more than 2 orders of magnitude during this time. *P. falciparum* was not detected by microscopy in the latter part of the study when infection was dominated by *P. vivax*. During this period PCR genotyping revealed that the same *P. falciparum* infection was maintained at low density throughout the *P. vivax* episode. In contrast to *P. falciparum*, the *P. vivax* episode was the result of infection with more than 1 genotype with variable durations.

P. falciparum dynamics resembled more those of *P. vivax* in children older than 4 years, being more complex and of shorter duration. The greatest number of *P. falciparum* genotypes, 9, was detected in child 31, aged 10 years (Fig. 6C). Recurrent behaviour was seen in *P. falciparum* genotypes 13 and 14. They were detected at the beginning of the study period (from days 0–6 and 3–18, respectively) and again towards the end (day 57 and days 39 and 42, respectively). *P. vivax* infection was not as complex as *P. falciparum* in this child, mirroring the paucity of *P. vivax* smear-positive samples.

The numbers of different *P. falciparum* and *P. vivax* genotypes were more equitable in other

Table 4. Median duration (in days) of *Plasmodium falciparum* and *P. vivax* genotype episodes by age group, estimated using the moderate and maximum methods

(Median duration is obtained from Kaplan-Meier plots (Fig. 5); 95% confidence intervals in parentheses.)

	4 years	5–14 years
<i>P. falciparum</i> moderate	> 60 (9–> 60)	9 (6–12)
<i>P. falciparum</i> maximum	> 60 (39–> 60)	15 (9–21)
<i>P. vivax</i> moderate	6 (6–18)	6 (3–6)
<i>P. vivax</i> maximum	12 (3–33)	6 (3–15)

children, e.g. child 22 (Fig. 6D). Here the duration and density associated with the 4 *P. falciparum* genotypes was variable. *P. falciparum* genotype 4 was present from day 15, was seen at consecutive points from day 27 to 42 and recurs on day 51. During this time a second *P. falciparum* infection was detected (genotype 16). This genotype was present from day 30 to 39. These dynamics show that the immune response(s) responsible for clearing genotype 16 were not acting on genotype 4, which continues throughout the genotype 16 infection.

The 6 day periodicity of detection of *P. falciparum* genotypes is illustrated in data from child 33, aged 10 (Fig. 6E). *P. falciparum* genotype 5 was detected on days 9 and 15. It was out of phase with genotype 4, which was present on days 12, 18 and 24. This genotype was not detected on day 30 but remains in phase with the first episode when it recurred on days 36 and 42. During this period there was substantial variation in the density associated with this genotype.

P. falciparum and *P. vivax* infection detectable by microscopy was reduced both in the number of time points and density in child 10, aged 8 (Fig. 6F). Despite this, multiple genotypes of each species were detected in this child but the pattern of detection was more sporadic. Even in this case, the same genotypes were maintained for a substantial period of time.

DISCUSSION

This study presents for the first time an analysis of the dynamics of co-infections of multiple genotypes from multiple species of *Plasmodium* in naturally infected children. The diversity and dynamics of individual infections of both *P. falciparum* and *P. vivax* are highly complex and vary considerably between but also within children. Despite this, some generalizations can be made which provide insights into the burden of multiple asymptomatic malaria

infections and how these are regulated by host immune reactions in individuals with some degree of naturally acquired immunity.

Different infections were distinguished by allelic diversity at species-specific polymorphic loci. The number of alleles distinguishable at the *P. vivax* Msp3 α locus was almost as high as that detected at the *P. falciparum* Msp2 locus despite the reduced sensitivity of the *P. vivax* PCR amplification. This is because of RFLP determination used in the analysis of Msp3 α detects point mutations whereas the Msp2 method relies upon detection of major size polymorphisms. Size polymorphism was also observed in the Msp3 α gene but the extent of this may be limited by functional constraints on this molecule (Galinski *et al.* 1999).

The mean multiplicity of *P. falciparum* infections per sample was less than in other highly endemic regions (Babiker, Ranford-Cartwright & Walliker, 1999) but was similar to results of other surveys in the Madang area of PNG (Paul *et al.* 1995). *P. vivax* multiplicity per sample was similar to that estimated in another study in PNG (Kolakovich *et al.* 1996) and one from India (Joshi *et al.* 1997) but major differences in sample collection and genotyping techniques makes such comparisons of limited epidemiological value. Similarly, the mean multiplicity of infection of *P. falciparum* and *P. vivax* in this study cannot be compared because of the difference in PCR sensitivity (*P. vivax*, 100 parasites/ μ l (Bruce *et al.* 1999), *P. falciparum* 10 parasites/ μ l) in the genotyping method and possible differences in the actual allelic diversity at each locus. The absence of correlation between either *P. vivax* or *P. falciparum* multiplicity per sample and species-specific parasite density in this data set is similar to results from children aged 3–7 years in Tanzania (Smith *et al.* 1999a) and is in correspondence with the acquisition of density-dependent regulation of parasitaemia by the age of 4 years in PNG (Bruce *et al.* 2000a).

Although mean multiplicity values per sample are often calculated from longitudinal data, such statistics are biased by the temporal correlation structure within multiple samples. A more useful statistic is the number of infections per child during the study. This gives a better estimate of the burden of infection. All but 1 child had multiple infections as discerned by either species- or molecular typing. Multiple *P. vivax* infections were commonplace in children of all ages. The reduction in the complexity of *P. falciparum* infections in 4-year-old children compared with older children is in line with the age-dependency of the complexity of this species in Tanzania (Smith *et al.* 1999a).

Periodicity in the detection of *P. falciparum* genotypes is likely to be a result of the synchronous replication and the sequestration characteristics of this species in combination with the 3 day sampling

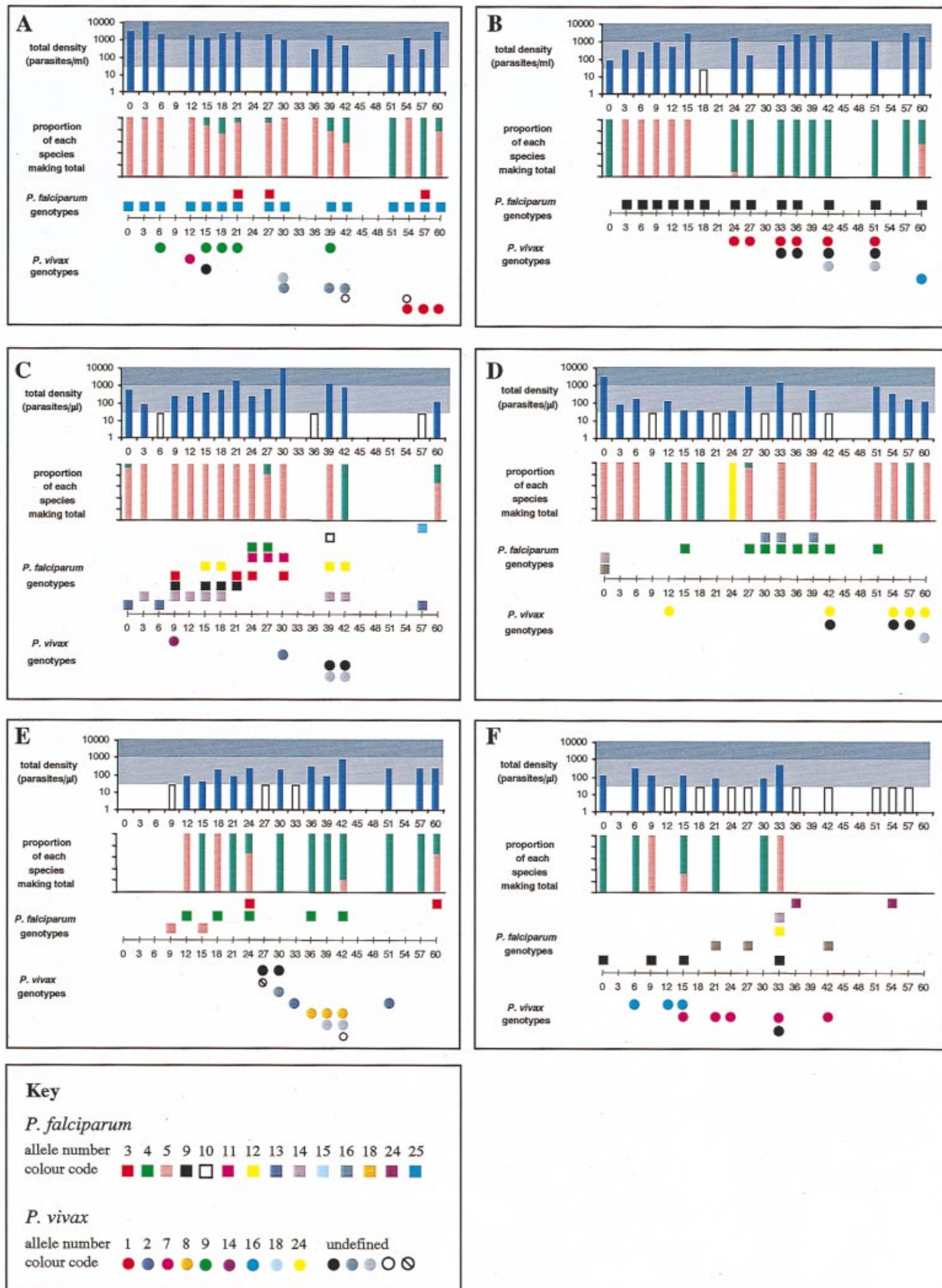


Fig. 6. Dynamics of *Plasmodium falciparum* and *P. vivax* genotypes in relation to parasite density are shown for (A) child 5; (B) child 1; (C) child 31; (D) child 22; (E) child 33 and (F) child 10. Bars on upper graphs (blue) show total parasite density on a log scale. The proportion of each species is shown in the second graph, *P. falciparum*, red; *P. vivax*, green; *P. malariae*, yellow. Light grey shading indicates density between the microscopy sensitivity level (40 parasites/ μ l) and the approximate fever threshold (1000 parasites/ μ l (Cox *et al.* 1994)). Dark grey shading shows

protocol. Daily periodicity in *P. falciparum* genotype detection has been previously demonstrated in asymptomatic patients in Tanzania (Färnert *et al.* 1997). Statistical analysis of the periodicity in this study provides quantitative evidence that single genotypes within individuals show a tendency for synchronous replication. The periodicity of individual genotypes underlies the periodicity detected by microscopy in the same children (Bruce *et al.* 2000b). The absence of synchrony amongst multiple co-infecting *P. falciparum* genotypes suggests that the maintenance of periodicity is governed mainly by alignment of the parasite replication cycle with host diurnal rhythms (Coatney & Stubbs, 1940; Hawking, Worms & Gammage, 1968) rather than killing of parasites by fever, which would synchronize the cycles of all genotypes present (Kwiatkowski & Greenwood, 1989). Epidemiological studies of *P. falciparum* must take the tendency for synchronous replication into account in the design of sampling protocols. This is especially important when detection of individual genotypes is used as an end point measure in longitudinal drug or vaccine trials.

The observed dynamics of infection showed that host immunity which results in reduction of parasite density to below PCR detectable levels, was acting against genotypes of a single species independently and that clearance of one genotype was not linked with clearance of others. This is in line with observations that show that the immunity that clears parasites in humans is predominantly species- and genotype-specific (Taliaferro, 1939; Jarra & Brown, 1985).

Episodes of each species have been shown to follow a sequential rather than concurrent pattern in this data set (Bruce *et al.* 2000a). The sequential pattern of infection is statistically different to that expected by chance alone and total parasite density has been shown to follow density-dependent dynamics in highly parasitized children (Bruce *et al.* 2000a). Density-dependent regulation, which is species- and genotype-transcending in nature, is thought to play a role in the regulation of parasitaemia and be responsible for interactions between different species. To be able to detect interactions between different genotypes of a single species the density of each genotype is required. The non-quantitative genotyping methods used in this study preclude any conclusions about genotype interactions.

The dynamics of parasitaemia from blood smears in combination with PCR genotyping in children

with single *P. falciparum* genotypes show the occurrence of major changes in the density of a single genotype over short periods. Such changes may correspond with specific clearance of parasites expressing a particular RBC surface antigenic variant. PCR detection has demonstrated the persistence of the same genotype below microscopy detection levels following such clearance, during the rise of parasitaemia of another species. Survival of a minority of parasites expressing a different antigenic type from the majority of the same population can explain these dynamics. These data are the first to show that dynamics consistent with antigenic variation (Reeder & Brown, 1996) occur in naturally infected humans carrying multiple *Plasmodium* infections.

Two measures of the duration of infection of genotypes termed moderate and maximum were made. Moderate estimates used the same rules as those from blood smears (Bruce *et al.* 2000b) and provide a measure of individual episodes of infection. Using this measure, episodes of *P. falciparum* and *P. vivax* were longer in younger children. Decreasing duration of episodes of individual genotypes underlies the similar trend with age for both species in microscopy data (Bruce *et al.* 2000b). Selection of the most parasitized children for genotyping may have biased genotype durations towards slightly longer estimates but the comparison of relative duration across age groups remains valid. *P. falciparum* episodes showed the greatest difference in duration, between 4-year-old and 5 to 14-year-olds. This longer duration explains the absence of a correlation of the number of *P. falciparum* genotypes with the proportion of smear positive samples in 4-year-olds (Bruce *et al.* 2000a).

The reduction of the duration of infection of individual genotypes with age demonstrated here is in agreement with data from children of similar ages in Tanzania, where the duration of infection (calculated as the reciprocal of the recovery rate) decreases slightly in older children and more significantly into adulthood (Smith *et al.* 1999a) although the authors did not interpret their data in this way (Smith *et al.* 1999b).

Surprisingly, moderate estimates of episode duration of individual genotypes did not vary significantly from those made from blood smear results for either species and all age groups. The greater sensitivity of *P. falciparum* genotyping compared to microscopy was expected to result in longer estimates for this parasite from genotyping. The small number

parasite density above the fever threshold. Open bars, represent smears which were negative by microscopy. Where bars are missing samples were absent. Individual genotypes of *P. falciparum* are shown as squares and *P. vivax* genotypes as circles. Different colours represent genotypes defined by alleles of *Msp2* and *Msp3 α* as in the key. *P. vivax* genotypes shown in black or grey are undefined.

of infections analysed may have been insufficient to allow detection of differences using these two methods. The similarity of episode duration estimated from microscopy and genotyping may not hold for areas of higher endemicity where a greater percentage of children will be constantly smear-positive.

The duration of episodes of infection with *P. falciparum* and *P. vivax* genotypes cannot be directly compared because of the difference in sensitivity of the PCR genotyping. Microscopy does not have the same limitation because the sensitivity of detection of both *P. falciparum* and *P. vivax* infections is equal. *P. vivax* infections measured by microscopy tended to be shorter in duration than *P. falciparum* infections in all age groups (Bruce *et al.* 2000b). This is likely to be a result of a real difference in the duration of *P. vivax* genotypes. This difference could be explained by faster acquisition of specific immunity to *P. vivax*. Supporting evidence for this comes from induced infection experiments in which fewer inoculations were required to produce immunity to homologous *P. vivax* than to *P. falciparum* infections (discussed by Taliaferro, 1939). It has also been suggested that faster acquisition of immunity to *P. vivax* explains the quicker decline in age prevalence curves for this species in regions where both species exist (Maitland *et al.* 1996; Maitland, Williams & Newbold, 1997).

Maximum estimates of genotype duration accounted for recurrence in the detection of genotypes. Recurrence of the same genotype can be due to either recrudescence of the same infection or to re-infection with a different infection that carries the same allele. The probability of re-infection with the same genotype can be approximated from the sum of the squares of genotype frequency per child (0.060 for *P. falciparum* and 0.064 for *P. vivax* calculated from Tables 2 and 3, respectively). This assumes that (1) the frequency of genotypes in blood is equal to that transmitted from mosquitoes, (2) the probability of re-infection is equal between individuals and (3) infection with a genotype is a random event. These assumptions may not hold and therefore, these values should be viewed only as rough estimates. They do, however, suggest that recrudescence rather than re-infection is more likely to explain the majority of recurrences. In children with multiple infections, recurrence may also be due to the limitation of the PCR genotyping techniques that can fail to detect a genotype if others are present at a ratio greater than 1:10 (unpublished observations).

The maximum measure is a better estimate of duration of genotypes than the moderate estimate as it accounts both for recrudescence and possible PCR anomalies that prevent detection of genotypes in multiply infected samples. However, the total duration of infection with each genotype may be much

longer than the 61 day duration of this study (James, Nicol & Shute, 1932; Roper *et al.* 1998). It has been suggested that increased multiplicity of *P. falciparum* in older children is the result of the cumulated chronicity of multiple infections over long periods of time (Smith *et al.* 1999b). Recurring chronic infections that become undetectable between episodes cannot be distinguished from newly inoculated parasites when transmission is ongoing. Similarly, relapses from dormant liver stages of *P. vivax* cannot be distinguished from new infections. Measurement of the total duration of malaria infections therefore poses a significant challenge in endemic regions where the high rate of multiple infections prevents the use of multi-locus genotyping to more accurately distinguish parasite populations detected at different times.

We would like to thank the people of Gonoa for their longstanding co-operation and for their tolerance throughout the study period. Our thanks also to the staff of the Papua New Guinea Institute of Medical Research at Madang for their assistance, and especially to the members of the field team who assisted with sample collection. This work is dedicated to the memory of Helena Vrbova and Nicky Gibson, whose contribution to malaria research in PNG was substantial. Field work was funded by a grant from The European Commission, STD 3, to D. W., K. P. D. and M. P. A. K. D. and C. A. D. are funded by The Wellcome Trust. M. B. was funded by a studentship from The British Medical Research Council and by The Wellcome Trust. The research conducted here was also supported by National Institutes of Health (NIH) grant AI24710 awarded to J. W. B., and NIH grant AI37545 and WHO/TDR grants (950440 and 910495) awarded to M. R. G.

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