Seasonal changes in the *Plasmodium falciparum* population in individuals and their relationship to clinical malaria: a longitudinal study in a Sudanese village

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SUMMARY

Residents of Daraweesh village in Sudan were monitored for *Plasmodium falciparum* infection and malaria morbidity in 3 malaria seasons from 1993 to 1996. Malaria parasites were detected microscopically and by polymerase chain reaction (PCR) in a series of cross-sectional surveys. PCR revealed submicroscopical infections during the dry season, particularly among individuals who had recovered from a malaria episode following successful drug treatment. Clinical and subclinical infections were contrasted by assaying for allelic polymorphism at 2 gene loci, MSP-1 and GLURP and 2 hypotheses examined with reference to these data: that clinical malaria is associated with infection with novel parasite genotypes not previously detected in that host, or alternatively, that clinical malaria episodes are associated with an increased number of clones in an infection. We detected more mixed infections among clinical isolates, but people carrying parasites during the dry season were not found to have an increased risk of disease in the following malaria season. There was a clear association of disease with the appearance of novel parasite genotypes.

Key words: *Plasmodium falciparum*, asymptomatic infection, PCR genotyping, longitudinal field studies, Sudan.

INTRODUCTION

Malaria is unstable in the semi-arid savannah of central and northern Sudan and the great majority of infective bites take place in September and October, immediately prior to the seasonal peak of malaria cases during these months (Omer & Cloudsley-Thompson, 1970). Individuals in Daraweesh have around 1 episode of clinical malaria every 2–3 years and do not show the pattern of age-dependent acquired immunity found in holo- or hyper-endemic malaria situations (Theander *et al*. 1990). Slide detectable parasitaemias are generally associated with clinical malaria episodes and are largely confined to the September–December transmission season. The underlying parasitological situation is more complex. Anti-malarial antibody titres were found to rise over the malaria season in the majority of individuals although only one third had had a clinical attack of malaria in that particular year (Elhassan *et al*. 1995). PCR detection of low level *Plasmodium falciparum* infections (< 10 parasites/μl) has shown that some individuals harbour asymptomatic, submicroscopically patent infections during the dry season (Roper *et al*. 1996). Thus, although it is obvious that the major factor controlling the seasonal outbreaks of disease is the increase in transmission after the rains, given that infections are present through the dry season, why this effect is so pronounced is less clear and why only a minority of infections actually appear to lead to clinical illness is not understood.

In this study 2 hypotheses for the mechanism by which disease might be linked to the role of superinfecting *P. falciparum* inoculations have been tested by PCR genotyping a longitudinal series of infection samples from a cohort of Daraweesh residents under malaria morbidity surveillance. (1) The 'strain' or antigenic variant-specific immunity may operate in such a way that exposure to novel variants leads to

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infections not suppressed by existing antigen-specific immune responses. (2) That the number of PCR-detectable genotypes *per se* may be linked to disease. These 2 hypotheses are not mutually exclusive and may be particularly hard to disentangle in data gathered from areas of high malaria endemicity where the turnover of parasites in individuals is high. The exceptionally restricted transmission season and low entomological inoculation rates of eastern Sudan make Daraweesh a particularly suitable place to study the effects of a low pressure of *P. falciparum* infection on the incidence of asymptomatic infection and factors precipitating episodes of disease.

MATERIALS AND METHODS

The study area

Daraweesh is a farming village (1996 population ~ 430), 18 km south of the town of Gedaref, in Gedaref State, eastern Sudan. Its major agricultural activities are the rain-dependent cultivation of sorghum and sesame. Irrigation of vegetable gardens from deep wells is also carried out close to the hut compounds. These wells are thought to be sites where mosquitoes can persist through the long, hot, Sudanese dry season (Omer & Cloudsley-Thompson, 1968). The area is mesoendemic for malaria and the great majority of cases occur in the September–November period after the July–September annual rains. Although a few cases of *P. vivax* and *P. malariae* are seen, around 95 % of all cases of malaria are caused by *P. falciparum* and transmitted by *Anopheles arabiensis* (Haridi 1972).

Malaria case detection

The study is based upon longitudinal sampling and observation of a volunteer cohort of 106 residents of Daraweesh village, none of whom carry the sickling allele of the a-haemoglobin gene. Both passive and active case detection methods are used to monitor experience of clinical malaria and asymptomatic *P. falciparum* infection in both wet and dry seasons (Elhassan *et al*. 1995; Roper *et al*. 1996). While the cohort constitutes the main focus of the study, village-wide monitoring of *P. falciparum* infection is maintained in parallel. The blood sampling plan has been granted ethical clearance by the Research Board of the Faculty of Medicine, University of Khartoum and national clearance by the Sudanese Ministry of Health.

Cross-sectional screening of the cohort and all available village residents is carried out at the beginning and end of the malaria season (early September and early-mid January respectively) and again in the middle and at the end of the dry season. In the course of the screening, residents donate a blood sample from which a thick blood film slide is made. At the same time temperature is measured, a spleen examination is carried out, and verbal reports of fever or other symptoms of febrile disease are recorded. During the malaria season residents report medical complaints to the study team which includes a health worker who lives in the village. Upon reporting any symptoms suggestive of malaria a thick blood film is taken and examined after Giemsa staining. Films were considered negative after examination of 200 microscopical fields without detection of parasites. Clinical malaria is defined as a positive blood slide together with either reported experience of fever or measured temperature equal to or greater than 37·5 °C. Patients are treated with chloroquine and the minority who fail to respond to chloroquine are further treated with sulphadoxine/pyremethamine. In addition to this passive detection system, members of the cohort are monitored throughout the malaria season by a bi-monthly health enquiry, temperature measurement and spleen examination.

Malaria case definition

Malaria case definitions in areas of high endemicity use indices of parasite density adjusted for age as the criterion for attributing the cause of febrile illness to malaria (Alonso *et al*. 1994; D'Alessandro *et al*. 1995). In Daraweesh, case definition is decided on the basis of 2 criteria, firstly symptoms reported by the patient (constant or intermittent fever, headache, joint pain, vomiting, and diarrhoea) and, secondly, the measured signs of temperature and blood film. A relational database (FoxProTM) containing clinical and parasitological records of all village inhabitants has been established. The database can be 'queried' such that every person/time data-point is allocated into 1 of the following 6 categories. Type 0. No malaria. No symptoms and a measured temperature of less than 37·5 °C. Type 1. Asymptomatic infection. Blood slide positive. Symptoms and signs of malaria absent. Type 2. Symptomatic but non-febrile disease. Blood slide positive. Symptoms reported, but no fever either measured or reported. Type 4. Symptomatic febrile malaria. Blood slide positive. Reported fever, but measured temperature less than 37·5 °C. Type 5. Symptomatic febrile malaria. Blood slide positive. Measured temperature over 37·5 °C. Type 9. Other disease. Blood slide negative. Reported fever or measured temperature greater than 37·5 °C.

We have used this approach because in Daraweesh, slide-detectable asymptomatic carriage of malaria parasites is uncommon, in contrast to the situation in highly endemic areas (Smith, Armstrong-Schellenberg & Hayes, 1994). We there-

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fore attribute the detection of parasites in a blood film from a febrile individual to a clinical malaria attack.

P. falciparum *DNA extraction*

All parasitized red blood cells were separated from plasma in the field and mixed with an equal volume of parasite freezing solution (28% glycerol, 3% sorbitol, 0·65% NaCl) before being shipped from the field in liquid nitrogen, then stored at −70 °C. PCR-quality DNA was prepared using a modified version of Foley's rapid extraction protocol (Foley, Ranford-Cartwright & Babiker, 1992). Twenty ml of thawed red blood cells were vortexed in 0·5 ml of ice-cold 5 mM sodium phosphate, pH 8·0. Insoluble material, including parasite nuclei was then pelleted by micro-centrifugation for 10 min at 10000 ***g***. This procedure was repeated twice before re-suspending the final pellet in 50 ml of sterile water and boiling for 10 min. After a final centrifugation the DNA-containing supernatant was removed and stored at −20 °C.

PCR analysis

rDNA genes. All samples were initially screened for the presence of *P. falciparum* DNA using nested PCR amplification of the small subunit ribosomal RNA gene using previously described primers and cycling parameters (Snounou *et al*. 1993). Outer PCR reactions were carried out with 4 μl of DNA prepared as above in 20 ml reactions containing 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl, pH 8·3, 0·1 mg/ml of gelatin, and 125 mM of each of the 4 deoxynucleotide triphosphates. Each primer was present at a concentration of 250 nM and 0·5 units of AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, CT) per reaction were used. One microlitre of the product of the 'outer' reaction was used as template for the second, 'inner' reaction.

Genotyping using GLURP locus primers. Alleles at the GLURP gene locus were detected in a nested PCR reaction using outer and inner primers derived from the published gene sequence (Borre *et al*. 1991). Outer primers are G4 (5′ACATGCAAGTGTTGATCC 3′) and G5 (5′GATGGTTTGGGAGTAACG 3′) (Paul *et al*. 1995) used for 30 cycles with parameters of 94 °C for 25 sec, 45 °C for 1 min and 68 °C for 2 min. One μl of the outer reaction was used as template in an inner reaction using primers G1 (5′TGAATTTGAAGATGTTCACACTGAAC 3′) and Gs (5′TGTAGGTACCACGGGTTCTTGTGG 3′) (Viriyakasol *et al*. 1995) in a reaction of 30 cycles with parameters of 94 °C for 1 min, 55 °C for 2 min and 70 °C for 2 min. Outer and inner reaction conditions were carried out in a final concentration of 2·5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl, pH 8·3, with 1 μM of each primer.

Genotyping using primers for 3 major types of MSP-1 block 2 type sequences (*3D7/Palo Alto/K1*, *Mad 20/Wellcome and RO33*)

Genotyping at the MSP-1 locus was carried out as a nested PCR reaction. The outer PCR primers O1 (5′CACATGAAAGTTATCAAGAACTTGTC 3′) and O2 (5′GTACGTCTAATTCATTTGCACG 3′) were used in the initial amplification reaction of 30 cycles at 94 °C for 25 sec, 50 °C for 35 sec and 68 °C for 2·5 min with a final extension at 68 °C for 10 min (Ranford-Cartwright *et al*. 1993). One μl of the outer reaction was then transferred to a second 'inner' reaction tube for amplification using the published primers and conditions of Cavanagh & McBride (1997). All inner reactions were carried out with cycling parameters of 95 °C for 1·5 min, 50 °C for 15 sec and 72 °C for 30 sec for 35 cycles. 3D7 type alleles were amplified with primer 1 (5′CTGGATCCAATGAAGAAGAAATTACT3′) and primer 2 (5′GGGAATTCTTAGCTTGCATCAGCTGGAGG 3′). Mad 20 type alleles were amplified using primer 3 (5′CTGGATCCAATGAAGGAACAAGTGGA 3′) and primer 4 (5′GGGAATTCTTAACTTGAATTATCTGAAGG 3′). RO33 type alleles were amplified by primer 5 (5′CTGGATCCAAGGATGGAGCAAATACT 3′) and primer 6 (5′GGGAATTCTTAACTTGAATCATCTGAAGG 3′). These primers match the 5′ and 3′ end of the 3 main types of MSP-1 and block 2 sequences (Miller *et al*. 1993) in addition to containing *Bam*H 1 and *Eco*R 1 cloning sites.

Statistical analysis

The measure of infection complexity used was minimum clone number. This value was estimated as the higher number of alleles detected at either locus in any given isolate. Thus if 2 alleles were detected at the MSP-1 locus and 1 allele at the GLURP locus the minimum clone number was 2. For comparison of symptomatic and asymptomatic infections, isolates were classified bivariately as having a clone number either equal to or greater than 1. The effect of age and symptoms was assessed by analysis of covariance carried out using a generalized linear model with binomial error structure (Crawley, 1993). All other analyses were performed using SPSS for Windows, version 6.1 (SPSS Inc., Chicago).

RESULTS

The prevalence of infections in Daraweesh from 1993 to 1995

P. falciparum infections were detected by both microscopical examination of blood films and by nested PCR amplification of parasite DNA at 10 cross-sectional survey points between September

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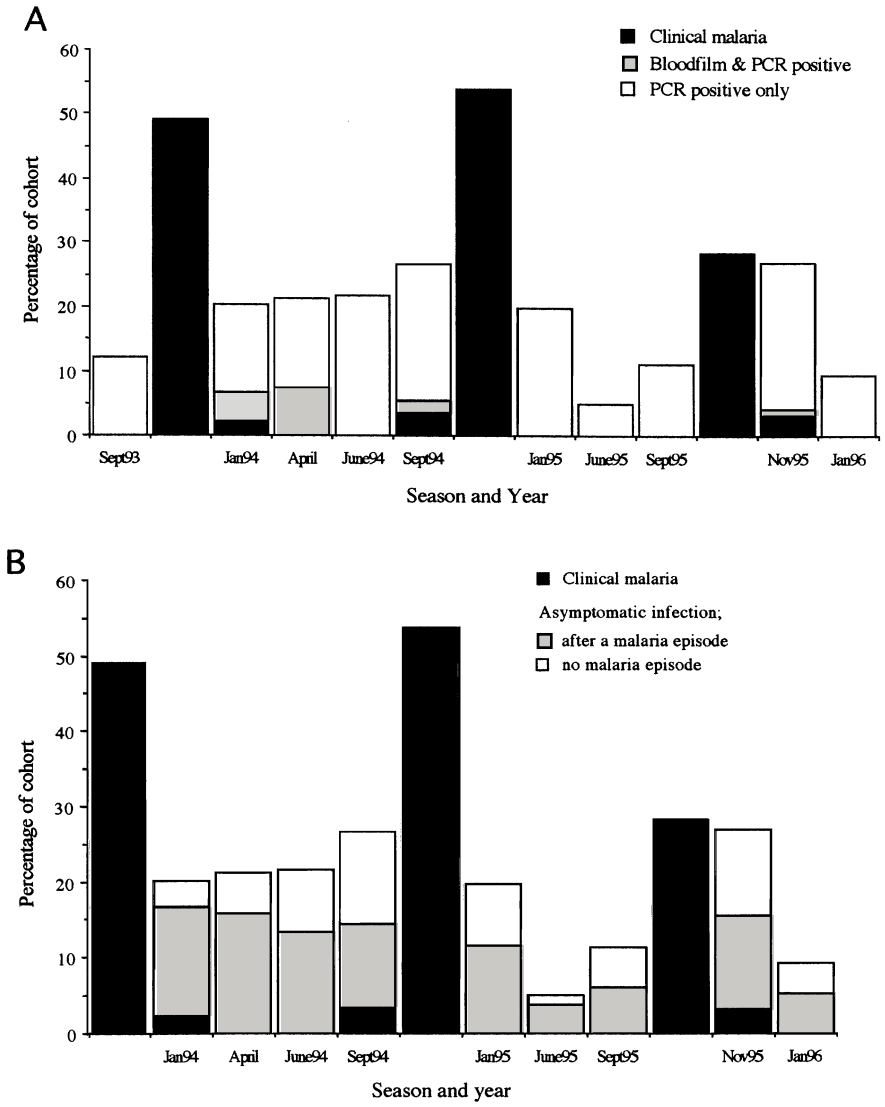

Fig. 1. The history of symptomatic and asymptomatic *Plasmodium falciparum* infection in a cohort of 106 people. (A) The incidence of malaria and the prevalence of asymptomatic infection. The percentage of the cohort which had malaria during September–January is shown as a black column. Point prevalence surveys conducted between malaria seasons are also shown and illustrate the prevalence of infection as detected by blood film examination (grey) and PCR (open). The small number of clinical infections overlapping with the January, November and September surveys are also shaded in black. (B) The relationship between clinical malaria and subsequent asymptomatic infection. As in (A) the percentage of people who experienced clinical malaria each malaria season is shown in black and the percentage of all asymptomatic infections at each survey point (whether detected by blood film or PCR) are shaded to indicate that proportion which had malaria in the preceeding malaria season (grey) and that which did not (open).

1993 and January 1996. Point prevalence was measured using these 2 methods and the results are shown in Fig. 1A. PCR amplification of parasite DNA confirmed microscopical parasite detection and, in addition, revealed substantial numbers of submicroscopical infections. The overall proportion of the cohort who experienced clinical disease during the malaria season each year is also shown. Half of the cohort suffered at least 1 clinical malaria episode during the 1993 malaria season. A similar proportion was affected in 1994. In 1995 the malaria outbreak was smaller and of shorter duration affecting only 33 members of the cohort. Small numbers of clinical cases were found at either end of the malaria season in September 1993 and January 1994 and these are indicated on the bar chart. Type 1 infections (asymptomatic, blood film positive) were occasionally recorded outside the malaria season but were never seen in the late dry season (June) yet PCR assays demonstrated that the reservoir of infected individuals exists year round. The sensitivity of parasite detection by nested PCR is greater than by thick blood films read under field conditions (Roper *et al*. 1996). Thus there is a trend for measured parasite densities to decline as the dry season progresses.

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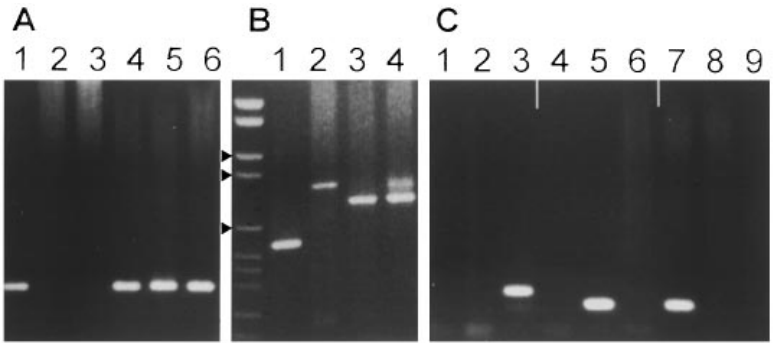

Fig. 2. Detection and genotyping of *Plasmodium falciparum* infections by PCR amplification of rDNA, GLURP and MSP-1. (A) PCR of DNA isolated from 6 individuals' blood samples. Reaction products were separated on a 1·5% agarose gel after 'outer' and 'inner' nested PCR reactions using primers derived from the *P. falciparum* small subunit rRNA gene. (B) DNA from 4 clinical isolates amplified in a nested PCR reaction using primers which flank a region with a variable number of tandemly repeated sequences in the GLURP gene. The 3 arrows indicate marker bands of 1230, 1033 and 653 bp. (C) DNA from 3 clinical isolates amplified with primers specific to MSP-1 block 2 allele classes. Lanes 1–9 show the product of a common 'outer' PCR re-amplified with 'inner' primers specific for 1 of 3 classes of Block 2 alleles. The reactions in lanes 1, 4 and 7 were carried out with the RO33 group specific inner primers. Lanes 2, 5 and 8 used the inner primers specific for the Mad 20 type alleles and lanes 3, 6 and 9 the 3D7 type-specific 'inner' primers.

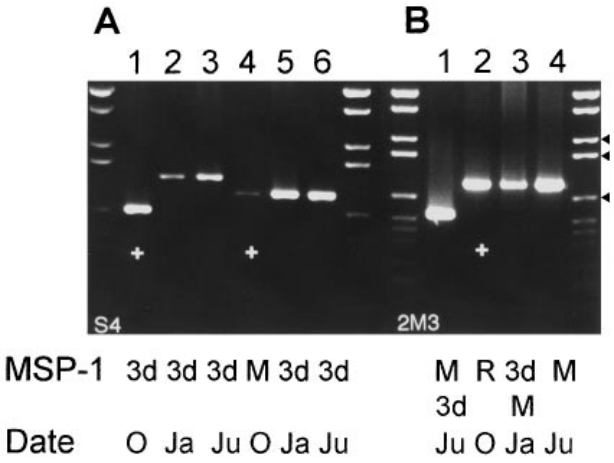

Fig. 3. PCR genotyping of longitudinal collections of blood samples from 2 cohort members. Product from nested GLURP amplification of DNA from a series of isolates collected between October 1993 and June 1995 are shown. (A) PCR-positive samples were taken from cohort member S4 at the 6 dates indicated and amplified with the GLURP primers. MSP-1 allele types of the same DNA sample are shown underneath each lane. The white crosses underneath lanes 1 and 4 indicate that these were samples taken during clinical episodes of malaria. (B) GLURP gene amplifications and corresponding MSP-1 allele types from cohort member 2M3. The 3 arrows indicate marker bands of 1230, 1033 and 653 bp.

The effects of recent infection upon subsequent susceptibility

The risk of suffering a clinical attack was the same for people who had an attack the previous year as for those who did not. Relative risk (R) from 1993 to 1994 was 0·85 and from 1994 to 1995 was 1·13. Neither value was significantly different from 1. Likewise the risk of a malaria attack was not increased in those individuals found to be asymptomatically infected during the January, April, or June preceding the malaria season ($R = 0{\cdot}91$ in 1994, and 0·94 in 1995).

Chronic infection after clinical malaria attacks

Fig. 1B shows the relationship between the experience of clinical infection and subsequent asymptomatic infection. Asymptomatic infections at each survey point are shaded to indicate the proportion who had malaria the previous malaria season. The relative risk of being asymptomatically infected in January after having a treated clinical malaria episode in the previous season was 4·89 in 1993–4, 1·16 in 1994–5, and 2·79 in 1995–6. Each year the direction of difference was the same but R was only significantly different from 1 in the 1993–4 period. Chronic infections do not always arise through this route. In 1994, 14% of the cohort were found to be asymptomatically infected in dry season (January–June) without having had a clinical malaria attack in the previous malaria season and in 1995, 7% of the cohort became infected under the same circumstances.

Detection and genotyping of P. falciparum *infections*

Fig. 2 illustrates the nested PCR results obtained when detecting and genotyping malaria infections. After DNA extraction, all samples were amplified using an 'outer' and then 'inner' pair of primers based on the *P. falciparum* small subunit rRNA gene. Fig. 2A, lanes 1–5 show the result of nested rDNA PCR on DNA extracted from 20 μl of blood from 5 individuals in the Daraweesh cohort during

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Table 1. Seasonal variation in the minimum number of *Plasmodium falciparum* clones detected

	Minimum clone number					
	0	1	2	3	4	Mean
September 93	0	6	2	1	0	*1·44*
Clinical 93	0	14	11	2	0	*1·56*
January 94	0	9	6	0	0	*1·40*
April 94	0	6	7	5	1	*2·05*
June 94	0	6	10	4	1	*2·00*
September 94	0	14	3	3	0	*1·45*
Clinical 94	0	18	18	9	0	*1·80*
January 95	0	10	8	1	0	*1·53*
June 95	0	1	2	1	0	*2·00*
September 95	0	8	3	0	0	*1·27*
Clinical 95	0	19	13	0	0	*1·41*
November 95	0	19	4	0	0	*1·17*
January 96	0	8	1	0	0	*1·11*
Total	0	138	88	26	2	

November 1995. All of these individuals were asymptomatic without visible parasites in a standard 200 field examination of a Giemsa stained slide. However, 4 of the 5 samples amplified a 205 bp band with the rDNA primers. Allele typing was carried out with the GLURP and MSP-1 assays. Fig. 2B illustrates size polymorphism among the products of the 'inner' GLURP nested PCR reaction. Lane 4 contains 2 products of different size indicating that there were at least 2 distinct genotypes of parasite present in the blood of this patient. Fig. 2C shows MSP-1 genotyping of 3 isolates using a nested PCR assay. The product of the outer PCR reaction was re-amplified in 3 separate 'inner' reactions with primers based on sequences unique to each of the 3 Block 2 types RO33, Mad 20 and 3D7. The isolate in lanes 1, 2 and 3 was positive only for parasites with a 3D7 type MSP-1 allele, while the isolates in lanes 3–5 and 7–9 were positive only for parasites with Mad 20 and RO33 alleles respectively.

Longitudinal sampling and genotyping of blood samples from the Daraweesh cohort

All PCR-positive *P. falciparum* infections whether clinical or asymptomatic, were genotyped for allelic variation at the MSP-1 and GLURP loci. Fig. 3 shows an example of this longitudinal sampling and PCR typing for 2 individuals S4 (Fig. 3A) and 2M3 (Fig. 3B). S4 donated a blood sample while suffering from malaria in October 1993. Lane 1 shows a 650 bp GLURP product amplified from this sample. The patient recovered following chloroquine treatment yet remained PCR positive in the following January and June surveys. In both samples (lanes 2 and 3) the patient S4 was asymptomatically infected with parasites containing a different GLURP allele ($\sim$ 900 bp) from that detected in the clinical sample. MSP-1 allele typing indicated that S4 had parasites with the 3D7 allele at each of these 3 survey points. Therefore parasites of genotype 3D7/GLURP 900 either survived the chloroquine treatment or infected S4 after the cure of the first infection but did not give rise to a symptomatic malaria episode. Patient S4 suffered another malaria attack in the following malaria season (lane 4) with parasites carrying a new GLURP allele (705 bp) and a new MSP-1 allele (Mad 20). This individual remained chronically infected following treatment and was PCR positive in January and June 1995, although again the 'chronic phase' parasites were not genetically identical to the 'acute phase' parasites. We cannot distinguish between the possibilities that a clone originating in a mixed inoculum was not successfully cleared and that of asymptomatic re-infection following treatment.

These complex population turnovers were also observed in the apparently simple GLURP PCR results seen in a series of samples from patient 2M3 (Fig. 3B). 2M3 was asymptomatically infected with parasites with a GLURP product of approximately 530 bp in June 1994 (Fig. 3B, lane 1). There were at least 2 clones in this infection as this sample was also positive for the MSP-1 Mad 20 and 3D7 alleles. A sample taken from this patient during a malaria episode in October 1994 revealed parasites with a larger GLURP allele and the RO33 allele. Patient 2M3 was treated and recovered yet remained asymptomatically infected and positive for the GLURP 750 bp allele in the following January and June 1995. By January 1995 the RO33 allele had disappeared but 2 parasite genotypes with Mad 20 and 3D7 alleles were detected. By June 1995 (late dry season) the asymptomatic infection had only GLURP 750/MSP-1 Mad 20 type parasites.

The complexity of infections through the seasons

Table 1 shows the frequency with which 0–4 alleles/infection were detected at the GLURP and MSP-1 loci in each survey. The numbers of alleles detected in each infection were used to estimate minimum clone number and these are also summarized. Clinical isolates collected during the malaria transmission seasons are italicized. The average 'minimum clone number' was calculated for each time-point and is shown in each graph. The 2 highest mean minimum clone numbers occurred in June of 1994 and 1995. Each had a value of 2. January mean values were all lower than those of the preceding malaria season. The relative complexity of infection in clinical isolates correlates with the size (in numbers of cases) of the malaria outbreak which occurred each year i.e. 1994 > 1993 > 1995.

To compare the complexity of symptomatic and asymptomatic infections, isolates from all 3 years

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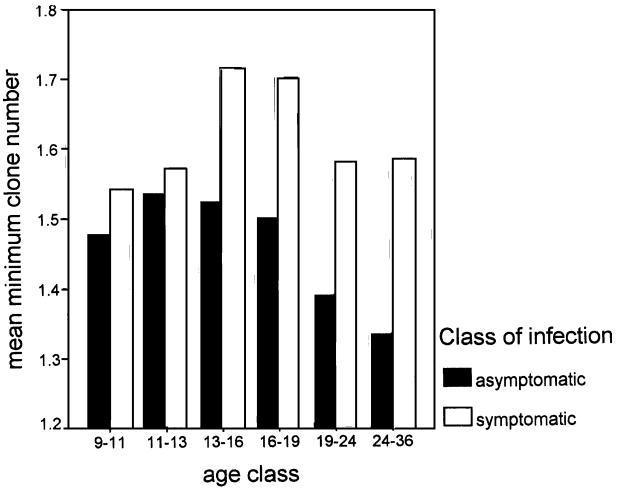

Fig. 4. The mean number of clones detected in symptomatic and asymptomatic *Plasmodium falciparum* infections across age classes.

were grouped. Fig. 4 shows the mean of numbers of clones in either asymptomatic or symptomatic infections over the range of age classes. The proportion of infections with more than 1 clone was greater in symptomatic infections (0·51) than in asymptomatic infection (0·38), though that difference was only marginally significant ($\chi^2 = 4{\cdot}05$, 1 D.F., $P < 0{\cdot}05$). There was no significant effect of age.

Parasite turnover during transitions from symptomatic and asymptomatic infection

To examine whether there are significant patterns of parasite population changes within individuals, we collated data on genotypic turnover from the entire cohort. Where a series of PCR detectable infections had occurred in 1 individual, the products amplified from successive infections were run in adjacent lanes on agarose gels (as seen in Fig. 3). If identically sized GLURP and MSP-1 alleles or classes of allele were common to 2 successive isolates these were classed as sharing a common infection.

Clinical isolates were compared with asymptomatic isolates taken from the same individual in January, April or June. Where asymptomatic infection followed clinical infection, MSP-1 and GLURP alleles were common to both isolates in 33% of cases. Where the asymptomatic infection preceded the symptomatic infection only 4% of cases had matching alleles although the likelihood of new infections being genetically identical by chance is the same in these 2 scenarios. A χ^2-test showed that the difference between the 2 types of transition was significant ($\chi^2 = 6{\cdot}37$, 1 D.F., $P < 0{\cdot}025$). This indicates that these clinical infections have a significant tendency to become chronic despite a successful outcome of drug treatment.

Chronic infections arising in this way are gradually cleared. Of the cases where clinical malaria was followed by an asymptomatic infection the proportion of matches diminished with time since the illness. In November, the survey closest to the peak malaria season, 64% of cases shared common alleles with the clinical isolate. By January this had fallen to 41%, by April to 40% and in June, 8 months after the malaria season it was 12%.

DISCUSSION

The seasonal infection cycle

PCR detection of malaria parasites has revealed that low parasitaemia, asymptomatic bloodstream infections persist through the long Sudanese dry season in a significant minority of Daraweesh residents. The combination of microscopical and PCR assays with their differing thresholds of detection sensitivity reveals an annual cycle in which bloodstream parasite density peaks during malaria episodes in the transmission season and then declines as the dry season progresses.

The tendency of malaria to become chronic

Asymptomatic infections are 1·2–4·9 times (depending on the year) more likely to be seen in January, in people who had clinical malaria during the transmission season. In 41% of such cases parasites apparently identical to those present during the clinical episode were still present 2–3 months later. Clinical cure, but frequently not parasite clearance was therefore achieved by chloroquine treatment but whether this is due to drug resistance or is in fact normal is unclear. The relatedness of clinical and subsequent asymptomatic infections decreased as the length of the time interval between them increased. This suggests that a process of gradual elimination occurs through the dry season, mirroring the trend towards diminished parasite densities over the same interval.

The appearance of 'novel' infection during the dry season

In 59% of cases where people were asymptomatically infected in the dry season following a clinical episode, the parasites in the 2 isolates were different. There are 2 possible explanations for this observation. First, novel parasite genotypes appearing during the dry season may represent clones present during the clinical episode but undetected because they were minority types. This interpretation is supported by the observation that PCR detection thresholds vary according to the relative abundance of the sequences 'competing' for primer molecules in the early stages of the chain reaction. When the ratio of 2 alleles is outside the 1:1 to 1:10 range, the PCR product of the rarer allele is obscured by the 'dominant' one (Arnot, Roper & Bayoumi, 1993; Contamin *et al*. 1995).

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An alternative possibility is that individuals become re-infected with novel parasites after successful clearance of a primary infection. This would imply that they were able to control this new infection and avoid clinical symptoms but not to prevent the establishment of chronic infection. This interpretation would be consistent with our observations that 7–14% of the cohort were infected in the dry season despite not having a preceding malaria episode. Newly apparent dry season infections probably arise via both of these routes but we do not know which is the more important in the Sudanese situation. This question is not trivial since either explanation supports different assumptions about immunity, the true entomological inoculation rate and the length of the transmission season. Longer term and more frequent follow-up sampling of individual infections may resolve this question.

The complexity of infections in symptomatic and asymptomatic individuals

If the number of clones/infection *per se* increases the risk of symptomatic disease, then chronic infections lingering through the dry season might increase the risk of malaria in the following transmission season. This is not supported by our data and we estimate the relative risk of malaria in those known to be asymptomatically infected in the preceding dry season to be 0·91 in 1994 and 0·94 in 1995.

In comparisons between the 2 infection states we find a small but significant tendency for infections to contain a greater number of clones during clinical episodes than during asymptomatic carriage. There is a considerable difference in parasite density between these 2 infection states and one explanation of the observed difference is that sampling a greater number of parasites increases the likelihood of detecting mixed infections. Another effect noted in this study, however, was that in April and June, when parasite densities are at their lowest, the average number of detected clones was highest. Our proposed explanation for these apparently paradoxical results is that the relative proportions of different genotypes detected are more nearly equal in controlled asymptomatic infections, and consequently the rarer alleles are less likely to be obscured. If this is the case then it is probable that the number of clones in symptomatic infections is underestimated.

A study following 10 Senegalese individuals over a 4 month period of intense transmission found asymptomatic infections were more complex than symptomatic infections (Contamin *et al*. 1996); a trend in the opposite direction to that reported here. Although the asymptomatic infections assayed in Senegal were all blood film positive, the density of parasites was still substantially lower than in symptomatic infections. This finding would tend to support the explanation that it is the relative abundance of genotypes, rather than the numbers of parasites sampled, which is important in the detection of mixed infections by PCR. The disparate findings of these 2 studies may more closely reflect the density-dependent constraints upon PCR detection than real differences in genotype numbers.

The causes of clinical malaria and chronic infection

Even in areas of year-round infection, both mild and severe disease incidence are concentrated in the period of maximum sporozoite inoculation pressure, towards the end of the seasonal rains (Greenwood *et al*. 1987; Trape *et al*. 1994). The most popular hypothesis explaining this association is that it results from antigenic differences between the existing and superinfecting parasites leading to a situation where the new infection is not controlled by existing antigen-specific host immune responses (Lines & Armstrong, 1992). Recent studies have sampled children during periods of symptomatic and asymptomatic carriage and found that the parasites present at low density during asymptomatic phases differed from those parasites responsible for the clinical episode (Contamin *et al*. 1996). In the Daraweesh cohort, the genotypes of parasite associated with clinical malaria were also almost invariably found to be new to that individual. The few cases in which genotypes from clinical isolates had been previously detected were September survey samples which may have picked up pre-patent infections. Our results therefore support the view that clinical malaria is precipitated by novel infections.

Our data also show that there is a strong tendency for even drug-cured malaria episodes to develop into chronic but generally asymptomatic infections. However, we do not understand either the mechanisms employed by the parasite to evade immune clearance or the host's response to long-term infection. Studies on the seasonal changes in individuals' capacity to produce agglutinating antibody against infected erythrocytes indicate that during the transmission season, most individuals in Daraweesh seroconvert to produce antibodies, capable of agglutinating at least 1 isolate of parasites from clinical cases in the village that season (Giha *et al*. 1998). Surprisingly, this seasonal increase in the capacity to agglutinate parasitized cells was almost as pronounced in those who did not suffer a clinical attack as in those who did. Furthermore, 25 individuals have been found to be PCR positive in at least 1 of our surveys without having complained of any symptoms to our village health team in the last 3 years. These results indicate that asymptomatic infections are directly acquired and not solely the result of the decay of previous clinical infections. Mechanisms through which the parasite establishes

and maintains itself in the chronic phase, perhaps via antigenic variation, may be very important in epidemiological situations of seasonal vector scarcity such as the Sahel.

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