Population dynamics of *Plasmodium falciparum* in an unstable malaria area of eastern Sudan

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SUMMARY

The *Plasmodium falciparum* population in Asar village, eastern Sudan, where malaria transmission is markedly seasonal, was monitored monthly over a period of 15 months. A cohort of infected patients was treated and then followed monthly throughout the dry season until the next transmission season. Parasitaemia detected by microscopy among the cohort reduced dramatically following treatment, but remained sporadic during the dry season, and reappeared following the onset of the next wet season. However between 40 and 50% of the cohort retained a persisting parasitaemia detectable by PCR throughout the dry season. These parasites were genetically complex, consisting of multiple clones with a large repertoire of alleles of the studied genes. While the number of clones per host dropped significantly following treatment of acute cases during the transmission season, drug treated people nevertheless maintained an average of one clone throughout the dry season. Allele frequencies of MSP-1, MSP-2 and GLURP showed slight, statistically insignificant, fluctuations between the dry and wet seasons. A higher frequency of inbreeding was estimated among the parasites that survived the dry season compared to the wet season.

Key words: malaria, *Plasmodium falciparum*, dynamics, Sudan.

INTRODUCTION

In eastern Sudan, malaria is a disease of the short rainy season, with the rest of the year dry and almost malaria transmission-free. Such a pattern of epidemiology is common in the poor Savannah belt of sub-Saharan Africa, where rains are scant and markedly seasonal (Molineaux, 1988). However, the emergence of anopheline mosquitoes following rainfall can lead to major malaria outbreaks in the area. The origin of the *P. falciparum* parasites that precipitate these annual epidemics is not clear. Previously we have noticed fluctuations in frequencies of some polymorphic genes over 3 consecutive seasons (Babiker, Satti & Walliker, 1995). However, recent molecular surveys have demonstrated that a large reservoir of *P. falciparum* infections persists as asymptomatic subpatent infections during the dry season (Babiker *et al.* 1998; Roper *et al.* 1998).

In the present work we compare the parasite populations that occur during the dry season with those of the previous and the following wet season, in order to elucidate the stability of the parasite population and the role of the dry season parasite reservoir in the cyclic malaria epidemics in this area. The main aim was to examine whether *P. falciparum* in villages in this area comprises a continuous population or whether a significant reduction in population size (bottleneck) occurs during the lengthy dry season. We have estimated the mean number of clones per infection across this period, at 3 time-points, one of which was during the dry season. During this time of the year clinical malaria reports are rare, and no evidence of transmission has been observed (A. Hamad *et al.* unpublished observations). It has been suggested that in such an area mosquitoes retract to scanty breeding sites where they survive the dry season with an incomplete reproductive cycle (Omer & Cloudsley-Thompson, 1970).

MATERIALS AND METHODS

Study area

The study was carried out in Asar village in the eastern region of Sudan. The population of this village is approximately 4000. Malaria endemicity in this area matches the hypo-endemic classification, and transmission is markedly seasonal following the rainy season (July–November), lasting approxi-

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mately 8–12 weeks. *P. falciparum* is the predominant parasite species, accounting for more than 90% of malaria cases, while *P. vivax* occurs at a frequency of around 5%. The parasite rate among schoolchildren is less than 5%. *Anopheles arabiensis* is the main mosquito vector, and the entomological inoculation rate (EIR) is less than 1 bite per person during the transmission period (Babiker *et al*. 1997). However, the risk of malaria infection is not uniform in the village and some families are at greater risk than others.

P. falciparum *isolates*

For this study a cohort of 83 patients, belonging to 18 families distributed throughout the village, were recruited in October 1993. All harboured *P. falciparum* parasites diagnosed microscopically by blood smear. The families consented to participate in the study after the aims and the protocols were clearly explained. Following diagnosis, patients were treated with chloroquine or Fansidar, then monitored fortnightly for the first 2 months and subsequently each month throughout the dry season until the end of the next transmission season (December 1994).

Detection of malaria parasites

For microscopy, thick and thin blood smears were collected from each patient, at each time-point during the survey. Smears were stained with Giemsa's stain and examined microscopically. A film was diagnosed negative after examination of 100 thick fields.

For PCR, parasite DNA was first extracted from 10–20 μl of each blood sample using a resin buffered suspension matrix as described by the manufacturer (Insta Gene, Bio-Rad). PCR was then carried out using outer and nested primers to enhance the sensitivity of detection of subpatent parasitaemia (Ranford-Cartwright *et al*. 1997; Babiker *et al*. 1998). The primers amplified respectively a polymorphic region (block 2) of MSP-1 (Tanabe *et al*. 1987), most of MSP-2 (Smythe *et al*. 1991) and the central region of the GLURP gene (Borre *et al*. 1991).

Variation in the length of the amplified fragments of each gene was identified as size differences of the PCR products. Due to the continuous nature of size variation of the amplified fragments of the examined region in these genes, alleles with close sizes were binned around an average size. In addition, sequence variations in the amplified regions of MSP-1 and MSP-2 were detected using oligonucleotide probes (Babiker *et al*. 1994). Three sequence classes of the amplified block 2 of the MSP-1 gene, denoted K1, MAD20 and RO33 (Kimura *et al*. 1990), and 2 of block 3 of MSP-2, denoted IC1 & FC27 (Smythe *et al*. 1991), were examined.

Estimates of mean number of clones, allele frequencies and inbreeding coefficient

Three time-points were chosen for the analysis of the above parameters among the examined isolates, the wet season 1993, the dry season 1994 and the following wet season 1994. Parasites representing the wet season of 1993 were those that caused the initial infections (October 1993) among 65 patients who completed the study. The dry season parasites were represented by infections among individuals found to maintain their parasitaemia in June 1994. If a patient was not infected in June then an infection that occurred close to June, during the dry season between February and July 1994, was considered as representative. The next transmission season was represented by infections that occurred during October and November 1994.

Estimates of allele frequencies and mean number of clones were based on the analysis of the sequence variants of block 2 of MSP-1 (K1, MAD20 and RO33) and the central region of MSP-2 (IC1 and FC27). Maximum likelihood methods were used to estimate the mean number of clones and allele frequencies, as described by Hill & Babiker (1995). The method uses data from infected people only and so yields a conditional mean (μ) that excludes PCR-negative records. However, an unconditional mean (m) can be calculated from μ to give an estimate of the mean number of clones in all patients, including those which are PCR negative. A Poisson distribution of number of clones per individual was assumed.

Monitoring the monthly minimum mean number of clones among the cohort

To examine persistence of multiplicity among infections that survived the lengthy dry season, we examined the change in the minimum number of clones (*mm*) throughout the study period (an average of 12 records for each patient). Clone number was defined as the largest number of alleles at any one locus. Thus, an infection with 1, 2 and 2 alleles of MSP-1, MSP-2 and GLURP, respectively, was scored as 2 clones. Note that this measure is an underestimate of the number of clones likely to be present, unlike that used above where the actual number of clones was estimated using information on population frequencies of alleles (Hill & Babiker, 1995). A logistic regression model was fitted to the data with fixed effects for age, sex and month of measurement, a random effect for family, and another random effect for individual to allow for correlations between repeated records from the same individual. After preliminary analysis the data were split into 2 parts – 1 for October 1993 until February 1994, and 1 from March 1994 onwards – in order to estimate separate time trends for the wet and dry seasons.

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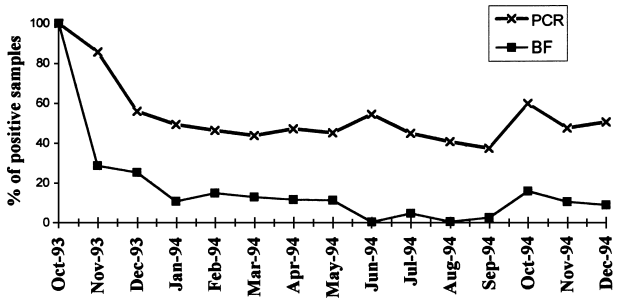

Fig. 1. Monthly *Plasmodium falciparum* infection detected by microscopy (BF) and polymerase chain reaction (PCR) among a cohort of 83 patients, following chloroquine treatment in October/November 1993.

RESULTS

Dry/wet parasite prevalence

Among the cohort. All patients included were initially infected with *P. falciparum*, as detected by microscopy. Their initial parasite count ranged from 0·36 to 264×10^3 parasites/μl of blood. The initial parasitaemia dropped dramatically following chloroquine treatment and progress of the dry season. By June 1994 no patient had parasitaemia detectable by microscopy (Fig. 1).

However, during the next transmission season (October–November 1994) the number had increased slightly (Fig. 1). On the other hand, parasite rate among the cohort, as detected by PCR, dropped progressively from December 1993 to January 1994 and then remained fluctuating between 40 and 54 % during the dry season, January to September 1994. It then increased slightly in October 1994 (transmission season).

In the village. Independent cross-sectional surveys were carried out between April 1994 and December 1995 to assess the overall parasite rate, detected by microscopy, in the village. In April 1994 (dry season) few individuals were found to harbour patent parasitaemias (1·4 %). This increased to 9·0 % during November/December 1994 (transmission season). A similar pattern was observed during the next dry season of 1995, when in February, March, April and June the parasite rate dropped to 2·7, 2·0, 1·4 and 1·1 %, respectively. However, it started to rise in August 1995 (beginning of the wet season) and peaked at 9·9 % in October 1995 (middle of the transmission season).

Complexity of P. falciparum *during the dry and wet season*

Mean number of clones and estimated inbreeding. The mean number of clones per patient was estimated among the studied cohort using sequence data on MSP-1 and MSP-2 only. During the 1993 wet season when the study began, patients carried a mean of 2·2 clones (2·4 excluding uninfected patients). Assuming that these clones were equally frequent and infective to mosquitoes, the frequency of zygotes formed by fertilization between identical clones, the inbreeding coefficient (f), was estimated as 0·55 (Hill & Babiker, 1995). By the middle of the dry season the mean number of clones per patient dropped to 1·2 clones (1·7 excluding uninfected patients) and f increased to 0·72. During the following wet season in 1994, despite the occurrence of transmission, the mean number of clones in this cohort did not change (1·1 for all patients, 1·6 excluding uninfected patients) corresponding to f of 0·76 (Table 1).

Stability of minimum number of clones during the dry season. Following drug treatment of acute infections in October and November 1993, the mean minimum number of clones dropped from 2·1 to 1·0 per infection in February. It then remained stable ($P > 0{\cdot}05$), with an average of one clone per patient throughout the dry season until the beginning of the next transmission season when there was a slight but insignificant increase (Fig. 2). The results on the minimum number of clones (mm) are similar to those from analysis of the actual number of clones (m), i.e. are not severely biased downwards. No effects of age or sex on the mean number of clones was observed ($P = 0{\cdot}10$ and $P = 0{\cdot}29$, respectively). The within-individual and within-family correlations between repeated records (i.e. the repeatability) were 0·3 and 0·2 respectively, indicating consistency of number of clones per individual over time, and correlation among family members in the mean number of clones carried.

Gene frequencies during the wet season 1993, and the dry and wet seasons 1994

For this analysis a single sample per individual included in the cohort was taken at 3 points during the survey, the transmission season of 1993 October/November, the dry season 1994 (February to July) and the next transmission season of 1994 (October/November). Changes in frequencies over time for each locus are described below.

MSP-1

The frequencies of the sequence variants of the MSP-1 (K1, MAD20 and RO33) fluctuated between seasons, but their differences were not significant ($P > 0{\cdot}05$) (Table 1).

During the wet season of 1993 (October/November), 22 alleles of MSP-1, distinguishable by size and sequence differences, were detected. During the dry season (June 1994), the number reduced to 17 alleles. All of these alleles, except one (MAD20 [440 bp]), were detected previously in October/

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Table 1. Estimates of allele frequencies, the mean number of clones and inbreeding coefficient in Asar for data from the wet season 1993, the dry season 1994 and the following wet season 1994, using a Poisson distribution fitting 2 loci

(m, Unconditional mean number of clones (including patients who became uninfected during the follow-up), calculated from the conditional mean (μ); *mm*, unconditional minimum mean number of clones; μ, conditional mean number of clones (records from infected patients only); *f*, the inbreeding coefficient.)

	Wet season 1993	Dry season 1994	Wet season 1994
MSP-1			
(K1)	0·41	0·32	0·33
(MAD20)	0·29	0·40	0·43
(RO33)	0·30	0·28	0·24
MSP-2			
(IC1)	0·61	0·46	0·63
(FC27)	0·39	0·54	0·37
m	2·16	1·22	1·05
mm	2·00	1·04	1·05
μ	2·40	1·73	1·60
(support limits)	(2·06–2·90)	(1·36–1·99)	(1·44–2·15)
f	0·55	0·72	0·76
(support limits)	(0·47–0·63)	(0·85–0·65)	(0·82–0·61)

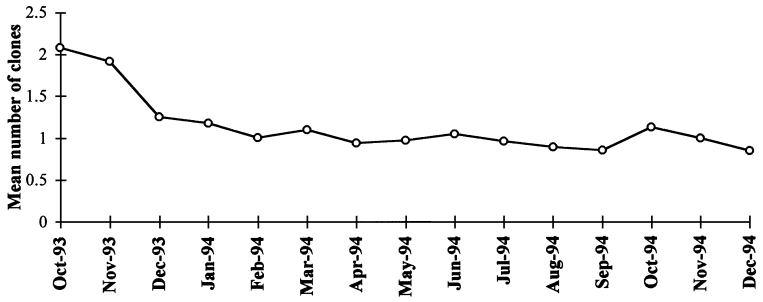

Fig. 2. The monthly minimum mean number of *Plasmodium falciparum* clones detected by polymerase chain reaction (PCR) among the studied cohort during the transmission season 1993, the dry season 1994 and the following transmission season in 1994. The open circles are the monthly means after adjustment for differences between families.

November 1993, but occurred at a lower frequency in the dry season than in the wet season. During the next transmission season (October/November 1994), 20 alleles of MSP-1 were seen among patients who had remained infected within the cohort. This included all the 17 alleles that were seen during the dry season, except for one rare allele, K1(400 bp). However, four new alleles, MAD20 (580 bp), MAD20 (520 pb), MAD20 (460 pb) and RO33 (460 bp) were seen.

MSP-2

The 2 sequence types of MSP-2, IC1 and FC27, were detected during the wet season of 1993, the dry season in 1994 and the next transmission season in 1994. As with MSP-1 the frequency of these alleles fluctuated across seasons, but these changes were not significant ($P > 0·05$) (Table 1).

Twenty-one alleles were detected, by size and sequence differences, in October/November 1993, 19 of which persisted during the dry season. During the next transmission season in October/November 1994, only 17 alleles were detected, all except one (FC27 [620 bp]) having been detected in the dry season (Fig. 3).

GLURP

For GLURP, 21, 16 and 21 alleles were detected during the wet season 1993, the dry season 1994 and the wet season 1994 respectively. Three alleles detected in the dry season of 1994 were not seen in the wet season of 1993. Similarly, 8 alleles detected during the wet season of 1994 were not detected in the dry season of 1994. However, 5 of these alleles were seen among the original parasites in the wet season of 1993.

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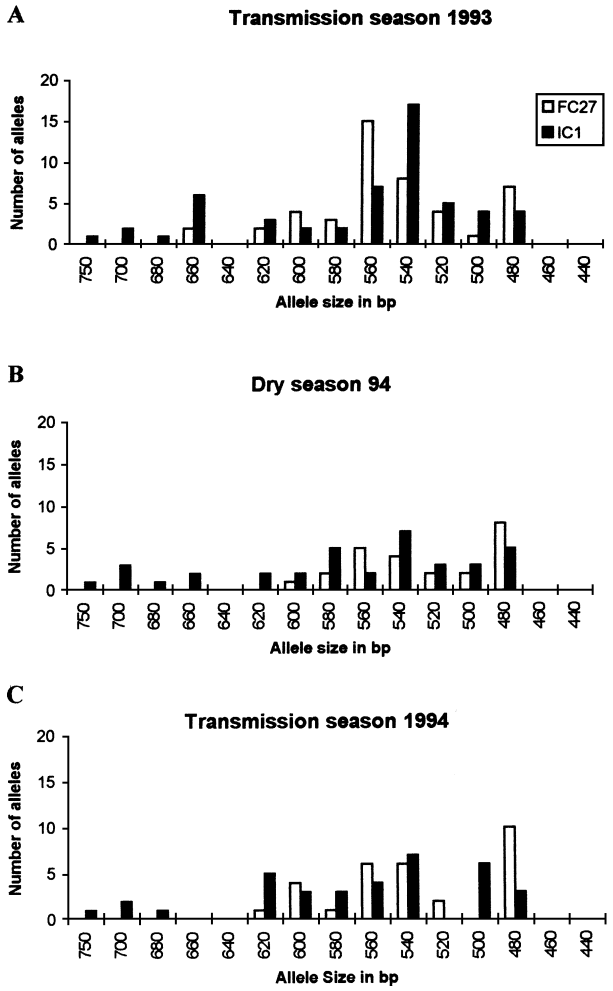

Fig. 3. Alleles of the MSP-2 gene, classified by size (base pairs [bp]) and sequence variants detected by allele-specific probes identifying the two major sequence types on block 3 (IC1 and FC27), among *Plasmodium falciparum* detected in the cohort, (A) during the transmission season 1993, (B) the middle of the dry season (June 1994) and (C) the following transmission season in 1994.

DISCUSSION

Malaria endemicity in central and eastern Sudan lies at the edge of the transmission strata, where transmission is unstable, pausing over a lengthy period during the hot dry season (A. Hamad *et al.* unpublished observations). Nevertheless, malaria is endemic in much of this region. Previous surveys have demonstrated a large gene pool among the parasites that cause the clinical malaria during the wet season. It has been inferred from these studies that the parasite population in this area is large (Babiker *et al.* 1995). However, it is not clear how the parasites (mainly *P. falciparum*) and the vector survive the lengthy dry and transmission-free period of this area. Longitudinal surveys in Asar revealed that many inhabitants who became infected during the transmission season retain the same *P. falciparum* clones as subpatent asymptomatic parasitaemias throughout the dry season (Babiker *et al.* 1998). Thus, the aim of this study was to investigate whether *P. falciparum* in Asar comprised a continuous population or whether it experienced severe contraction in size and consequently a 'bottleneck' and random genetic drift following the dry season. If the latter were true, the following transmission season could be initiated from only a limited number of clones surviving the dry season among inhabitants in the village or from founder clones brought into the village by immigrant people. Small populations are subjected to relatively large stochastic fluctuation in gene frequencies (Hartl & Clark, 1989). The maintenance of more than one *P. falciparum* clone per patient during the dry season, coupled with the non-significant changes in frequencies of polymorphic genes across seasons suggest that a substantial number of genotypes persist throughout the year in Asar. It is therefore most probable that the parasite populations in villages in this area are continuous, and undergo cyclic fluctuations in size, but not in genetic composition. In contrast, the disease has a marked seasonal appearance following rains and the onset of mosquito transmission. The persistence of parasites during the dry season cannot be attributed to drug resistance since only about 15% of *P. falciparum* in this village show resistance to the most commonly used antimalarial (chloroquine) (Babiker *et al.* 1995; and unpublished data).

The above is relevant to areas with low and unstable malaria not only in Sudan but also in other sites in sub-Saharan Africa (Zwetyenga *et al.* 1999). A similar conclusion is reached in areas with high transmission. In these areas the parasite rate peaks during the rainy season, then reduces slightly with reduction in rainfall (Greenwood *et al.* 1987; Smith *et al.* 1993). It appears that this seasonal fluctuation in parasite prevalence does not influence the effective population size and its gene pool (Conway, Greenwood & McBride, 1992; Kyes *et al.* 1997). In these areas the actual parasite rate, including subpatent parasitaemias, can be consistently high all year round (Bottius *et al.* 1996). Among such large and randomly mating parasite populations allele frequencies are expected to remain stable from generation to generation in the absence of selection. On the other hand, in the unstable malaria areas of eastern Sudan, the patent parasite rate reduces significantly during the lengthy dry season. However, it appears that the continuously parasitaemic inhabitants maintain a large number of clones throughout the year.

Previously, we have noticed fluctuations in frequencies of some polymorphic genes and stability of others among *P. falciparum* populations in 3 consecutive transmission seasons in Asar (Babiker *et al.* 1995). These fluctuations could have arisen due to the limited data set rather than genetic drift following contraction of the parasite population size during the dry season. One of the difficulties of these analyses is

the calculation of allele frequencies when infections with multiple clones are common. The analysis presented here overcomes this problem by applying maximum likelihood methods to estimate allele frequencies. Using a bigger sample size and a more powerful analysis, the present study made a comparison between the parasites of the wet season and the consecutive dry season, and has provided evidence suggesting that *P. falciparum* parasites in this village comprise a stable population.

Recent molecular surveys have demonstrated that a high proportion of malaria parasites are maintained in the tropics as chronic subpatent subclinical infections, which are not accounted for when control measures are considered (e.g. Bottius *et al*. 1996). The role of these chronic infections in the epidemiology, pathology and immunology of malaria has not been well studied, so it is important to understand how they arise and are maintained, especially in areas of seasonal and unstable transmission such as eastern Sudan (Babiker *et al*. 1998; Roper *et al*. 1998). In this area antibody responses to malaria infections, acquired during the transmission season, can be short lived following elimination of the parasite by drugs (Cavanagh *et al*. 1998). It appears that these chronic infections stimulate persistent antibody responses throughout the dry season (J. S. McBride, personal communication). Furthermore, it has been suggested that chronic infections can be maintained by clonal antigenic variation (Reader & Brown, 1996). The continuous interaction between the parasite and the human immune system is most likely to generate an immune response that controls these parasites. This response might be maintained by density-dependent mechanisms that operate, throughout the dry season, following the drop of the initial parasitaemia by the drug and acute-phase immune responses. However, when the next transmission season starts, some of the patients who had asymptomatic infections in the dry season develop fever. The majority of patients showed new clones at the time of these episodes (Babiker *et al*. 1998). The appearance of new genotypes or 'strains', different from pre-existing clones, at the beginning of the transmission season seems likely to cause the malaria epidemics. These observations support the notion that protective immunity against malaria can be strain-specific (Day & Marsh, 1992; Lines & Armstrong, 1992; Cadigon & Chaicumpa, 1969).

A limiting factor to the intensity of malaria transmission in this area is the level of annual rain (A. Hamad *et al*. unpublished observations). Seasonal fluctuation in the extent of multiplicity has previously been observed in Asar and a neighbouring village (Babiker *et al*. 1997; Roper *et al*. 1998). This has been attributed to the amount of annual rain in this area (Babiker *et al*. 1997). The lower multiplicity during the 1994 transmission season compared to the dry season of the same year is unexpected and cannot be explained by a low level of rain that year. Estimates of multiplicity for that transmission season were obtained from the chronically infected individuals within the studied cohort. It is possible that those individuals had mounted an immune response that protected them from super-infection during that transmission season of 1994. However, some of these patients had experienced new infections during that transmission season and had shown new clones not present during the dry season.

The continuous parasite populations in areas of marked seasonal transmission highlights a challenge to control strategies. It seems likely that these stable parasitaemias provide a reservoir of gametocytes to initiate the transmission season when anopheline mosquitoes become abundant. Work is currently under way to examine gametocyte production by *P. falciparum* clones that exist at submicroscopical level during the lengthy dry season of eastern Sudan.

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