Local differentiation in *Plasmodium falciparum* drug resistance genes in Sudan

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SUMMARY

Studies of population genetic structure of parasites can be used to infer which parasite genes are under selection. Here, the population structure of 4 genes associated with drug resistance of *Plasmodium falciparum* (the chloroquine resistance transporter, *pfcrt*, dihydrofolate reductase, *dhfr*, dihydropteroate synthase, *dhps*, and multi-drug resistance, *pfmdr-1*) were examined in parasite populations in 3 villages in eastern Sudan and in an urban area of Khartoum, the capital. In order to differentiate the effects of drug selection from neutral influences on population structure, parasites were also genotyped for 3 putatively neutral microsatellite loci (*polyα*, *TA81* and *pfg377*), and for 2 antigenic loci that are either under balancing selection or neutral, merozoite surface protein 1 and 2, (*MSP-1* and *MSP-2*). Cross-sectional surveys were carried out during the peak transmission (wet) season and in the ensuing dry season. No significant variation in frequencies of *MSP-1* and *MSP-2* alleles was seen among villages in the eastern region and between the villages and Khartoum, nor between the wet and dry season. However, the drug resistance genes, *pfmdr-1*, *pfcrt* and *dhfr* and to a lesser extent the microsatellite loci showed high F_{ST} values when comparing villages with Khartoum, indicating strong geographical differentiation at these loci. Moreover, variation in frequencies of the drug resistance genes, *pfmdr-1*, *pfcrt* and *dhfr*, was observed between the wet and dry season. These differences most probably reflect the variation in drug pressure between each region, and in drug usage between the wet and dry season in a given region.

Key words: malaria, *Plasmodium falciparum*, antimalarials, selection, drug resistance genes.

INTRODUCTION

Recent studies on *Plasmodium falciparum* have described the genetic structure of natural parasite populations in areas with different levels of endemicity, and have highlighted epidemiological forces that influence this process. These studies have demonstrated structuring of *P. falciparum* populations between and within continents (Anderson *et al*. 2000). The question of whether *P. falciparum* populations in different communities form distinct non-overlapping foci of infection or whether there is gene flow between them has practical implications for control strategies for this parasite. For example, in considering the origin and spread of drug resistance, a resistant mutant arising in a parasite population as a result of local drug use would not spread beyond this community if the parasites were reproductively isolated. The frequency of drug resistance mutations is also an important parameter for rational and effective drug treatment.

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Most such studies have examined either neutral polymorphic loci (microsatellites) (Anderson *et al*. 2000; Conway *et al*. 2000) or antigenic loci (e.g. Babiker *et al*. 1997; Kyes *et al*. 1997) in *P. falciparum* populations. Studies on microsatellite loci have revealed significant geographical variation in allele frequencies between continents and within continents, the latter being less apparent within Africa than within South America and Asia (Anderson *et al*. 2000). The even distribution of allele frequencies of some antigenic loci has been attributed to balancing (diversifying) selection (Conway *et al*. 2000; Polley & Conway, 2001), while skewed distributions in other loci have been attributed to positive selection (Drakeley *et al*. 1996; Conway *et al*. 2001).

In the present study we examined levels of population differentiation in drug resistance genes to address the question as to whether differences in type and magnitude of antimalarial drug usage in different endemic sites in central and eastern Sudan, driven mainly by the history and distribution of drug resistance, have imposed different selection pressures on local *P. falciparum* parasite populations. There is huge drug pressure on parasite populations during the seasonal malaria outbreaks in this region. Chloroquine resistance emerged here

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in the mid-1980s: low grade resistance was first suspected in the Khartoum area (Al Tawil & Akood, 1983) and highly resistant *P. falciparum* parasites were later detected in eastern Sudan (Bayoumi *et al.* 1989). Fansidar and quinine are used when chloroquine fails. The impact of diverse antimalarial usage in Sudan on drug resistance genes may parallel the differential pyrethroid insecticide selection on tobacco budworm populations, which has been found to generate significant geographical heterogeneity in its molecular target (sodium channel marker allele) compared with neutral loci (Taylor, Shen & Kreitman, 1995). Such an approach has recently been used to identify antigen genes under immune selection (Conway *et al.* 2000).

The genes we studied here were *pfcrt*, *pfmdr-1*, *dhfr* and *dhps*, all of which have been implicated as determinants of resistance to antimalarial drugs (Peterson, Walliker & Wellems, 1998; Wang *et al.* 1997; Fidock *et al.* 2000; Reed *et al.* 2000). For comparison, we also examined 3 microsatellite loci considered to be neutral, *polyα*, *TA81* and *pfg377*, and 2 merozoite surface protein genes *MSP-1*, and *MSP-2*, which may be neutral or under balancing selection. Neutral loci provide a background level of population structure against which the effects of drug selection on resistance loci can be compared.

MATERIALS AND METHODS

Study areas

The study was carried out in an urban area of Khartoum, the capital, in central Sudan, and in 3 villages, Asar, Ambassa and Kassab, in Gedarif State, eastern Sudan. The villages are situated respectively 14, 18 and 27 km south of Gedarif, the principal town in this region which is 420 km southeast of Khartoum. Malaria transmission in eastern Sudan is seasonal following the annual rains (July–November) reaching a peak in October. However, by January, the number of malaria cases drops substantially and entomological surveys showed no evidence of transmission during the dry season (Hamad *et al.* 2002). The predominant malaria parasite is *P. falciparum*, which accounts for more than 90% of all malaria infection. The principal vector is *Anopheles arabiensis*, and the average number of infectious mosquito bites per person is less than one per transmission season (Babiker *et al.* 1997). Malaria epidemiology in the Khartoum area is similar to that of eastern Sudan, except that a small peak of transmission occasionally occurs in December and January.

P. falciparum *samples*

Fingerprick blood samples were collected, with informed consent, from inhabitants of the 4 study areas at 2 sampling points – during the transmission season, October–November 1998 and the middle of the dry season, May–July 1999. Blood samples collected during the transmission season were from clinical cases while the dry season samples were from cross-sectional recruitment of asymptomatic inhabitants with no visible malaria parasites. Blood samples were dried, sealed individually in plastic bags, and stored at 4 °C. *P. falciparum* isolates were collected during the wet season in October 1998 and in the following dry season in June 1999 in Asar, Ambassa, Kassab and Khartoum.

DNA extraction, PCR analysis and allele designation

P. falciparum DNA was prepared from filter paper samples of blood as described elsewhere (Plowe *et al.* 1995). Specific conditions of PCR for each group of genes examined were as follows.

(*i*) *Drug resistance genes*. The *pfcrt* gene was analysed as described previously (Djimde *et al.* 2001). Parasite DNA was amplified by using primers flanking residue 76 in 2 rounds of PCR by using 2 pairs of outer and inner primers. The 145-bp nested PCR product was then digested with restriction enzyme *Apo*1 which cuts the *pfcrt*-Lys76 but not the *pfcrt*-Thr76 allele (Fidock *et al.* 2000; Djimde *et al.* 2001).

Analysis of the *pfmdr-1* gene was done as described previously (Duraisingh *et al.* 2000). Parasite DNA was amplified with primers flanking codon 86 in 2 rounds of outer and nested PCR reactions. The *pfmdr-1* alleles were then identified by using *Apo*1, which cuts the coding sequence of allele Asn86 but not Tyr86.

Alleles of the *dhfr* gene at codon 108 were detected as described by Plowe *et al.* (1995). In the first round of PCR, a 720 base pair fragment of the *dhfr* domain was amplified using outer primers. In the second reaction, 3 diagnostic primers paired with a common primer were used in 3 different reaction mixes to detect 108-Ser, 108-Asn and 108-Thr genotypes at this position.

Alleles of *dhps* were detected at codons 437 and 540 as described by Plowe *et al.* (1997). Two rounds of PCR were carried out and the 1100 bp product was analysed using the restriction enzymes *Ava*II for codon 437 and *Fok*I for codon 540. *Ava*II cuts the 437-lys allele but not 437-Gly, and *Fok*I cuts the 540-Ala allele but not 540-Glu.

(*ii*) *Merozoite surface protein genes* MSP-1 *and* MSP-2. Alleles of the merozoite surface proteins-1 and -2 (*MSP-1* and *MSP-2*) were analysed using sequence-specific primers. For *MSP-1* primers specific to the known 3 allelic types Mad20, 3D7 and RO33 were used as described by Cavanagh &

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McBride (1997), and alleles were identified based on their sequence types. Similarly, primers specific for the *MSP-2* family sequences IC1 and FC27 were used (Zwetyenga *et al*. 1998).

(*iii*) *Microsatellite loci*. Three single copy microsatellite loci denoted *polyα*, *TA81* and *pfg377*, located on chromosomes 4, 5 and 12 respectively (Su *et al*. 1999), were amplified and characterized as described by Anderson *et al*. (1999). Fluorescent PCR products were analysed in an ABI377 sequencer, and alleles were visualised and sized on Genescan (Applied Biosystems).

Statistical analysis

The overall objective of the statistical analysis was to determine whether there was significant differentiation between parasite populations sampled from different locations in Sudan, and in parasite populations sampled in the dry versus wet season within each of these locations, in drug resistance and other loci. Population differentiation was detected by measurement of allele frequencies in different populations and applying standard population genetics tests for population structure (see below). Note that, while we describe the dry season versus wet season comparison as 'seasonal differences', they may also reflect differences between symptomatic and asymptomatic patients, or differences due to a trend in time. In this study, the frequency of an allele was calculated as its proportion of all the alleles at a given locus detected among all the parasite isolates examined. Since human hosts often carry more than 1 parasite genotype, this method does not directly estimate the frequency in the parasite population, but instead measures the frequency of hosts carrying the particular parasite genotype. Using this method, the frequency of rare alleles in the parasite population is over-represented. Therefore a separate analysis was done using data from only single-clone infections (i.e. where only 1 allele was detected at all loci) to determine whether the use of data from multiple infections influenced the results. From these allele frequencies, levels of gene diversity (expected heterozygosities) between and within populations were calculated to yield Wright's F_{ST} statistic for population differentiation: this was done for all loci using Weir & Cockerham's method (1984) using the computer package GENETIX (Belkhir *et al*. 1996). A permutation test ($n=5000$) was applied (permuting alleles over populations) to test whether F_{ST} indices were significantly different from zero. Note that in the context of the malaria parasite which is haploid within the human host, the expected heterozygosity measures the theoretical proportion of heterozygotes produced in mosquitoes (when the parasite is diploid) assuming random mating among parasite clones.

For microsatellite loci, an additional fixation index (Slatkin's R_{ST} [Slatkin, 1996]) that accounts for variance in allele size was calculated using the FSTAT package (Goudet *et al*. 1996). R_{ST} is considered to be more reliable than F_{ST} for loci conforming to a stepwise mutation model (Slatkin, 1995), although it appears that microsatellites in *P. falciparum* do not conform to a strict stepwise mutation process (Anderson *et al*. 2000). As a further test of population differentiation, which, in contrast to tests based on F_{ST} and R_{ST}, does not depend on assumptions about the process generating population differentiation, the G-statistic (Goudet, 1995) was calculated using FSTAT with 15 000 randomizations. This test permutes alleles among population samples for all loci.

As a further indicator of population structure, linkage disequilibrium (LD values between all pairs of loci were calculated within each season for each village, using the method described by Black & Krafsur (1985). For these analyses, data from isolates with only a single allele at each locus, indicating clonal infections, were used. Data on all LD correlation coefficients were then pooled and examined for systematic differences between seasons, villages and the type of loci (i.e. involved in drug resistance or not) using ANOVA. Pairs of loci were classified as either DD for pairs where both loci were involved in resistance to the same drug (i.e. *pfmdr1-pfcrt* and *dhfr108-dhps437* pairs), NN for those that involved none of the 4 drug resistance loci, or DN for a mixture of these. Data on pairs involving *dhps540* were excluded from the analyses as this locus was in complete linkage disequilibrium with *dhps437*.

To gauge the level of within-population mixing among parasite genotypes (outcrossing), the numbers of clones per infected inhabitant were measured. These were estimated in 2 ways. First, the minimum number of clones present was estimated from the maximum number of alleles observed at *MSP-1* and *MSP-2* loci, or at microsatellite loci, or these combined. These data were analysed for season and location differences by logistic regression (SAS, 1990). Secondly, the maximum-likelihood method of Hill & Babiker (1995) was used on data from *MSP-1* and *MSP-2* loci: this method estimates the expected number of clones present given the population allele frequencies and assuming a Poisson distribution in the number of clones per infection. The former method yields a lower bound estimate of the latter.

RESULTS

Allele frequencies of all the genes studied, and the expected heterozygosities within each location in both the wet season of 1998 and the dry season of 1999 are given in Table 1. The microsatellite loci were the most polymorphic, the number of alleles

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Table 1. Allele frequencies and effective heterozygosity (H) in four *Plasmodium falciparum* populations in Sudan in the wet season of 1998 and the dry season of 1999

(Tyr, Thr, Asn, Gly and Glu are the mutant alleles for *pfmdr1*-86, *pfcrt* 76, *dhfr* 108, *dhps* 437 and *dhps* 540 respectively. *H*, effective heterozygosity, calculated as the sum of the product of frequencies of all possible pairs of different alleles.)

		Asar		Ambassa		Kassab		Khartoum	
Locus	Allele	Wet ($n=50$)	Dry ($n=32$)	Wet ($n=50$)	Dry ($n=32$)	Wet ($n=46$)	Dry ($n=31$)	Wet ($n=44$)	Dry ($n=33$)
pfmdr1-86	Tyr	0·65	0·69	0·70	0·83	0·55	0·88	0·88	0·88
	Asn	0·35	0·31	0·30	0·17	0·45	0·12	0·12	0·12
	H	0·46	0·42	0·42	0·29	0·44	0·22	0·21	0·21
pfcrt76	Thr	0·73	0·92	0·76	0·84	0·70	0·71	0·95	0·97
	Lys	0·27	0·08	0·24	0·16	0·30	0·29	0·05	0·03
	H	0·39	0·15	0·37	0·26	0·42	0·41	0·09	0·06
dhfr108	Asn	0·31	0·60	0·29	0·65	0·43	0·69	0·93	0·79
	Ser	0·69	0·40	0·71	0·35	0·57	0·31	0·07	0·21
	H	0·43	0·48	0·41	0·45	0·49	0·43	0·12	0·35
dhps437	Gly	0·12	0·13	0·16	0·13	0·09	0·11	0·08	0·07
	Ala	0·88	0·87	0·84	0·87	0·91	0·89	0·92	0·93
	H	0·21	0·22	0·28	0·23	0·17	0·26	0·14	0·13
dhps540	Glu	0·09	0·13	0·09	0·07	0·12	0·00	0·08	0·07
	Lys	0·91	0·87	0·91	0·93	0·88	1·00	0·92	0·93
	H	0·16	0·22	0·16	0·12	0·22	0·00	0·14	0·13
MSP-1	mad20	0·42	0·43	0·41	0·40	0·37	0·35	0·35	0·29
	3D7	0·39	0·30	0·31	0·34	0·44	0·46	0·49	0·42
	RO33	0·19	0·27	0·28	0·26	0·19	0·19	0·16	0·29
	H	0·64	0·65	0·66	0·66	0·64	0·62	0·61	0·65
MSP-2	IC1	0·53	0·56	0·69	0·48	0·50	0·41	0·55	0·54
	FC27	0·47	0·44	0·31	0·52	0·50	0·59	0·45	0·45
	H	0·50	0·49	0·46	0·50	0·50	0·48	0·49	0·50
polyα	132			0·02					
	141	0·04				0·02			
	144	0·02		0·02		0·04			
	147	0·15	0·16	0·09	0·14	0·10	0·11	0·36	0·24
	150	0·06		0·09		0·13		0·06	0·08
	153	0·11		0·07		0·13	0·17		
	156	0·08		0·16	0·05	0·06			0·08
	159	0·08			0·04	0·02			0·12
	162	0·16	0·11	0·09	0·19	0·15	0·11	0·06	
	165	0·04	0·21	0·09	0·09	0·04	0·11	0·05	0·04
	168	0·06	0·11	0·12	0·23	0·04	0·17	0·05	0·04
	171	0·02	0·11	0·02	0·04	0·08	0·06		0·04
	174	0·08	0·211	0·19	0·14	0·13	0·17	0·42	0·28
	177	0·06	0·05	0·02	0·09	0·02			
	180					0·02	0·111		0·08
	192					0·02			
	H	0·90	0·85	0·88	0·86	0·90	0·86	0·68	0·83
TA81	109			0·02		0·04			
	112							0·02	
	115			0·05		0·02			
	118	0·17	0·15	0·18	0·04	0·06	0·17	0·13	0·07
	121	0·10	0·25	0·25	0·30	0·23	0·22	0·30	0·18
	124	0·26	0·25	0·16	0·26	0·13	0·39	0·23	0·22
	127	0·33	0·15	0·29	0·30	0·44	0·22	0·23	0·52
	130	0·14	0·20	0·05	0·04	0·06		0·05	
	133				0·04			0·02	
	136					0·02		0·02	
	H	0·76	0·79	0·79	0·74	0·73	0·72	0·79	0·64
pfg377	92			0·02					
	95	0·04		0·12	0·12	0·15		0·15	0·17
	98	0·33	0·211	0·23	0·23	0·38	0·22	0·56	0·27
	101	0·60	0·68	0·58	0·61	0·48	0·78	0·23	0·56
	104	0·02	0·11	0·04	0·04			0·05	
	H	0·53	0·48	0·59	0·55	0·61	0·35	0·60	0·58

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Table 2. Levels of population differentiation (F_{ST}) between *Plasmodium falciparum* parasite populations sampled in the wet season versus the dry season in different locations in Sudan based on different loci

(R_{ST} values for microsatellite loci are shown in parentheses below the F_{ST} values. Asterisks indicate significant tests for whether $F_{ST}=0$; * $P<0{\cdot}05$, ** $P<0{\cdot}01$. Superscript letters indicate significant differences between populations based on the G-test. a, $P<0{\cdot}05$.)

Locus location	*MSP-1*	*MSP-2*	*pfmdr1-86*	*pfcrt76*	*dhfr108*	*dhps437*	*dhps540*	*polyα*	*TA81*	*pfg377*
Asar	−0·011	−0·028	−0·027	0·072*	0·126*a	−0·04	−0·034	0·013 (0·20)	0·001 (−0·02)	−0·008 (0·07)
Ambassa	−0·021	0·017	0·012	0·005	0·0213**	−0·023	−0·026	−0·001 (0·04)	−0·010 (0·01)	−0·030 (0·02)
Kassab	−0·019	−0·002	0·074*	−0·027	0·093*	−0·015	0·076	−0·007 (0·05)	0·076 (0·15)	0·048 (0·07)
Khartoum	−0·007	−0·026	−0·027	−0·025	0·055*	−0·031	−0·031	0·009 (−0·03)	0·038 (0·01)	0·121**a (0·012)

per locus ranging from 5 (*pfg377*) to 15 (*polyα*) in the wet season. Expected heterozygosity was highest for the microsatellite loci, ranging from 0·58 (*pfg377*) to 0·84 (*polyα*), intermediate for the merozoite protein genes, 0·64 (*MSP-1*) and 0·49 (*MSP-2*), and low for the drug resistance genes, 0·32 (*pfmdr-1*), 0·32 (*pfcrt*), 0·41 (*dhfr*) and 0·16 (*dhps*). Allele frequencies calculated from single-clone infections, of which there were 101 samples, were similar to those calculated from multiple-clone infections ($n=190$), varying by an average value of 0·004 (S.D. = 0·07) and average absolute value of 0·05 (S.D. = 0·05) (data not shown). Similarly, F_{ST} values based on data from single-clone infections were on average 0·02 less (maximum of 0·06) than those based on all data which was not significantly different from zero ($P>0{\cdot}10$). Results presented from here on are therefore from all data.

(*i*) *Seasonal variation in allele frequencies*

Allele frequencies of *MSP-1*, *MSP-2*, and 2 microsatellite loci remained stable between the wet and dry season, in each of the studied sites giving non-significant F_{ST} values (Tables 1 and 2). In contrast, significant variation in allele frequencies between seasons occurred among 1 or more of the drug resistance genes, and also for 1 of the microsatellite loci (*pfg377*). In general, mutant alleles of *pfmdr-1*, *pfcrt* and *dhfr* associated with drug resistance increased (Table 1), sometimes significantly (Table 2), at the expense of wild-type alleles during the dry season in all the locations (Table 1): *dhps*, on the other hand, showed no seasonal fluctuation in any of the locations. Data on single-clone infections gave qualitatively similar results (data not shown).

(*ii*) *Geographical variation in allele frequencies*

(*a*) *Drug resistance genes,* pfmdr-1, pfcrt, dhfr *and* dhps. Table 3 shows the F_{ST} values for pairwise comparisons of parasites in each location, in each season. The F_{ST} values for *pfmdr-1*, *pfcrt* and *dhfr* were high when comparing Khartoum and the 3 villages: this was due to higher frequencies of resistance alleles in Khartoum (Table 1). There were no significant differences between the villages themselves, except for Kassab, whose parasite population had different frequencies of *pfcrt* and *dhfr* during the dry season. In contrast with the other drug resistance genes, the F_{ST} values of the *dhps* alleles were small and not significantly different from zero in samples from both the wet and dry season (Table 3).

(*b*) *Microsatellite loci*. The majority of microsatellite alleles were shared among the parasites of the 3 villages in eastern Sudan. However, at least 4, 2 and 1 alleles of *polyα*, *TA81* and *pfg377* respectively, which were shared among the parasite populations of the villages, were absent from the Khartoum population (Table 1). Conversely, 1 *TA81* allele present in Khartoum was missing in the villages. The between-population variance (F_{ST}) was small for parasites in the villages (F_{ST} ranged from −0·009 to 0·021). However, a high degree of variation was observed when parasites in villages in eastern Sudan were compared to those in Khartoum, resulting in the higher F_{ST} indices and significant G-tests (F_{ST} ranged from 0·006 to 0·11) (Table 3).

Microsatellite loci were also studied using the R_{ST} index, which accounts for the allelic size variation due to gain or loss of repeat copies. The R_{ST} values among populations in the east were similar to F_{ST} values (ranging from −0·02 to 0·02). However, R_{ST} values were clearly lower than F_{ST} values when comparing the villages in the east with Khartoum, and when R_{ST} values were tested for significance no population differentiation was notices (R_{ST} ranged from −0·006 to 0·06) (Table 3).

(*c*) *Merozoite surface protein genes, MSP-1* and *MSP-2*. *MSP-1* had small F_{ST} values, −0·024 to

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Table 3. Levels of population differentiation (F_{ST}) between *Plasmodium falciparum* parasite populations sampled from different locations in Sudan

(Estimates were made within the wet season 1998 and the dry season 1999. R_{ST} values for microsatellite loci are shown in parentheses below F_{ST} values. Asterisks indicate significant differences from $F_{ST}=0$: * $P<0{\cdot}05$, ** $P<0{\cdot}01$. Superscript letters indicate significant differences between populations based on the G-test. a, $P<0{\cdot}05$; b, $P<0{\cdot}01$; c, $P<0{\cdot}001$.)

Location	Ambassa		Kassab		Khartoum		Khartoum vs villages	
Locus	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
pfmdr1-86							0·083^{*b}	0·002
Asar	0·012	0·009	−0·019	0·065	0·119^{**a}	0·072*		
Ambassa			−0·019	−0·025	0·066^{*a}	0·020		
Kassab					0·088^{*a}	−0·031		
pfcrt76							0·120^{**c}	0·069^{*a}
Asar	−0·019	−0·009	−0·019	0·098*	0·145^{**b}	0·013		
Ambassa			−0·012	0·019	0·120^{**a}	0·058*		
Kassab					0·180^{**a}	0·197^{**b}		
dhfr108							0·450^{**c}	0·0188
Asar	−0·026	0·037	0·008	−0·035	0·536^{**c}	0·039		
Ambassa			0·014	0·040	0·602^{**c}	0·305^{**c}		
Kassab					0·403^{**c}	0·013		
dhps437							−0·004	−0·003
Asar	−0·013	−0·050	−0·009	−0·049	−0·006	−0·033		
Ambassa			−0·004	−0·035	0·019	−0·014		
Kassab					0·012	−0·003		
dhps540							−0·014	−0·023
Asar	−0·027	−0·028	−0·015	0·113*	−0·023	−0·033		
Ambassa			−0·015	0·028	−0·024	−0·035		
Kassab					−0·013	0·033		
MSP-1							0·006	−0·009
Asar	0·0023	−0·024	−0·011	−0·005	−0·005	−0·007		
Ambassa			0·007	−0·007	0·022	−0·016		
Kassab					−0·016	−0·012		
MSP-2							−0·013	−0·012
Asar	0·006	−0·029	−0·015	0·016	−0·016	−0·037		
Ambassa			0·024	0·010	−0·009	−0·027		
Kassab					−0·008	0·012		
Polyα							0·078^{**c}	0·026^{b}
							(0·006)	(0·013)
Asar	0·002	−0·019	−0·009	0·018	0·089^{**c}	0·010		
Ambassa			−0·003	−0·005	0·071^{**c}	0·030		
Kassab					0·085^{**c}	0·019		
Pfg377							0·111^{**c}	0·006
							(0·060)	(0·086)
Asar	−0·008	−0·025	0·002	−0·032	0·132^{**b}	0·007^{a}		
Ambassa			0·006	−0·005	0·143^{**b}	−0·030		
Kassab					0·054^{*a}	0·027		
TA81							0·006	0·041
							(0·001)	(−0·006)
Asar	0·007	0·006	0·021	−0·012	0·017	0·076^{*a}		
Ambassa			0·003	−0·016	0·012	0·005		
Kassab					0·020	0·041		

0·022, among all the parasite populations, indicating similarities in the alleles of this gene and their frequencies in all the locations. Similarly, *MSP-2* allele frequencies were not significantly different between the villages or between the villages and Khartoum (Table 3).

(*iii*) *Linkage disequilibrium*

Results of LD analyses showed that there were significant differences between seasons, villages and the type of loci. Ambassa had significantly lower average LD than all other locations ($P<0{\cdot}01$), and LD was higher in the wet season than in the dry season ($P<0{\cdot}001$). There was much higher LD between pairs of loci involved in resistance to the same drug than between pairs of loci involving just 1 or zero drug resistance genes ($P<0{\cdot}001$, Table 4). There were no interactions among these main effects, i.e. the difference between drug resistance locus pairs and pairs involving neutral loci were consistent across villages and seasons.

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Table 4. Mean linkage disequilibrium correlation coefficients (S.E.M. in parentheses, n = number of observations per subgroup within season) between pairs of loci that are both involved in drug resistance to the same drug (DD), where neither locus is involved in drug resistance (NN) or a mixture of these (DN) within the wet and dry seasons

(Significance tests are after adjusting for village effects.)

Locus type	NN ($n=40$)	DN ($n=96$)	DD ($n=8$)	Overall
Season				
Wet	0·22 (0·01)	0·23 (0·01)	0·49 (0·12)	0·24 (0·01)
Dry	0·42 (0·03)	0·37 (0·02)	0·61 (0·13)	0·40 (0·02)
Overall	0·32 (0·02)	0·30 (0·01)	0·55 (0·08)	
Significance test for season				$P<0·001$
Significance test for locus type		$P>0·10$	$P<0·001$	

Table 5. Estimated number of *Plasmodium falciparum* clones per infected individual in each of the studied communities, using two independent methods (A and B)

(See text for details. Method A was based on the minimum clone number. Method B was that described by Hill & Babiker (1995) based on MSP-1 and MSP-2 combined.)

			Method A			
Location	Season	N	MSP-1 and MSP-2	Microsatellites	Both	Method B
Ambassa	Dry	34	1·27	1·16	1·32	1·42
	Wet	51	1·31	1·08	1·33	1·47
Asar	Dry	27	1·36	1·23	1·40	1·76
	Wet	50	1·56	1·24	1·64	1·87
Kassab	Dry	31	1·73	1·05	1·70	2·81
	Wet	46	1·48	1·20	1·50	1·72
Khartoum	Dry	33	1·48	1·14	1·18	1·39
	Wet	42	1·35	1·20	1·42	1·52
All locations	Dry	125	1·38	1·15	1·40	1·83
	Wet	189	1·43	1·18	1·48	1·65
All	Both seasons	315	1·41	1·17	1·44	1·71

(*iv*) *Multiplicity of infection*

Based on the minimum number of clones detected, infected inhabitants in Kassab and Asar had lower numbers of clones than those in Khartoum and Ambassa, estimated from *MSP-1* and *MSP-2* alleles ($P<0·05$). However, no differences were found when microsatellite loci were analysed (Table 5). The mean number of clones estimated by maximum likelihood methods based on *MSP-1* and *MSP-2* did not differ between the locations. There was a general tendency for lower clone numbers during the dry season than the wet season ($P<0·06$ based on minimum clone number), except in Kassab village.

DISCUSSION

This study has demonstrated strong geographic differentiation among *P. falciparum* drug resistance genes *pfcrt*, *pfmdr-1* and *dhfr* in 2 widely separated areas in Sudan, and an increase in frequency of resistance alleles from the transmission (wet) season to the ensuing dry season. The *pfcrt* and *pfmdr-1* alleles examined here have been shown to be involved in determining the response of *P. falciparum* to chloroquine, the first line drug used in this area, (Fidock *et al*. 2000; Reed *et al*. 2000; Djimde *et al*. 2001; Babiker *et al*. 2001), and *dhfr* alleles are involved in response to pyrimethamine which is one of the components of the second line drug, Fansidar (Peterson *et al*. 1998). In contrast, the *dhps*, *MSP-1* and *MSP-2* genes were similar in allele frequencies across locations, and between seasons, while microsatellite loci showed some limited differences. Other studies have shown that alleles at residues 437 and 540 and other sites of *dhps* are associated with resistance to the second component of Fansidar,

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sulfadoxine (Wang *et al*. 1987) and to Fansidar resistance in the field (Curtis, Duraisingh & Warhurst, 1998; Kublin *et al*. 2002). However, in our study, the *dhps* alleles did not show variation in frequency between parasite populations of eastern Sudan and Khartoum, indicating either that there is limited pressure on this locus, due to restricted usage of Fansidar and low level of resistance to the drug, or that the examined alleles are neutral to drug selection.

The differences in distribution of drug resistance genes between eastern Sudan and Khartoum are probably related to the different pattern of antimalarial drug usage and the history of emergence of drug resistance in each area. Low level chloroquine resistance was first noted in the north Khartoum area in the early 1980s (Al Tawil & Akood, 1983). However, it was not until the late 1980s when high-grade drug-resistant *P. falciparum* parasites were reported in eastern Sudan (Bayoumi *et al*. 1989): these parasites were thought to have arrived from Ethiopia following the vast refugee movement into eastern Sudan in the mid-1980s (Bayoumi *et al*. 1989). Fansidar then became frequently used for treatment of cases of chloroquine resistance. Fansidar resistance was first reported in central Sudan in the late 1980s (Ibrahim *et al*. 1991). However, pyrimethamine resistance was earlier noted in central and eastern Sudan (Abbott, 1955; Babiker *et al*. 1991). Community chloroquine and Fansidar usage is high in the Khartoum area due to multiple sources of drug distribution. In contrast, in the villages of eastern Sudan, outlets for antimalarials and other drugs are limited, and thus usage of antimalarials is somewhat restricted. However, reliable estimates of drug usage in either location are not available.

Direct evidence of *in vivo* selection of mutant genes of *dhfr* and *dhps*, following administration of antimalarials, has been demonstrated previously (Curtis *et al*. 1998). In an area of low and seasonal transmission, such as eastern Sudan, the semi-immune inhabitants rely heavily on antimalarials to treat the cyclical malaria outbreaks of this area, which occur across all age groups. Drug pressure, driven by high antimalarial usage, is therefore expected to be a major determinant in the selection and spread of drug resistance genes in the *P. falciparum* population of this region. In the present study, a high degree of linkage disequilibrium was seen among unlinked genes conferring resistance to the same drug (i.e. between *pfcrt* and *pfmdr-1*, and between *dhfr* and *dhps*), though not between loci involved in resistance to different drugs (data not shown), which is probably due to simultaneous selection by the same drug. Linkage disequilibrium between *pfmdr-1* and *cg2*, a gene which is closely linked to *pfcrt*, in The Gambia has been attributed to joint selection by chloroquine on both the *pfcrt* and *pfmdr-1* loci which are physically unlinked (Duraisingh *et al*. 2000; Sutherland *et al*. 2002). Similar association between *pfmdr-1* and *pfcrt* was previously observed among *P. falciparum* in Sudan (Babiker *et al*. 2001). A strong correlation between frequencies of mutant *dhfr* alleles and community Fansidar usage pattern has been observed in Mali (Plowe *et al*. 1996).

An interesting finding in this work was the divergence in the frequencies of alleles at the microsatellite loci *TA81*, *polyα* and *pfg377*. *TA81* had similar allele frequencies in all 4 sites. However, *polyα* and *pfg377* exhibited significant differences between Khartoum and the villages, although only when based on F_{ST} and G-statistics, and not R_{ST} statistics. Using single-clone infections data only *pfg377* confirmed the above differences. These discrepancies may reflect the problem of applying uniform population genetics tests on loci that differ in their mutational and population differentiation processes. An alternative explanation for the apparent geographical differentiation at these loci is that they are physically linked to, and hence co-segregate with, drug-resistance genes. *Polyα* is a microsatellite on chromosome 4 linked quite closely (215 kb) to the *dhfr* gene, and it would be expected to hitch-hike with this gene in selective sweeps caused by selection with antifolate drugs such as pyrimethamine and Fansidar (e.g. Wootton *et al*. 2002). *TA81* is on chromosome 5350 kb from the *pfmdr-1* gene (Su *et al*. 1999). Similar hitch-hiking events could thus be expected to apply in view of the involvement of *pfmdr-1* in high level chloroquine-resistance. Microsatellite *pfg377* is within the coding sequence of a gametocyte-specific gene (Menegon *et al*. 2000). Therefore, the variation in allele frequencies among the sites studied could be due to linkage to a putative drug-resistance gene, for example, or to the gene itself being under different types of positive selection in each site.

Recombination would be expected to break down this linkage, especially in high transmission areas. However, the counter-force of drug selection is almost always sufficient to counteract this recombination break-down and thus maintain linkage disequilibrium between loci under joint drug selection (Mackinnon & Hastings, 1998). In Sudan, the prevailing rate of outcrossing and subsequent recombination has been estimated to be low compared to other endemic sites in Africa (Babiker *et al*. 1997).

The even frequencies of the examined *MSP-1* and *MSP-2* alleles across different parasite populations in Sudan are in agreement with other surveys and the view that they may be maintained by balancing immune selection (Conway *et al*. 2000). Genes under balancing selection should exhibit less population structure because frequency-dependent selection maintains alleles at intermediate frequencies within populations. Consequently, loci under balancing selection should have lower estimates of F_{ST}

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compared to neutral genes whose frequencies are more affected by population structure and drift versus selection.

The local differentiation of drug resistance genes observed in this study indicates the need for small-scale mapping of these genes in malaria endemic areas. Drug pressure not only selects resistant mutants, but also may enhance parasite transmission (Targett *et al*. 2001; Buckling *et al*. 1997), and in turn the evolution of drug resistance (Mackinnon & Hastings, 1998). Therefore, control strategies that can limit transmission as well as drug pressure and thereby extend longevity of antimalarials in malaria endemic areas should be considered.

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