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Analysis of genetic polymorphism in select vaccine candidate antigens and microsatellite loci in *Plasmodium falciparum* from endemic areas at varying altitudes

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Abstract

Plasmodium falciparum parasites obtained from symptomatic patients attending clinics in Bindura (altitude 1,100 m), Chiredzi (600 m) and Kariba (< 600 m), previously reported to differ in malaria endemicity were genotyped on the *msp-1*, *msp-2* and *glurp* loci to examine the extent of parasite genetic diversity. While the parasites were monomorphic for *msp-1* allele RO33 from the three locations, the K1 allele was overrepresented in Kariba (p=0.02) and Mad20 alleles occurred at a higher frequency in Bindura. A similar PCR analysis for *glurp* and the two main allelic families of *msp-2*, i.e IC/3D7 and FC-27 revealed minimal differences in the parasite population. A total of 8 *msp-1* Block 2 and 11 *msp-2* genotypes were identified from the three locations combined. On the *glurp* locus, thirteen different genotypes ranging in size from 660 to 1160 bp were detected in parasites obtained from Bindura and Kariba. To gain further insight into *P. falciparum* genetic diversity in the three different geographical locations, parasites were examined for neutral microsatellite markers (C4M8, C13M30 and TA81). The number of microsatellite alleles ranged from 8 to 17 and the average expected heterozygosity (HE) for the three areas combined was 0.83 suggesting that the parasite population of Zimbabwe is genetically heterogeneous. These findings have implications in understanding the impact of genetic variation on immunity and possibly emergence of drug resistance.

Plasmodium falciparum parasites are highly genetically diverse and studies indicate that at any given time, humans or mosquitoes can harbor a number of different parasite clones(Babiker et al., 1991; Branch et al., 2001). The unique genetic characteristics of each parasite may determine clinical or parasitological outcomes and properties such as cytoadherence, immune evasion, resistance or susceptibility to drugs as well as infectivity to mosquitoes (Snounou et al., 1999). Understanding the distribution of genetically diverse *P. falciparum* parasites is important in malaria epidemiology as well as in designing vaccines as antigenic diversity continues to pose a big challenge to vaccine development. Additionally, the performance of

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vaccine candidates employed will need to be monitored by assessing switching of allelic types resulting from either natural or vaccine induced immune selection.

Msp-1 and *msp-2* are highly polymorphic single copy genes and have been employed to investigate parasite genetic diversity (Anders and Smythe, 1989; Smythe *et al.*, 1990). The examination of polymorphic genes is also useful in estimating the number of concurrent infections in a given individual, termed multiplicity of infection (MOI) (Smith et al., 1999). Evidence from several studies suggests that people who are constantly exposed to malaria caused by multiple parasite clones may have the advantage of clinical protection from future malaria attacks (Smith et al., 1999; Branch et al., 2001; Henning et al., 2004). In addition to genes such as *msp-1* or *msp-2* which are under extreme immune selection pressure (Cavanagh et al., 1998), neutral microsatellite markers have also been employed to demonstrate parasite genetic diversity.. Microsatellite analysis data has been used to estimate the expected heterozygosisty (HE) index which represents the probability of picking two parasites with different alleles at a given locus and it ranges in value from greater than 0 to less than 1 (Anderson et al., 1999; Su et al., 1999). Such HE calculations for parasites from South America, Africa and Asia revealed that the extent of genetic diversity reflected transmission intensity of the diseases with the highest diversity (HE=0.8) in Africa and the lowest diversity in South America (HE=0.3) (Anderson et al., 2000).

Zimbabwe lies on the southern border of malaria transmission in Africa and little is known about the genetic diversity of parasites in different endemic areas of the country. We describe an analysis of the genetic variation of parasites in three areas of Zimbabwe using *glurp*, *msp-1*, *msp-2* and neutral polymorphic microsatellite markers. These areas, Bindura, Chiredzi and Kariba, were described in a single study two decades ago to be of hypo-, meso- and hyperendemic transmission, respectively (Taylor and Mutambu, 1986). Transmission is seasonal in all these areas and the main mosquito vector is *Anopheles arabiensis.* National control for malaria at the time of sample collection consisted of chloroquine as first line treatment and second line treatment comprised of a combination of chloroquine and sulfadoxine/pyrimethamine. Clinical data for the three catchment areas for the years 1997 to 2003 revealed that the incidence of malaria was 152/1,000, 394/1,000 and 419/1,000 for Bindura, Chiredzi and Kariba respectively (Ministry of Health, Zimbabwe). Population characteristics and health center catchment sizes for the three areas has been described elsewhere (Mlambo et al., 2006).

Dried finger-prick blood on filter papers was obtained and a total of 112 samples from microscopy positive symptomatic patients from the three different locations were examined. These samples were collected between March and April of 2003 with ethical permission from the Medical Research Council of Zimbabwe and approval from Johns Hopkins School of Public Health IRB.

DNA was extracted from approximately 50 μl blood spotted on filter papers using the chelex method (Plowe et al., 1995). For PCR analysis of *glurp*, *msp-1* and *msp-2*, 2.5 μl of DNA was used as template in a 50 μl total reaction volume mix with 1.25 units of enzyme *Taq* polymerase, buffer (50 mM KCl, 10mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$), 200 μM dNTPs and 0.25 μM each of reverse and forward primer. The primer sequences in nested PCR amplification and cycling conditions for each genetic marker have been published elsewhere and modifications are as indicated, i.e *glurp* (Ranford-Cartwright et al., 1997), *msp-1* (Branch et al., 2001), first PCR annealing at 50°C, 30 cycles, Mad20 annealing at 55°C) and *msp-2* (Snounou et al., 1999) first PCR annealing at 50°C, 30 cycles, second PCR annealing at 55°C). In the second round of PCR, 2 μl from the first PCR amplified products was used as template and the PCR products were resolved on a 2.5% agarose gel stained with ethidium bromide. The *glurp*, *msp-1* and *msp-2* genotypes were grouped into bins ranging from 20 to 50 bp with the aid of

Kodak ID analysis image software version 2.0.2 (USA). The term genotype in this paper refers to the allele bins and not the specific allele size in bp. The mean multiplicity of infection (MOI) was calculated by adding up all the number of different *msp-1* genotypes (i.e allelic types, K1, RO33 and MAD20 as well as allele bin sizes) present per sample and dividing by the total number of samples analyzed. *Msp-1* genotypes were used to calculate MOI because in our analysis it revealed more mixed infections compared to *msp-2*. Expected heterozygosity (HE), a measure of diversity for *msp-1* and *msp-2* genotypes was calculated using the formula;

$$\text{Expected Heterozygosity (HE)} = 1 - \sum_{i=1}^{n} p^2 i$$

where *p* is the frequency of the *i*th genotype and *n* is the number of genotypes in the sample population.

DNA samples extracted by the chelex method (above) were used as template to amplify and examine neutral polymorphic microsatellite markers. The microsatellite markers that were analyzed are TA81, C4M8 and C13M30 (Gene Bank Acc. Nos. G3886, G37937 and G44421 respectively) and the primer sequences are published elsewhere (Jain et al., 2005). A hemi-nested PCR approach was employed to amplify microsatellite markers. Briefly, 2 μl of DNA was amplified in a total reaction volume of 15 μl containing 1.5 μl 10X buffer (50 mM KCl, 10mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$), 0.2 units *Taq* polymerase, 0.5 pM reverse and forward primer and 200 μM of dNTPs. For the second round of PCR, 2 μl from the primary reaction was used as template with an internal fluorescently labeled primer and the reverse primer used in the primary PCR. The cycling conditions for the primary PCR and the secondary PCR steps were as follows; initial denaturation at 94°C for 2 min followed by 25 cycles consisting of denaturation (94°C for 30 sec), annealing steps (42°C for 30 sec and 40°C for 30 sec), extension (68°C for 30 sec) and a final extension at 68°C for 2 min. PCR products were analyzed by capillary electrophoresis using the 3100 ABI capillary electrophoresis system in the presence of ROX500 size standards (Applied Biosystems, USA). Peak heights for each allele were captured with the aid of ABI GeneScan 3.7 software (Applied Biosystems, USA) and imported into Genotyper software, version 3.1 for further analysis. Alleles with peak heights of at least 500 arbitrary fluorescence units and base pair sizes corresponding to the expected allele sizes based on *P. falciparum* 3D7 from the PlasmoDB were considered for analysis. The HE was calculated as above. All the statistical analyses were performed with the aid of the statistical software package SAS version 9.1 (USA).

We first analyzed parasites from the three locations for polymorphisms at the *msp-1* locus for the presence of 3 possible allele families, RO33, Mad20 and K1 and the allele sizes in base pairs. As has been reported (Ariey, 1999; Mawili-Mboumba et al., 2003; Legrand et al., 2005), the RO33 allele was monomorphic (140 bp PCR product) whereas block 2 genotypes for K1 varied in size from 200 bp to 350 bp and the Mad20 alleles ranged from 160 bp to 220 bp in size. As shown in table 1, more than 50% of samples in all three areas revealed mixed *msp-1* genotypes. The presence of K1 alleles appeared to be influenced by location as there were statistically significant differences in the proportions between samples from Kariba (p=0.02) or Chiredzi (p=0.005) and Bindura (Table 1).

The data on *msp-1* block 2 genotypes was used to calculate mean MOI for all the samples and compared among the three areas. Values of MOI were approximately 2 for patients both below and above 15 years for Chiredzi and Bindura and children less than 15 years in Kariba. Interestingly, patients above 15 years (Table 1) in Kariba, an area previously reported to experience high malaria transmission (Taylor and Mutambu, 1986) revealed mean MOI of 1.3. It is not known why the adult sample population in Kariba had a lower MOI compared to the

younger age group; and though our data is preliminary, it is possible that MOI in adults might be regulated by strain specific immunity. Though the mean age of subjects less than 15 years for the three areas was similar (i.e 11, 8.5 and 10.5 years for Bindura, Chiredzi and Kariba, respectively), we did not see a similar trend in Bindura and Chiredzi probably because of low statistical power or likely malaria transmission differences.

Our analysis on *msp-1* genotypes and MOI did not reveal major differences in the genotypes of parasites analyzed from the three areas, therefore samples were pooled for all the subsequent genotype analysis. Since RO33 has only one allele size, it was excluded in the analysis of genetic diversity. For the K1 allele, a 250 bp genotype was detected in 50% of the samples while the 180 bp Mad20 genotype was the predominant genotype in all the three areas analyzed (Table 2). For *msp-2 locus,* a 400 bp genotype of the FC-27 type and a 600 bp genotype of IC/3D7 was common in all the three locations (Table 2). Combining all the three areas, a total of 8 *msp-1* and 11 *msp-2* genotypes were scored. *P. falciparum* samples from Kariba and Bindura were analyzed for polymorphism in the *glurp* gene and the results revealed a spectrum of 13 genotypes ranging from 660 bp to 1170 bp (Table 2). Based on *msp-1* block 2, *msp-2* and the *glurp* loci, our results revealed considerable genetic variation in parasites in the three locations.

We attempted to further analyze distribution of *msp-1* and *msp-2* genotypes in patients grouped below and above 15 years and did not find any significant differences for *msp-1* K1, R033 and *msp-2* genotypes. This analysis did reveal that the *msp-1* Mad20 allele prevalence was higher in patients less than 15 years compared to patients above 15 years (data not shown) supporting a previous suggestion that there could be host factors that help maintain Mad20 genotypes in children below 15 years (Ntoumi et al., 1997).

After analyzing *msp-1*, *msp-2* and *glurp* loci which code for immunogenic antigens, we hypothesized that genetic diversity based on these markers would differ from neutral microsatellite loci analysis. We therefore employed 3 neutral polymorphic microsatellite markers to examine parasite samples in a hemi-nested PCR approach to gain insights into the population structure and genetic diversity. Our results revealed that C4M8 was the most polymorphic marker which had 17 alleles while C13M30 and TA81 had 14 and 8 alleles, respectively. These results were then used to calculate HE and the HE was 0.80, 0.80 and 0.90 for TA81, 13M30 and C4M8, respectively. The mean HE for the three locations was 0.83. After calculating the HE based on microsatellite markers, we went back and calculated the HE based on *msp-1* block 2, *msp-2* and *glurp* genotypes which was 0.70, 0.86 and 0.89 for *msp-1* block 2 locus, *msp-2* and *glurp*, respectively.

Finally, we compared MOI values based on *msp-1*, *msp-2* and microsatellite data. For this analysis we included only those samples for which we had data for the three markers. The 3 markers revealed similar MOI values for parasites analyzed from the three different locations (data not shown). One simple interpretation of our data is that the 3 areas at present do not differ in malaria transmission intensity and a single parameter like MOI alone may not be sufficient to assess transmission differences. Studies in Tanzania have previously revealed very similar MOI among areas differing by over 1000 fold in EIR values (Babiker et al., 1997; Bendixen et al., 2001). Thus our study although revealing high genetic diversity in the three areas of Zimbabwe will need to be backed up by direct EIR data to evaluate any correlation between MOI and transmission intensity differences.

The data reported here provides a first comprehensive attempt to define genetic diversity of malaria parasites in Zimbabwe. Parameters assessed here such as MOI, age biased distribution of *msp-1* Mad20 genotypes, microsatellite markers and the percentage of mixed infections have been investigated in many other malarious areas and suggest that transmission conditions in Zimbabwe are comparable with that of other medium to high transmission areas such as

Tanzania, Papau New Guinea and Senegal (Paul et al., 1995; Babiker et al., 1997; Ntoumi et al., 1997; Zwetyenga et al., 1998).

In conclusion, our analysis suggest high parasite genetic diversity as assessed by examining *msp-1*, *msp-2* and *glurp* loci plus neutral polymorphic microsatellite markers in the 3 areas that differ in terms of altitude. Although these three areas differed markedly in patterns of transmission as reported 2 decades ago, this study on genetic diversity neither corroborate nor dispute this previous report. Furthermore, we do not know whether migration among the three sites may impact overall parasite genetic diversity. More studies are warranted to examine whether these areas indeed still differ in transmission and if there are any biological implications such as development of protective immunity for the differences in the distribution of genotypes observed in this study.

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Table 1

Percentage distributionof MSP-1 block 2mixed and single alleleinfections ofK1 (K), RO33 (R) and Mad20 (M) and multiplicity of infection (MOI) in the two age groups (<15 and >15 years) in samples from Bindura, Chiredzi and Kariba.

Health Centers	Allele(s)							MOI ± SEM	
	M	R	K	R/M	K/M	K/R	K/R/M	<15 years	> 15 years
Bindura (n=16, 20)*	36	0	0	30	22	0	11	1.9 ± 0.2	1.8 ± 0.1
Chiredzi (n=16, 15)	10	7	7	13	35	0	29	2.2 ± 0.2	2.0 ± 0.2
Kariba (n= 18, 27)	10	15	25	8	25	5	12	2.2 ± 0.2	1.3 ± 0.1**

*(n = subjects < 15 years, subjects > 15 years)

**For Kariba samples, mean MOI was higher for patients less than 15 years compared with those above 15 years ($p < 0.05$ by the *t*-test).

Table 2

Distribution of *m*sp-1, *msp*-2 and *glurp* genotypes (bp) in samples pooled from the three locations. Number of samples for a given allele size is indicated in parenthesis.

Marker	MSP-1		MSP-2		GLURP*
Allele type	**K1**	**Mad 20**	**FC-27**	**IC/3D7**	**GLURP**
	200 (10)	160 (1)	300 (13)	450 (9)	660 (1)
	250 (31)	180 (75)	350 (9)	500 (17)	720 (2)
	300 (17)	200 (9)	400 (16)	550 (15)	750 (3)
	350 (4)	220 (6)	450 (5)	600 (40)	780 (4)
			500 (10)	650 (4)	830 (8)
				700 (12)	880 (1)
					900 (9)
					930 (3)
					950 (5)
					980 (5)
					1030 (2)
					1050 (5)
					1170 (4)

*Data on *glurp* genotypes was for samples pooled from Bindura and Kariba.