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RESEARCH ARTICLE

ALLELIC POLYMORPHISM OF MSP1 AND MSP2 GENE IN SEVERE *P. falciparum* MALARIA IN AN AREA OF LOW AND SEASONAL TRANSMISSION

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ABSTRACT

The severe malaria (SM) and uncomplicated malaria (UM) infections are expected to have different genetic makeup. In this study, blood samples were obtained from 268 patients with SM and UM infection. from Central Sudan Sennar state. The SM group included patients with cerebral malaria (CM), severe malarial anemia (SMA), and other complications. The MSP! And MSP2 locus was exploited for parasite genotyping. We found that the genetic diversity within the parasite population was marked (21 genotypes). The multiplicity of infection (MOI) was 62, and it was alike between SM and UM. The ratio of the IC1 to FC27 allele families was comparable between SM and UM, and differ between Mad20, K1 and Ro33 and SM and UM .The distribution of the allele sizes was. correlated P<0.001). Positive correlations evidenced between parasitemia with the allelic family of Mad20 and IC (P value= 0.004 and 0.001). Positive correlations evidenced between multiplicity of infection and Hb (P= 0.008), and between MOI and parasitemia (P= 0.00) . Also between MOI an allelic family of Mad 20,K1 and Ro33 (P= 0.00) .Relation between distinct alleles on one side and parasitemia on the other, as indicators for malaria severity were assessed by plotting different alleles against parasitemia. The results obtained show that, alleles of IC and Fc27 associate with severity of disease since those alleles were represented in the parasitemia (>5000 parasites/ µl blood).

Key words: Falciprum, Allelic, polymorphism, MSP2, MSP1, genotypes.

INTRODUCTION

Malaria is a vector-borne infectious disease caused by protozoan parasites. It is widespread in tropical and subtropical regions, including parts of the Americas, Asia, and Africa. Each year, there are approximately 350–500 million cases of malaria, killing between one and three million people, the majority of whom are young children in Sub-Saharan Africa. (Snow 2005) predominantly, because of severe malaria (SM). However, after appropriate treatment, many SM patients recover, but some patients die for unknown reasons (Greenwood *et al*., 1991), which is largely because the pathogenesis of SM is not well understood. The molecular epidemiology of SM is the first step toward the molecular basis to the disease (Brown *et al*., 2000). The term SM is applied for diverse clinical syndromes such as cerebral malaria (CM) and severe malarial anemia (SMA), which are both leading causes of mortality in sub-Saharan Africa (WHO 2000). Generally, malaria severity is attributed to factors related to the human host, the parasite, and the environment (Molineaux 1996). The later is a major determinant for acquired immunity and clinical outcome of malaria infections in different localities (Snow *et al*., 1997). The parasite factors that might modulate the infection outcome includes the multiplicity of infection (MOI) and the parasite genotype. The recombination during the sexual life of the parasites is believed to determine the genotypes in the parasite population (Ranford-Cartwright *et al*., 1993). On the other hand, the MOI could be due to injection of multiple clones in a single bite of mosquito or closely spaced injections of single clones (super infections). The two mechanisms are not mutually exclusive, although the later is rather more sensible, as multi-colon infection correlates well with malaria transmission intensity (Conway *and* McBride 1991). In areas of high compared to low malaria transmission, the parasite population diversity is relatively broad, and the MOI is high (Babiker *et al*., 1997; Konate *et al*., 1999). Genotyping of highly polymorphic genes is ideal and generally acceptable for the determination of the MOI; the gene coding for merozoite surface protein MSP1 and MSP2 was found to be the most suitable single marker, as it posses variable sequences with different lengths (Felger *et al*., 1994; Ntoumi *et al*., 1995). However, what is estimated is usually the minimal clone number. While MSP1 and MSP2 alleles are not expected to be virulence markers, the predominance of a specific parasite genotype in SM proclaims the association of a certain parasite subpopulation with the development of SM in a specific community. In this study, we aimed to describe the genetic polymorphism of parasites causing the different complications in SM in a unique epidemiological setting.

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MATERIALS AND METHODS

This is a crossectional hospital-based study, carried out in Abyay clinical center. Sennar town, Central Sudan. The malaria transmission is high seasonal, and markedly unstable. The inhabitants from the town 1000,000 individuals) are of

multiethnic origin. This study was carried out over peak malaria transmission season (September–October 2006). The clinical and epidemiological pattern of SM was presented elsewhere (Giha *et al*., 2005). The P. *falciparum* is the predominant species (98%), and the Anopheles arabiensis is the sole vector.

Study population

434 patients screened for malaria infection 268 were malaria positive, 32 patients had SM according to the WHO (2000.) definition, were admitted to the clinic center, and enrolled into this study after their (or guardian) consent. Data and samples were available from 268 patients. The controls malaria-free (apparently healthy) individuals (MF, n=20), matched for age, sex, residence, tribe, etc. to SM patients and sometimes were patient's relatives. The mean age of the study groups SM (9.5 years), UM (22.5 years), and MF (15 years) was comparable. The SM patients were further subdivided into SMA (n=8, age 5.6 years), convulsion's associated malaria (CAM, n=3,5.9 years), CM (n=15 9.5 years), and the remaining patients had multiple complication's (n=6). Malaria definition and diagnosis and blood sampling uncomplicated malaria was defined as measured (>37.5°C) or reported fever with microscopically detectable asexual parasitemia. In SM, in addition to the parasitemia, there were other syndrome-specific signs/symptoms. For sub grouping of patients with SM, we strictly adhered to the WHO description of complications (WHO 2000). Blood samples (five ml and a drop in filter paper) were obtained from all patient's (n=268), Parasite detection and genotyping by polymerase chain reaction.The deoxyribonucleic acid (DNA) was extracted by the chelex100 (Bio-Rad Laboratories, Sigma) method as described before (Snounou *et al*., 1993), and stored at −20°C until use.

For genotyping at the MSP1 and MSP2 locus, the polymerase chain reaction (PCR) was run as described previously (Snounou *et al*., 1993), in brief, 2 µl of extracted DNA were used for amplification with PCR premix (deoxynucleotide triphosphate, MgCl2, primers, buffer, and Taq DNA polymerase). In order to increase sensitivity, nested PCR technique was employed (Snounou, 1993); two pairs of primers were used for each marker, an "outer" pair and a nested pair. The outer pair was used for the initial amplification, and the product obtained is used as a template for the next amplification using the nested pair of primers. In the outer reaction, 1 µl of DNA template suspension was used to amplify the region spanning both Blocks 2 and 4 for MSP1 and Block 3 for MSP2. For inner reactions, Five separate additional amplification reactions were carried out using primer pairs specific for the allelic families of block 2 polymorphic regions of MSP1 (Mad 20, K1 and RO33); two allelic families of block 3 polymorphic regions (FC27 and IC/3D7). The oligonucleotide primers used for this study, were designed from published sequences as listed in the UNDP/World Bank/WHO-TDR Malaria Database compiled by Ross Coppel... These primers were kindly gifted at Dr. G. Snounou (Imperial College of Medicine, UK). Amplification was performed using a Thermocycler (Techne serial no.84682-11). The profile for outer PCR for all markers was 30 cycles as follows: 95ºC, 5 minutes; 58ºC, 2 minutes; 72ºC, 2 minutes; 94ºC, 1 minute followed by 1 cycle of 58ºC for 2 minutes and 72ºC for 5 minutes.The profile for all nested reaction was 35 cycles of: 95ºC for 5 minutes; 61ºC for 2 minutes; 72ºC for 2 minutes and 94ºC for 1 minute then followed by one cycle of 61ºC for 2 minutes and 72ºC for 5 minutes (Snounou *et al*., 1993). The control was DNA from 3D7 for MAD20, K1, RO33 IC1 and FC27, respectively. Electrophoresis was performed on 5 ul of the nested amplification product on 2 % for MSP1 and MSP2 MetaPhor® agarose gel (FMC Bio Products, Maine, USA) stained with 3 ul ethidium bromide (10 mg/ml) (GiBcoBRL, Scotland) and run for 1-2 hrs in 1x TBE buffer (mM Tris, mM EDTA, mM Boric acid) at 80 V, and photographed under a standard UV transilluminator. DNA fragment sizes were determined using standard-size markers (100 bp DNA Ladder Plus- MBI Ferment as Ltd., Vilnius, Lithuania). Due to continuous nature of size variations of the amplified fragments of these genes, alleles with close sizes were binned around an average size. (Snounou *et al*., 1993).

Statistical analysis

The correlations between the variables were analyzed by SPSS.

RESULTS

Clone number, parasite density, and age. All blood samples were screened by PCR for P. *falciparum* detection and genotyping at MSP1 and MSP2 locus. The parasite DNA was detected in all positive blood samples obtained from malaria patients (UM and SM). The number of clones in the individual infections (MOI) was correlated with the parasite count (correlation coefficient [CC]=0.05, P=0.48, Also between MOI an allelic family of Mad 20, K1 and Ro33 (P= 0.00). Similarly, there was no correlation between MOI and age for all study groups (SM, UM, and ASUM; P=0. 58).The prevalence of single-clone and multicolone malaria infections as seen in Table 1, the single-clone infections in SM accounts for 31.3 %, which was not. Significantly different from that in UM (34.8 %) neither in frequency nor in genotypes (IC1, 10.6 %; FC27, 12.8 % Mad 20 14.6%, K1 11.0 % and Ro33 11.5 % respectively).

Table 1. The frequency of single allele and multi-allele infections in patients with severe malaria (SM) and uncomplicated malaria (UM) and in sever malaria (SM)

Severity of malaria	*MSP1*			*MSP2*	
	Mad20	K1	Ro33	FC27	IC
Uncomplicated	4 (4.9%)	3 (3.3%)	2 (2.3%)	5 (4.0%)	11 (6.1%)
Complicated	66 (80.5%)	78 (85.7%)	73 (85.9%)	104 (83.2%)	150 (83.3%)
Sever	12 (14.6)	10 (11.0%)	10 (11.8%)	16 (12.8%)	19 (10.6%)
Total (563)	82	91	85	125	180

The multiclone infections in SM (23.0 %) and UM (28.6 %) were slightly differ and basically of two types: either composed of clones of one allele family, IC1, FC27, Mad 20, Ro33,K1 (homogenic), or multy allele families, IC1, FC27, Mad 20, Ro33 and K1 (heterogenic). The difference between the frequency of the homogenic and heterogenic infections was significant (P=0.005) whether or not SM and UM were considered together. The maximum number of clones in individual infection was five in SM and three in UM. When MOI in UM (4.0) and SM (5.5), were compared the difference was not significant.

Table 2. The frequency of single clone and multi-clone infections in patients with severe malaria (SM) and uncomplicated malaria (UM) and in sever malaria (SM)

MOI	*Severity of Malaria*		
	UM	CM	SM
1 Strain	6 (42.8%)	64 (28.8%)	10 (31.3%)
2 Strains	4 (28.6%)	77 (34.7%)	11 (34.4%)
3 Strains	4 (28.6%)	61 (27.5%)	6 (18.8%)
4 Strains	–	18 (8.1%)	4 (12.5%)
5 Strains	–	2 (0.9%)	1 (3.0%)
Total (268)	14	222	32

The parasite genotypes in the different study groups

The overall frequency of IC1, FC27, K1, Ro33 and Mad20 families in all malaria infections was 59%. 41%, 35.4%, 32.8% and 31.9% respectively. The total number of allele types (size polymorphism, bp) in the IC1 and FC27, K1, Ro33 and Mad20 families were 4, 5. 4, 3, and five types, respectively. The number of allele types in UM Mad, K1, Ro33, Fc27 and Ic1 are 66, 78,73,104 and 150 respectively and SM 12, 10, 10, 16, and 19 respectively) were differed. The generally more recognized alleles were IC1 (400 bp), FC27 (200 bp, Mad20 (100bp) . K1 (150 bp) and Ro33 (150bp).

DISCUSSION

The prevalence of malaria during the study period was found to be 38%, which were obtained by parasite point prevalence carried out at Abyay Primary school. The parasitological data obtained for this study indicated that malaria is highly endemic and still a major health problem for the area, in spite of intensive control measures efforts carried out in the area, this in agreement with previous entomological studies (Almahdi, 2006). This indicates further assessment of control measures applied to the area is needed.Genetic diversity of *P. falciparum* is common in high transmission areas. The diversity is associated with the transmission intensity (Badiker *et al* 1997) and can induce a high level of severe malaria, especially in cities where the pressure against malaria is lower than other disease-endemic areas. (Robert *et al* 1996 and Schleiermacher *et al* 2001). The genomic DNA of the *P. falciparum* isolates were investigated for genetic diversity at highly polymorphic loci (Merozoite surface protein 1 [MSP1] and MSP2). The MSP 1 and MSP 2 loci have been genotyped using the nested polymerase chain reaction (PCR) strategy and conditions previously described.

We have observed high genetic diversity in P. falciparum in Sennar with a mean of 3.8 parasite populations (maximum _5) this result is a consistent study Gadarif western Sudan, they found the maximum number of clones in individual infection was four in SM and five in UM (Ishraga. 2007). The MSP2 gene shows a higher polymorphism than the msp1 gene. Our results are in consistent with those of (Robert *et al* 1996 and Leclerc *et al* 2002) That reported extensive genetic diversity analyzed by antigenic markers in isolates collected in Dakar. The results for this study confirmed the relation between genetic diversity and occurrence of severe malaria. Furthermore, the results are supporting and reinforcing the notion that some *P. falciparum* strains might cause more severe infections than others (Legrand *et al*., 2005). The results reported in the present work supported former reports, which pointed out a relation of certain alleles and severity of malaria (Ariey *et al*., 2001, Legrand *et al*., 2005, Amodu *et al*., 2005, Ranjit *et al*., 2005). The present results are consistent with other results reported by Engelbrech *et al*., (1995) in Papua New Guinea, that clinical malaria was associated. with MSP-2 FC27 alleles. (Marshall *et al*., 1994) also noted a predominance of FC27 allelic type in patients from Irian Jaya. The results indicate that both MSP2 (FC27) and Mad20 are good indicators for determination of MOI, since high numbers of bands were encountered with genotypes of these two genes. However, this observation is supported by the finding that, genotyping of MSP2 is a standard method for assessing MOI (Viriyakosol *et al*., 1995), as it is highly polymorphic in length and sequence (Ntoumi *et al*., 1995). MOI was found to be significantly higher at the end of transmission season for the study area. The majority of PCR positive samples had multiple infection, these constitute 62 % of the total samples from the study, correlated with parasite density and distributed regardless of age.

A high prevalence of multiple infections was consistent with the reports by (Creasey *et al*., 1990), (Manijeh *et al*., 2008) and (Prescott *et al*., 1994), those authors observed 83%, 64% 64% and 40%, respectively, of mixed infections in Zimbabwe, Gabon, and in the Solomon Islands. (Conway *et al*., 1991) Furthermore, observed mixed infections in Gambian malaria patients, with an average of two distinct clones per isolate. It has been proposed by previous studies (Contamin *et al*., 1995, Farnert *et al*., 2001) that, high parasite densities increase the probability of detecting concurrent clones in an individual. The present study which is conducted in meso endemic malaria region is consistent with the above proposed observation since a positive correlation between MOI and parasite density on one hand and MOI and Hb, on the other hand, were observed.(P=0.00 and 0.008 respectively) However, three subjects from this study were found to harbor five strains of *P. falciparum* but associated with high parasitemia. MOI does not vary over age and show a significant correlation with transmission density, as the higher transmission seasons increase the probability of having multiple infections. The result from this study was on consistency among the results reported in a recent work in which MOI was reported to be not age-dependent, but was correlated with parasite density (Vafa *et al*., 2008).

The SM is a multifactorial disorder. With diverse clinical presentation, the parasite characteristics are likely contributing to this clinical diversity. The MOI and allelic Polymorphism of MSP2 is more frequently investigated in UM and in malaria-hyperendemic regions (Felger *et al*., 1994; Ntoumi *et al*., 1995; Konate *et al*., 1999). However, little is known about the parasite allelic polymorphism in the individual complications of SM and in areas of low transmission. We carried out this descriptive study in an area of strictly seasonal and markedly unstable and high malaria transmission in Sudan. In such situation, genotypes, multiplicity, and dynamics of infecting parasites are less influenced by confounding factors, such as age and immunity (Ntoumi *et al*., 1995). Although in individual infections, the numbers of clones were few, the overall circulating allele types were numerous (>20). We found no correlation between MOI and age, unlike in previous reports from malaria-hyperendemic regions (Konate *et al*., 1999; Ntoumi *et al*., 1995). However,

in this area, both UM and SM affect all age groups, but SM is more common among younger patients (Giha *et al*., 2005). Densities Densities on MOI is likely to be immunity dependent, so their influence is modest in areas of low transmission. Furthermore, the MOI was comparable between UM and SM, and it was not different between the individual complications of SM. The frequency of single-clone infections was ranging from 12.5 in individuals with in SM and 80% in UM. SM was mostly due to IC1 clonal infections. Fatal CM was exclusively due to IC1 single-clone infections. In this setting, it was previously reported that the risk of development of UM increases with the increased number of clones in a given infection (Roper *et al*., 1998). The clinical consequences of multiclonal infections vary considerably between epidemiological settings (Arnot 1998) and in different ages and immune status (Henning *et al*., 2004; Smith *et al*., 1999). In Tanzania and Papua New Guinea, the higher MOI was found to be associated with reduced incidence of clinical malaria (Farnert *et al*., 1999; al-Yaman *et al*., 1997). While in other studies conducted in Tanzanian (Smith *et al*., 1999), western Kenya (Branch *et al*., 2001), Ghana (Ofosu-Okyere *et al*., 2001), and Mozambique (Mayor *et al*., 2003), the increased MOI was found to be a risk factor for the development of clinical malaria.

However, most of these studies compared UM with asymptomatic infections. Generally, there was great similarity in the prevalence of the two MSP2 families, the IC1 and FC27, in UM and SM infections, although more IC1 parasites were recognized in UM. The size polymorphism of both MSP2 families was comparable between UM and SM, not only in the total number of alleles but also in the distribution of the individual allele types. The frequency of FC27 single-clone. Infection was relatively low in this setting (41%) as in French Guiana (Ariey *et al*., 2001), while in other areas, e. g., Papua New Guinea, the FC27 genotypes predominate (Felger *et al*., 1994; Engelbrecht *et al*., 1995). The frequency of the multiclonal infections, homogenic (consists of either IC1 or FC27) or heterogenic (IC1 and FC27), were comparable between SM and UM in this study; however, most of the mixed infections were heterogenic (82.7%). That means infections with a certain genotype may possibly elicit stronger immunological. Protection from infection with the same genotype family; that is, there was cross-immunity between alleles of different sizes but of the same family. For MSP1 families, the Mad20. K1 and Ro33 were differed, in UM and SM infections, although more Mad20 parasites were recognized in SM while K1 is high in UM.

In conclusion, while the MOI was higher in clinical malaria compared to UM infections, it was not significantly different between UM and SM. The low MOI was associated with UM infection and fatal CM. The predominant genotype was IC1 and Mad20. The IC1, FC27., Mad20, K1 and Ro33 alleles were differed distributed in SM and UM. The results from the different epidemiological settings support the notion that SM is not usually caused by specific phenotypes but varies in place and time as a result of the host/parasite co evolution. An acknowledgment The patients, Sennar Teaching Hospital Staff, and our field team: Dr Mohamed Fadl-Elsseed, Mohamed Mhy-Eldeen, Abyay Clinical Center Staff and Sennar Malaria Center Staff, are all acknowledged for the unlimited cooperation.

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