

## PARASITE DIVERSITY IN ADULT PATIENTS WITH CEREBRAL MALARIA: A HOSPITAL-BASED, CASE-CONTROL STUDY

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**Abstract.** Thirty adult patients with cerebral malaria (CM) were recruited for this study. Two clinical groups were used as controls: those with mild malaria (n = 20) and asymptomatic volunteers (n = 20). Thick and thin blood smears were examined for detection of *Plasmodium falciparum* and estimating infection intensity. A nested polymerase chain reaction (PCR) using allele-specific primers for merozoite surface protein gene was used to determine the parasite diversity of *Plasmodium falciparum* causing CM. *Plasmodium falciparum* was detected in blood smears of all malaria patients. No significant difference in parasite count was found between the groups. Thirteen (65%) of the asymptomatic volunteers had a positive PCR for *P. falciparum*. Multiple alleles were found in 17 (58.6%) patients with CM, but only in 7 (35.6%) with uncomplicated malaria. Multiple alleles were also found in 6 (46.2%) of the 13 PCR-positive asymptomatic individuals. We could not identify a specific strain or strains of *P. falciparum* that showed a significant association with disease severity. Therefore, we assume that the development of CM in adults residing in endemic areas is more dependent on strain multiplicity rather than on a specific strain or strains of *P. falciparum*, and that the parasite intensity has no relationship with disease severity. Asymptomatic adults may repeatedly be exposed to low levels of a wide range of different strains during low transmission season and acquire sub-patent parasitemia. This may also confer premunition that renders them relatively resistant to CM.

### INTRODUCTION

Cerebral malaria (CM) is the most serious and potentially fatal presentation of all the various clinical syndromes caused by *Plasmodium falciparum* infection. Cross-sectional and longitudinal genetic epidemiologic studies in community-based surveys in areas of high perennial transmission have persistently showed concomitant multiple infections with different strains occurring at the individual level. An infection can have several genotypes due to superinfections and inoculation of multiple genotypes during a single mosquito bite. Such genetic heterogeneity within the parasite population and host defense response influence the ultimate clinical manifestations of the disease.<sup>1,2</sup> It has also been shown that certain *P. falciparum* genotypes appear to be associated with more virulent infections,<sup>3,4</sup> and different *in vitro* isolates express significant and stable differences in their potency to induce tumor necrosis factor *in vitro*.<sup>1</sup>

The merozoite surface protein 2 (*msp-2*) is a major polymorphic protein expressed on the surface of infected erythrocytes.<sup>5,6</sup> The N- and C- terminals of the protein are conserved, whereas the large central region is highly variable in different *P. falciparum* strains. This extensive polymorphism of *msp-2* has been widely exploited as a marker of great use to determine the diversity of *P. falciparum* in field studies.<sup>7</sup> Allelic sequencing of the *msp-2* gene has shown corresponding strain-specific length polymorphism of the two tandem repeats encoding the variable central region of the protein, namely the FC27 and 3D7 alleles.<sup>8</sup> It was found that parasites carrying the FC27 genotype are more virulent, twice as likely to be found in symptomatic than asymptomatic patients, and are associated with higher mortality.<sup>2,9</sup>

In this hospital-based prospective study, we sought to demonstrate the parasite diversity of *P. falciparum* in blood samples collected from adult patients with CM, compared with that in patients with uncomplicated mild malaria (MM) and in clinically healthy asymptomatic (AS) volunteers residing in a malaria-endemic region in Sudan. We used a nested polymerase chain reaction (PCR) for amplification and de-

tection of the polymorphic region FC27 of the *msp-2* gene, and used it as a genetic marker for different clone infections.

### MATERIALS AND METHODS

**Study area.** The study was conducted in Khartoum Teaching Hospital, the largest referral hospital in the region, located in Khartoum, the capital of Sudan. In Sudan, the winter months of October to February are characterized by an outbreak of *P. falciparum* malaria due to peak transmission following the preceding rainy season from July to September. Low-grade transmission and case incidence are maintained throughout the rest of the year.

**Patients.** Seventy adult men and women, all from the same area, were recruited in the different study groups during the period from October 1998 to November 1999. The groups included patients with CM (n = 30), patients with MM (n = 20), and the AS group (n = 20). Patients with CM were recruited at presentation to the emergency department and followed-up in the hospital. The diagnosis of CM was made according to the World Health Organization (WHO) definition.<sup>10–12</sup> Patients who had other manifestation of severe malaria, (e.g., renal failure, circulatory collapse, pulmonary edema, hyperpyrexia, hypoglycemia, severe electrolyte disturbance) and those who had a recent history of alcohol or neuroleptic drug intake were excluded. The MM group presented to the emergency department with fever, had positive blood films for asexual *P. falciparum*, and had no other explanation for their illness. They exhibited no WHO criteria for severe malaria and were treated as outpatients. The AS group included clinically healthy asymptomatic volunteers attending the emergency department at the same time and coming from the same residing area as the other groups. The results of blood film testing for malaria in the AS group were negative by light microscopy. The MM and AS groups were age and sex matched with the CM group. Informed consent was obtained from all participants either directly or from attending close relatives if the subjects were unconscious. The

study was reviewed and approved by the ethical committee of the Graduate Medical Studies Board of the Faculty of Medicine of the University of Khartoum.

**Detection and counting of malaria parasites.** Giemsa-stained thick and thin blood films were prepared for detection and identification of *Plasmodium* species and for parasite count. The parasite density/microliter was calculated from the number of parasites present per high-power microscopic field and the assumed the volume of blood present in one high-power field.<sup>13,14</sup>

**Sample collection and extraction of DNA.** Drops of venous blood were blotted on Whatman (Brentford, United Kingdom) 3 MM chromatography paper, air-dried, individually placed in plastic bags, and stored at room temperature. Parasite DNA was extracted with Chelex.<sup>15</sup> Briefly, 180  $\mu$ L of a 5% Chelex-100 solution (Bio-Rad Laboratories, Hercules, CA) was added to a 1.5-mL microcentrifuge tube and placed in heating block at 100°C for five minutes. Each filter paper sample was excised and added to the hot Chelex solution, and the tube was gently vortexed and returned to the heat block for 10 minutes. The samples were centrifuged twice and the final supernatant was removed to a new microcentrifuge tube and stored at 4°C until used in the amplification reaction.

**Amplification by PCR.** Primary and nested PCRs for *msp-2* amplification were completed in all except one CM blood sample because of a sampling error. For the primary PCR, outer primer pairs corresponding to the flanking sequence of the conserved regions of *msp-2* were used. The primary products were re-amplified by a nested PCR using allele-specific primer sets corresponding to the inner hypervariable FC27 allele.<sup>15-17</sup> The following oligonucleotide primer sequences were used: *msp-2s1* (5'-GAA GGT AAT TAA AAC ATT GTC-3'), *msp-2s2* (5'-GAG GGA TGT TGC TGC TCC ACA G-3'), *msp-2s3* (5'-GAA GGT AAT TAA AAC ATT GTC-3'), and *msp-2s4* (5'-ACA TGC AAG TGT TGA TCC-3'). For the reaction, 2.5  $\mu$ L of primer mixture (10 mM each) was added to a mixture of 5  $\mu$ L of 10  $\times$  MgCl<sub>2</sub>-free reaction buffer, 2  $\mu$ L of 20 mM dNTP mixture (AB gene-0196 DNA polymerase mixture; ABgene House, Epsom, Surrey, United Kingdom), and 0.25  $\mu$ L of 5 units/ $\mu$ L of thermostable DNA polymerase. For the primary PCR, 20  $\mu$ L of template DNA was added to the reaction mixture. An automated 30-cycle PCR was performed using a Perkin Elmer (Wellesley, MA) 480 sequencer. The cycle conditions for the outer PCR were denaturation at 94°C for 25 seconds, annealing at 50°C for 60 seconds, and extension at 65°C for 120 seconds. Similar conditions were used for the inner PCR, except that the extension temperature was 70°C and an additional 10-minute extension was done at the end of the cycles to insure that all products were full-length. The PCR products were stored at 4°C until they were analyzed by electrophoresis on a 1.5% agarose gel.

**Statistical analysis.** Statistical analysis was carried out using the software Statistical Package for Social Sciences (SPSS Inc., Chicago, IL). The chi-square and Student *t*-test were applied with statistical significance of  $P < 0.05$ .

## RESULTS

All CM and MM patients had microscopically detectable malaria parasites in their blood films. None of the AS indi-

viduals had a positive blood film for malaria. Table 1 summarizes the parasite count in the two clinical groups. The majority (60% of those with CM and 50% of those with MM) of the patients had a low-profile parasitemia. There was no correlation between the parasite density in the blood samples and the severity of clinical presentation ( $P > 0.05$ ).

The nested PCR for *msp-2* amplification was completed on all samples from the three groups, except for one CM blood sample in which the inner PCR data were missing. Predictably, *P. falciparum* DNA was detected in all (100%) 29 CM patients and all 20 MM patients. In the healthy AS group, the PCR showed the presence of *P. falciparum* in 13 (65%) individuals, indicating sub-clinical infections. Agarose gel electrophoresis of the product generated a number of discrete DNA bands with different sizes that ranged between 400 and 700 basepairs. These were predominantly of 500 and 600 basepairs in the study and control groups (Figure 1). One band was above the expected length range for *msp-2* and had a size of 900 basepairs. This result could not be explained and was considered unusual and disregarded. No significant association was found between the size of a band and the clinical presentations ( $P > 0.05$ ). The number of allelic variants for *msp-2* in each sample ranged from one to four. Multiple alleles, indicating simultaneous complex infections by more than one strain, were found in 17 of 29 CM patients (58.6%). More than two alleles were found in five patients (17.2%). In MM patients, multiple alleles were found in 7 of 20 patients (35%), and more than two alleles were observed in only one patient (5%). The difference between the two groups was statistically significant ( $P < 0.02$ ). It was of interest that multiple alleles were also found in 6 (46.2%) of the 13 PCR-positive AS individuals. This indicated that they were harboring several parasite clones without any presenting symptoms (Table 2).

## DISCUSSION

In endemic areas, adults are less vulnerable to CM than children because of the acquisition of partial immunity. Notwithstanding, adults constitute less than 10% of all cases of life-threatening malaria in Africa, the mortality rate is higher, and CM is the most common cause of death among them.<sup>18</sup> This prevalence difference is perhaps one of the reasons why we see fewer epidemiologic and case studies for adult CM. Since the spectrum of disease significantly changes with age, more focus on adult studies is necessary for a better understanding of the dynamics of infection complexity and immune interaction.

Parasite density, as determined by light microscopy, did not show significant differences between patients with CM and

TABLE 1

A sexual parasite count in peripheral blood films of patients with cerebral malaria (CM) and mild malaria (MM)\*

Parasite count (n/ $\mu$ L)	No. of patients (%)	
	CM	MM
< 5,000	18 (60%)	10 (50%)
5,000-50,000	6 (20%)	6 (30%)
> 50,000	6 (20%)	4 (20%)

\*  $\chi^2 = 0.714, P > 0.2$ .

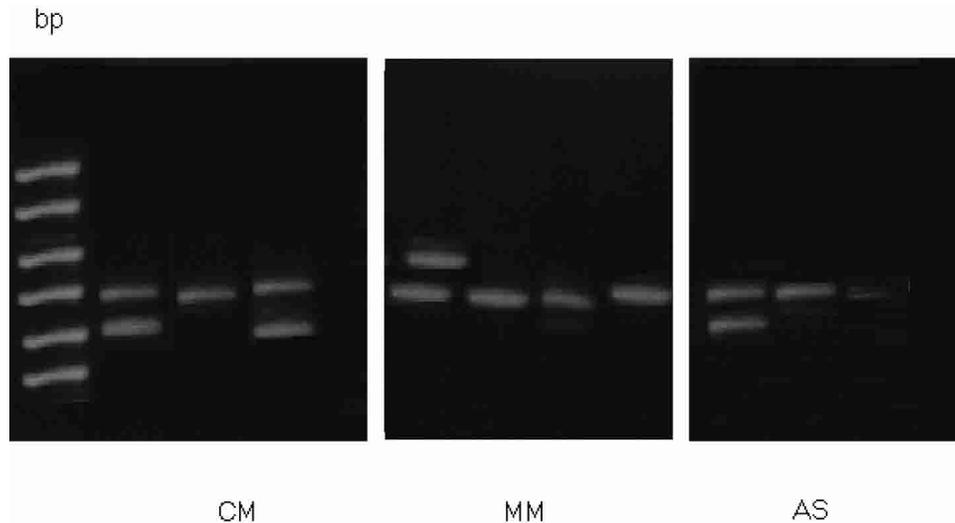


FIGURE 1. Polymerase chain reaction products obtained from amplification of a polymorphic merozoite surface protein 2 gene separated by agarose gel electrophoresis and stained with ethidium bromide. Several bands were seen in some of the lanes, indicating multiple clone infection. CM = cerebral malaria, MM = mild malaria, AS = PCR-positive asymptomatic individuals. Lane bp = basepair ladder.

those with MM. It is known that in native inhabitants of endemic areas the correlation between parasite count and disease severity diminishes with age and intensity of transmission.<sup>19</sup> It is believed that disease severity is related more to the number of parasites sequestered than to the number circulating.<sup>20,21</sup> It is the predominance of older parasites in the circulation that results in a poor prognosis because it reflects a relatively greater sequestered biomass at the tissue level.<sup>21</sup>

The PCR amplification of *msp-2* did not display any significant frequency for certain strain or strains in our patients with CM. There was concomitant existence of multiple clone infections in CM and MM groups, the pattern and complexity of which varied even among those within the same group. A significant increase in band number was observed in the more severe cerebral form. Such increased complexity of infection was most likely a reflection of a state of high transmission intensity in those patients.<sup>17</sup> It is well recognized that factors that influence clonal multiplicity in semi-immune adults living in mesoendemic and hypoendemic areas include intensity of transmission and host immunity. Novel parasites inoculated during the transmission season are less prone to elimination by host immune mechanisms and are thus more likely to be detected in blood samples.<sup>19,22,23</sup> In a cohort followed for 10 years at Darawish village in eastern Sudan, longitudinal genetic monitoring had clearly shown that clinical malaria episodes were almost always associated with parasite genotypes

that had not been previously detected.<sup>24</sup> Increased morbidity in Ghanaian children had been shown to be associated with both increasing multiplicity and novel clone infections during transition into high transmission season.<sup>25</sup> The experimental model for malaria in rodents has also provided evidence that mixed clone infections are more virulent than single clone infections.<sup>26</sup> It is therefore reasonable to assume that increased clone number and infections with novel strains resulted in a greater risk for our patients to develop CM.

The presence of diverse mixed infections in asymptomatic inhabitants of endemic areas is another intriguing example of the complex nature of *Plasmodium* infection. Paradoxically, there is a trend towards increased clone counts in asymptomatic individuals than in clinically ill patients in areas of high endemicity.<sup>27</sup> Similarly, most of our PCR-positive AS individuals harbored multiple alleles that tended to be more complex than those in MM patients and approximately one-third had more than three-band multiplicity. Those individuals lived in a holoendemic area and were subject to repeated exposure to relatively low levels of a wide range of different strains during low-grade transmission seasons. Such exposure may lead to sub-patent parasitemia that is not detectable by light microscopy.<sup>28</sup> It is also probable that they are capable of clearing parasitemias to below detection levels by innate or specific immunity. The immunity developed tends to target antigenic determinants expressed by most of the transmittable parasite genotypes available in the area, which will account for the resistance to parasitemia despite having more complex infections.<sup>2</sup> This portion of the community acquires a premunition, at least against certain strains, and serves as a parasite reservoir. They behave as asymptomatic carriers capable of infecting others, but they may also convert to clinical disease if their premunition is breached, e.g., by debility or malnutrition.<sup>29,30</sup> Since all patients and controls were recruited from the same geographic area and during the same season, the occurrence of different patterns of presentation, despite uniform exposure, indicates the presence of some host factors operating in determining the clinical type of disease.

TABLE 2

Number of polymerase chain reaction product bands visualized with an ultraviolet transilluminator in patients with cerebral malaria (CM), mild malaria (MM), and asymptomatic healthy volunteers (AS)\*

No. of bands	CM	MM	AS
1	12 (41.4%)	13 (65%)	7 (53.8%)
2	12 (41.4%)	6 (30%)	2 (15.4%)
3	4 (13.8%)	0 (0%)	0 (0%)
4	1 (3.4%)	1 (5%)	4 (30.8%)

\*  $\chi^2 = 15.368, P < 0.2$ .

In addition to the recognized inherited hemoglobin defects and the structural abnormalities, there are at least three human genes in the major histocompatibility complex that have been isolated and shown to influence the outcome and presentation of malaria infection.<sup>31</sup>

In conclusion, in adult malaria it is clonal multiplicity, rather than parasite diversity or density of parasitemia, that tends to carry a risk for developing CM. Since the host defense interaction is another important determinant, larger scale studies including the immune profile in different clinical categories are needed.

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## REFERENCES

- Mendis KN, Carter R, 1995. Clinical disease and pathogenesis in malaria. *Parasitol Today* 11: 1–15.
- Branch OH, Takalo S, Kariuki S, Nahlen BL, Kolczak M, Hawley W, Lal AA, 2001. *Plasmodium falciparum* genotypes, low complexity of infection, and resistance to subsequent malaria in participants in the Asembo Bay cohort project. *Infect Immun* 69: 7783–7792.
- Engelbrecht F, Felger I, Genton B, Alpers M, Beck H-P, 1995. Malaria morbidity is associated with *Plasmodium falciparum* merozoite surface antigen 2 genotypes. *Exp Parasitol* 81: 90–96.
- Ariey F, Hommel D, Le Scanf C, Ducemin JB, Peneau C, Hulin A, Sarthou JL, Reynes JM, Fandeur T, Mercereau-Puijalon O, 2001. Association of severe malaria with a specific *Plasmodium falciparum* genotype in French Guiana. *J Infect Dis* 184: 237–241.
- Borst P, Bitter W, McCulloch R, Leewen FV, Rudenko G, 1995. Antigenic variation in malaria. *Cell* 82: 1–4.
- Kemp DJ, Cowman AF, Walliker D, 1990. Genetic diversity in *Plasmodium falciparum*. *Adv Parasitol* 29: 75–149.
- Felger I, Irion A, Steiger S, Beck H-P, 1999. Epidemiology of multiple *Plasmodium falciparum* infection. Genotypes of merozoites surface protein 2 of *Plasmodium falciparum* in Tanzania. *Trans R Soc Trop Med Hyg* 93: S3–S9.
- Smyteh JA, Peterson GM, Coppel R, Soul KJ, Kemp DJ, Anders R, 1990. Structural diversity in the 45-kilodalton merozoite surface antigen of *Plasmodium falciparum*. *Mol Biochem Parasitol* 39: 227–234.
- Al-Yaman F, Genton B, Reeder JC, Mokela D, Anders RF, Alpers MP, 1997. Humoral response to defined *Plasmodium falciparum* antigens in cerebral and uncomplicated malaria and their relationship to parasite genotype. *Am J Trop Med Hyg* 56: 430–435.
- Teasdale G, Jennet B, 1974. Assessment of coma and impaired consciousness. A practical scale. *Lancet* 2: 81–84.
- Warrell DA, 1989. Cerebral malaria. *Q J Med* 265: 369–371.
- Warrell DA, Molyneux ME, Beales PF, 1990. Severe and complicated malaria. *Trans R Soc Trop Med Hyg* 84 (Suppl 1): 1–65.
- Gilles HM, 1993. Diagnostic methods in malaria. Gilles HM, Warrell DA, eds. *Bruce Chwatt's Essential Malariology*. Third edition. London: Edward Arnold, 78–95.
- Greenwood BM, Armstrong JRM, 1991. Comparison of two simple methods for determining malaria parasite density. *Trans R Soc Trop Med Hyg* 85: 186–188.
- Kain KC, Lanar DE, 1991. Determination of genetic variation within *Plasmodium falciparum* by using enzymatically amplified DNA from filter paper disks impregnated with whole blood. *J Clin Microbiol* 29: 1171–1174.
- Cavanagh DR, Elhassan IM, Roper C, Robinson VJ, Giha H, Holder AA, Theander TG, Arnot DE, McBoride JS, 1998. A longitudinal study of type-specific antibody responses to *Plasmodium falciparum* merozoite surface protein-1 in an area of unstable malaria in Sudan. *J Immunol* 161: 347–359.
- Contamin H, Fandeur T, Bonnefoy S, Skourri F, Ntoumi F, Mercereau-Puijalon O, 1995. PCR typing of field isolates of *Plasmodium falciparum*. *J Clin Microbiol* 33: 944–951.
- World Health Organization, 2000. Severe falciparum malaria. *Trans R Soc Trop Med Hyg* 94 (Suppl 1): 1–18.
- Bendixen M, Msangeni HA, Pedersen BV, Shayo D, Bodkar R, 2001. Diversity of *Plasmodium falciparum* populations and complexity of infections in relation to transmission intensity and host age: a study from the Usambara Mountains, Tanzania. *Trans R Soc Trop Med Hyg* 95: 143–148.
- Okheallialam TC, Kilama WL, Ramji BD, 1972. The clinical significance of malaria parasitemia in children. *East Afr Med J* 49: 962–982.
- Silamut K, White NJ, 1993. Relation of the stage of parasite development in the peripheral blood to prognosis in severe falciparum malaria. *Trans R Soc Trop Med Hyg* 87: 436–443.
- Babiker HA, Abdul-Mohsin AA, Ranford-Cartwright L, Satti G, Walliker D, 1998. Characteristics of *Plasmodium falciparum* parasites that survive the lengthy dry season in eastern Sudan where malaria transmission is markedly seasonal. *Am J Trop Med Hyg* 59: 582–590.
- Zwetyenga J, Rogier C, Spiegel A, Fontenille D, Trape J-F, Mercereau-Puijalon O, 1999. A cohort study of *Plasmodium falciparum* diversity during the dry season in Ndiop, a Senegalese village with seasonal, mesoendemic malaria. *Trans R Soc Trop Med Hyg* 93: 375–380.
- Roper C, Richardson W, Elhassan IM, Giha H, Hviid L, Satti GM, Theander TG, Arnot DE, 1998. Seasonal changes in the *Plasmodium falciparum* population in individuals and its relationship to clinical malaria in an area of unstable transmission in Sudan. *Parasitology* 116: 501–510.
- Ofose-Okyere A, Mackinnon MJ, Sowa MP, Koram KA, Nkrumah F, Osei YD, Hill WG, Wilson MD, Arnot DE, 2001. Novel *Plasmodium falciparum* clone multiplicities are associated with the increase in malaria morbidity in Ghanaian children during the transition into the high transmission season. *Parasitology* 123: 113–123.
- Taylor LH, Mackinnon MJ, Read AF, 1998. Virulence of mixed-clone and single-clone infections of the rodent malaria *Plasmodium chabaudi*. *Evolution* 52: 583–591.
- Al-Yaman F, Genton B, Reeder JC, Anders RF, Smith T, Alpers MP, 1997. Reduced risk of clinical malaria in children infected with multiple clones of *Plasmodium falciparum* in a highly endemic area: a prospective community study. *Trans R Soc Trop Med Hyg* 91: 602–605.
- Ferreira MU, Kimura EAS, Katzin AM, Santos-Neto LL, Ferrarri JO, Villalobos JM, DeCarvalho ME, 1998. The IgG subclass distribution of naturally acquired antibodies to *Plasmodium falciparum* in relation to malaria exposure and severity. *Ann Trop Med Parasitol* 92: 245–256.
- Roper C, Elhassan IM, Hviid L, Giha H, Richardson W, Babiker H, Satti GMH, Theander TG, Arnot DE, 1996. Detection of very low level *Plasmodium falciparum* infection using the nested polymerase chain reaction and a reassessment of the epidemiology of unstable malaria in Sudan. *Am J Trop Med Hyg* 54: 325–331.
- Singh B, Cox-Singh J, Miller AO, Abdullah MS, Snounou G, Abdul Rahman H, 1996. Detection of malaria by nested polymerase chain reaction amplification of dried blood spots on filter paper. *Trans R Soc Trop Med Hyg* 90: 519–521.
- Jepson A, Sisay-Joof F, Banya W, Hassan-King M, Frodshman A, Bennett S, Hill AVS, White H, 1997. Genetic linkage of mild malaria to the major histocompatibility complex in Gambian children: study of affected sibling pairs. *BMJ* 315: 96–97.