Introduction

Malaria is a major health-threatening disease that is responsible for 0.8 million deaths; the vast majority of these deaths occur in Africa in children <5 years old. Plasmodium falciparum is the most virulent species of the human malaria and is responsible for most malaria-related deaths. To control and eventually eradicate malaria, an effective vaccine is considered to be needed in addition to the existing strategies such as insecticide-treated bed nets (ITNs) and chemotherapy. Extensive genetic diversity in the parasite field isolates have been reported in several studies. This diversity hampers development of effective vaccine as it limits the efficacy of protective immunity (i.e., antibody-mediated parasite inhibition). Therefore, it is important to determine the genetic diversity of antigens that are vaccine targets in different transmission settings to assist the development of effective malaria vaccines.

Merozoite surface protein 1 (MSP1) and 2 (MSP2) of P. falciparum are principal blood-stage malaria vaccine targets. MSP1 is major surface protein of approximately 190 kDa size. It plays a pivotal role in erythrocyte invasion by merozoite. The protein is a major target of immune responses. MSP1 contains 17 blocks of sequence flanked by conserved regions. Block 2, which is the most polymorphic part of MSP1, is grouped into...
three allelic families namely K1, MDA20, and RO33 type.\textsuperscript{[31]} MSP2 is a glycoprotein consisting of 5 blocks, where the central block is the most polymorphic. The MSP2 alleles are grouped into two allelic families, FC27 and 3D7/IC1.\textsuperscript{[12,13]} Due to the polymorphic features, MSP1 and MSP2 have been widely exploited as markers of great use to determine the diversity of \textit{P. falciparum} in field studies\textsuperscript{[14‑18]} and also to determine association with disease severity.\textsuperscript{[17,18]}

Malaria incidence in Sudan is estimated to be about 9 million episodes and the number of deaths is about 44000 every year.\textsuperscript{[19]} The morbidity and mortality rates due to malaria have been declining steadily in Sudan during the last recent years, yet malaria remains one of the major health problems in the country.

Extensive work on the genetic diversity of \textit{P. falciparum} field isolates in Gadaref state, eastern Sudan, an area characterized by seasonal and low malaria transmission has been thoroughly investigated.\textsuperscript{[3,20]} Parasites were genetically complex, consisting of multiple clones with large repertoire of MSP1 and MSP2 alleles. In these studies, fluctuations in allele prevalence from 1 year were also reported.

This study presents the genetic diversity and multiplicity of infection of \textit{P. falciparum} field isolates collected from Kosti site, in the White Nile state, central Sudan, in 2003. Malaria in the White Nile region and, in general, in central Sudan is characterized by low to moderate malaria transmission with highly seasonal outbreaks from July to November.\textsuperscript{[20]} Genotyping of the polymorphic loci of the MSP1 and MSP2 was performed and data was used to examine the relationship between genetic diversity and multiplicity of infection according to parasite density and age.

**Materials and Methods**

**Study site**

This study was conducted in Kosti, White Nile state in central Sudan. The study region is situated on the west bank of the White Nile, approximately 340 km south of Khartoum. Kosti region experiences one long rainy season, which usually extends from June to September with slight year-to-year variation.

**Samples collection and processing**

Blood samples were collected from a total of 121 individuals during a cross-sectional survey following transmission season in March and April 2003. All patients with malaria complaints reported to Kosti Teaching Hospital and El‑Tigani Health Center in Kosti city were interviewed. Clinical data was recorded including patient age. Microscopy thick and thin blood film slides were prepared using 10% Giemsa solution for 30 min. The stained slides were examined under a light microscope using 100x oil immersion by an experienced laboratory technician. Parasitaemia was calculated per 200 white blood cells (WBC) assuming 8000 WBC/μl of blood stage.\textsuperscript{[22]} Based on the microscopy results, the study subjects were classified into two groups: Group 1 that had hyperparasitaemia (total number of parasite >5000 parasites/μl of blood), group 2 that had hypoparasitaemia (total number of parasite <5000 parasites/μl of blood).\textsuperscript{[21]} Malaria case was diagnosed if a patient had fever (auxiliary temperature >37.5°C) and microscopically detectable asexual parasites. The study was approved by the Institute of Endemic Diseases Ethical Committee. Informed consent was obtained from each participant or guardians for the children prior to recruitment.

**DNA preparation**

Genomic DNA was extracted from frozen blood samples (approximately 200 μl) using 0.5% saponin (Gimsa‑Aldrich, Germany) to free parasites from red blood cells, followed by phenol extraction and ethanol precipitation as previously described.\textsuperscript{[24]} DNA was suspended in 100 μl 1X TE and kept frozen at −20°C until needed.

**Identification of plasmodium species**

All 121 DNA samples were genotyped using \textit{P. falciparum} genus and species-specific primers in a nested PCR approach (rPLUS1 and rPLUS5; rFAL1 and rFAL2). Details of the methodology have been described elsewhere.\textsuperscript{[24]}

**Allelic typing of \textit{P. falciparum} MSP1 and MSP2**

The primers specific for the polymorphic regions (block 2 of \textit{MSP1} and block 3 of \textit{MSP2}) were described previously.\textsuperscript{[25]} The two genes were amplified using nested-PCR. An initial amplification of the outer regions of the two genes was followed by a nested-PCR with allelic family specific primer pairs. All PCR reactions were carried out in a total volume of 50 μl containing 0.2 mM dNTPs, 2 mM MgCl\textsubscript{2}, 1 μM each primer, and 1 unit of AmpliTaq Polymerase (Perkin Elmer, England). In the first round reaction, 2 μl of genomic DNA was added as a template. In the nested reaction, 1 μl of the outer PCR product was added.

The cycling parameters for both the outer and nested PCR were 39 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min, followed by an additional extension cycle of 72°C for 10 min. Positive controls and negative controls were incorporated in each set of reaction.
PCR analysis
PCR products were analyzed on 3% agarose gels. The DNA was stained with ethidium bromide and visualized by UV trans-illumination (BioDoc-It UVP, Cambridge, UK). The number and size of resulting DNA bands were analyzed using Gene Ruler 50 bp DNA ladder marker (Thermo scientific, Germany).

Multiplicity of infection
The mean multiplicity of infection was estimated by dividing the total number of distinct MSP1 or MSP2 genotypes detected by the number of positive samples for the same marker.[26,27]

Statistical analysis
The R software version 2.13.1 (R foundation for Statistical Computing, Vienna, Austria) was used for all statistical analyses. For descriptive analysis, proportion was used to present the distribution of different allelic families. Comparison of means was performed using non parametric Mann-Whitney U test. Continuous variables were compared using Spearman’s rank correlation coefficient. Furthermore, linear regression (glm) was performed to test for independent association between age, parasite density, and multiplicity of infection. P values ≤ 0.05 were considered to indicate statistical significance.

Results
Detection of malaria infections and parasite densities
Of the 121 blood samples collected, 39 (32%) samples were confirmed as *P. falciparum* positive by nested-PCR. In contrast to the number of 30 microscopically positive samples, an additional 9 positive samples were detected by PCR resulting in frequency of 23% as submicroscopic parasites. One individual was positive for *P. vivax*, another was positive for *P. malariae*. Eighty samples (66%) were considered negative for all *Plasmodium* species by both PCR and microscopy. For the samples that were microscopically positive, the mean parasite density was 7302 parasites/μl of blood with the range of 165–19800. Children <10 years of age had the highest mean parasite density (10285 parasites/μl ± 8061). The mean parasite density in adults >20 years was 2663 parasites/μl (±4151) [Table 1].

Allelic diversity of *P. falciparum* MSP1 and MSP2
All 39 isolates were all successfully examined for the presence of MSP1 allelic families (MAD20, K1, and RO33) and MSP2 allelic families (3D7/IC1 and FC27). Alleles of MSP1 and MSP2 were classified according to the size of their PCR amplified fragments [Figure 1]. Both genetic markers and corresponding allelic family were very diverse [Table 2]. In MSP1, 11 different MSP1 alleles were identified by size differences on agarose gels representing MAD20 (3 alleles), K1 (4 alleles), and RO33 family (4 alleles). Allelic family RO33 was positive in 16 of the isolates (41%) and yielded four different fragments (150–250 bp). Amplification of the allelic family MAD20 was positive in 15 isolates (39%) and yielded three different bands (200–300 bp).

Table 1: Mean of *plasmodium falciparum* density (parasite/μl) and multiplicity of infection in MSP1 and MSP2 genes stratified by age group (N=39)

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>N</th>
<th>Parasite Density</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10</td>
<td>18</td>
<td>10285</td>
<td>1.93</td>
</tr>
<tr>
<td>10–20</td>
<td>9</td>
<td>7636</td>
<td>1.6</td>
</tr>
<tr>
<td>&gt;20</td>
<td>12</td>
<td>2663</td>
<td>1.6</td>
</tr>
</tbody>
</table>

N: Number of malaria cases; MSP1: Merozoite surface proteins 1
Allelic family K1 was positive in 12 isolates (31%) and produced up to four different fragments (150–300 bp). Some 15% (N = 6) of the infections carried two allelic types (MAD20/K1, RO33/K1, MAD20/RO33), whereas only three samples (7%) contained all three allelic types.

In MSP2, a total of 16 different alleles were identified [Table 2], of which 8 alleles belonged to each allelic family (3D7/IC1 or FC27). Alleles sizes ranged from (400-750 bp) for 3D7/IC1 and (200-700 bp) for FC27 allelic families. The FC27 family was more prevalent with 25 samples positive (64%) of overall detected MSP2 alleles as compared to 23 samples (59%) positive for 3D7/IC1 family. Eighteen of the isolates (46%) carried both MSP2 allelic families. A significant decrease in number of alleles with increase in age was observed for 3D7/IC1 (P = 0.05) and FC27 (P = 0.002). Parasite densities were decreased with age (correlation coefficient = −0.408, P = 0.025) [Figure 2].

### Table 2: Number of *plasmodium falciparum* alleles and base pair ranges observed per allelic family in *MSP1* and *MSP2*

<table>
<thead>
<tr>
<th>Gene/Allele</th>
<th>Number of positive samples</th>
<th>Frequency (%)</th>
<th>PCR product size</th>
<th>Number of observed alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MSP1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAD20</td>
<td>15</td>
<td>39</td>
<td>200–300 bp</td>
<td>3</td>
</tr>
<tr>
<td>K1</td>
<td>12</td>
<td>31</td>
<td>150–300 bp</td>
<td>4</td>
</tr>
<tr>
<td>RO33</td>
<td>16</td>
<td>41</td>
<td>150–250 bp</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td></td>
<td></td>
<td>11</td>
</tr>
<tr>
<td><strong>MSP2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3D7/IC1</td>
<td>23</td>
<td>59</td>
<td>400–750 bp</td>
<td>8</td>
</tr>
<tr>
<td>FC27</td>
<td>25</td>
<td>64</td>
<td>200–700 bp</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td></td>
<td></td>
<td>16</td>
</tr>
</tbody>
</table>

*For MSP1, 10 (26%) samples showed more than one type of allelic family; **For MSP2, 18 (46%) samples showed more than one type of allelic family; ***estimated sizes of alleles in base pairs (bp)*

**Association between mean multiplicity of infection, parasite density, and age**

Of the 39 positive samples, 24 (62%) harbored more than one parasite genotype identified by the presence of two or more alleles of one or both genes. The overall mean multiplicity of infection was 1.94 (CI 95% 1.1-2.76). When considering *MSP1* and *MSP2* separately, the estimated multiplicity was 1.86 (CI 95% 1.46–2.26) and 2.0 (CI 95% 1.60–2.39) for *MSP1* and *MSP2*, respectively. Thirty patients (30/39) had parasitaemia detectable in blood film. Nineteen isolates (63%) harbored multiclonal infection (mean parasite density of 9463 parasites/μl) as compared to 11 isolates (37%) with monoclonal infection (mean parasite density 4478 parasites/μl).

In *MSP1*, the mean parasite density was significantly higher (9368 parasites/μl) in patients (N = 15) carrying multiple *MSP1* genotypes as compared with patients (N = 15) with monoclonal infections (mean density of 5238 parasites/μl) (P = 0.05). Furthermore, in *MSP2*, much higher parasite density was observed in patients (N = 17) harboring multiple clonal infections as compared to patients (N = 13) with single *MSP2* genotype infections, mean parasite densities were 9463 parasites/μl versus 4478 parasites/μl, respectively (P=.025).

For the parasite densities between the different allelic families type significantly higher parasite densities were observed for *MSP2* 3D7/IC1 allelic families (P = 0.015). The relationship between mean parasite density and number of alleles was analyzed [Figure 3]. The parasite density was correlated with number of *MSP2* alleles (Spearman rank coefficient = 0.470; P = 0.009). The
correlation was less pronounced when the two genes are taken together (Spearman rank coefficient = 0.420; \(P = 0.022\)).

Parasite density and mean multiplicity of infection in \(MSP1\) and \(MSP2\) categorized by age groups is presented in Table 1.

**Discussion**

The present study provides the first report on genetic diversity and multiplicity of \(P. falciparum\) isolates in an area characterized by low and highly seasonal malaria transmission in central Sudan. Such knowledge will build up on the existing knowledge on genetic characteristics and multiplicity of \(P. falciparum\) parasite population in Sudan. Genetic diversity has been reported to play key role in the acquisition of anti-malaria parasite immunity.\(^{[6,28,29]}\) Therefore, identifying the genotypes circulating in different geographical locations would facilitate the development of effective control strategies (i.e., vaccine or drug).

The allele specific \(MSP1\) and \(MSP2\) genotyping data reveal considerable allelic diversity in \(P. falciparum\) isolates in the study area. Nonetheless, the numbers of alleles (bands) detected may have been underestimated due to sensitivity of PCR technique as minor fragments (<50 bp) would not be detected on the agarose gel. Therefore, more robust techniques such as DNA sequencing are necessary to study in depth the molecular structure of the Plasmodium parasite. The number of alleles detected for \(MSP1\) and \(MSP2\) were 11 and 16, respectively. This data is consistent with that of previous studies in low and unstable malaria transmission regions in eastern Sudan\(^{[3,30]}\) and other areas of seasonal unstable malaria in Sudan (Alyamani L.Y., M.Sc thesis, University of Khartoum, 2002) and in central Africa.\(^{[31,32]}\)

In this study, we found that the predominant allele type for \(MSP1\) was RO33. This finding differs from those of previous studies in Asar area, eastern Sudan,\(^{[4,20]}\) where MAD20 was the most predominant allelic family. This discrepancy might be due to difference in the degree of transmission intensity. Regarding \(MSP2\), the predominant allele type was FC27; this is consistent with reports from other studies in eastern Sudan.\(^{[4,30]}\)

In this study, we have reported that almost two-third of the samples (62%) harbored multiple genotypes with an overall mean multiplicity of 1.94 clones (with 1.7 and 3.3 in \(MSP1\) and \(MSP2\), respectively. These findings are compatible with some previous studies that have shown increased multiplicity in areas of low and highly seasonal transmission in Senegal\(^{[27]}\) and Iran.\(^{[3]}\)

It has been shown in areas of low malaria transmission that patent parasitaemias decrease significantly during the long dry season (November to June).\(^{[33]}\) An interesting question arising out of this is whether infected people maintain the same multiplicity during the wet season; this will help to draw a complete picture of genetic profiles in this area including seasonal variations. In the current study, parasite density was found to be high in children aged <2 years and decreased with age ≥20 years. Similar result was recorded elsewhere.\(^{[34]}\)

The association between age and multiplicity of infection is not well understood. The present study found a significant association between age and the number of parasite genotypes (\(P = 0.003\)). Similar results were reported elsewhere,\(^{[35]}\) suggesting a key role of adaptive immunity (antibody and cell-mediated immunity).

This study found significant positive correlation between parasite density and the number of parasite genotypes (multiplicity of \(MSP1\) and \(MSP2\) (\(P = 0.022\)). This finding is well in agreement with that reported by Mayengue et al.,\(^{[36]}\) suggesting that individuals are exposed to different clones.

With regards to malaria parasites detection, nested-PCR detected 23% \(P. falciparum\), which were diagnosed as negative by microscopy, confirming high sensitivity of molecular tools in detecting sub-microscopic infection.

The reported study represents the first attempt to establish molecular technique for malaria research at the Institute of Endemic Diseases, University of Khartoum. Future studies should be designed to take larger sample size, use other \(MSP 1, 2\) genotype method, and to include different transmission seasons. Further studies should analyze genetic markers related to anti-malarial drug resistance will be taken to gain insight on \(P. falciparum\) molecular epidemiology in Sudan. In conclusion, this study suggested that \(P. falciparum\) in the White Nile region, central Sudan is highly diverse and mainly consisting of multiple clones.

**Conclusion**

Genetic diversity in \(P. falciparum\) field isolates in central was high and consisted mainly of multiple clones. Significant positive correlation between parasite density and age and parasite density and number of parasite genotypes were observed.

**Acknowledgments**

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References


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