LIMITED GENETIC DIVERSITY OF *PLASMODIUM FALCIPARUM* IN FIELD ISOLATES FROM HONDURAS

DIANA HADDAD, GEORGES SNOUNOU, DENISE MATTEI, IRMA GLORIA ENAMORADO, JULIO FIGUEROA, STEFAN STÅHL AND KLAVS BERZINS

*Department of Immunology, Stockholm University, Stockholm; Department of Infection and Tropical Medicine, Imperial College School of Medicine, Northwick Park Hospital, Harrow, Middlesex, United Kingdom; Unité de Parasitologie Expérimentale, Institut Pasteur, Paris, France; Department of Microbiology, Universidad Nacional Autónoma de Honduras, Tegucigalpa, Honduras; Department of Biochemistry, Royal Institute of Technology, Stockholm, Sweden*

**Abstract.** The genetic diversity displayed by *Plasmodium falciparum* field isolates, the occurrence of variant forms of the parasite at different frequencies in different geographic areas, and the complexity of the infections represent major obstacles for the development of effective malaria control measures. However, since most of the existing studies have been performed in regions where *P. falciparum* transmission is high, little is known about the diversity and complexity of parasite populations circulating in areas of low malaria endemicity. We investigated the extent of genetic polymorphism in *P. falciparum* field isolates from Honduras, a region where its transmission is low and seasonal. Allelic diversity was analyzed in the highly polymorphic parasite genes encoding the merozoite surface proteins-1 (MSP-1) and -2 (MSP-2) and the glutamate-rich protein (GLURP) by the polymerase chain reaction. Gene polymorphism was also assessed in the EB200 region derived from the highly size polymorphic Pf332 gene. Limited size polymorphism was detected in all genes analyzed, with four and three variants for the MSP-1 and MSP-2 alleles, respectively, and two size variants for the GLURP and Pf332 genes. Moreover, based on the studied genetic markers, most infections consisted of only a few genetically distinct parasite clones. These results suggest that the *P. falciparum* parasite populations circulating in this region are genetically homogeneous and point to an association between the extent of parasite genetic diversity and the intensity of malaria transmission.

*Plasmodium falciparum* parasite populations exhibit a large diversity in areas where malaria transmission is high.\(^1\)\(^-\)\(^7\) Comparatively few studies exist, however, where the extent of genetic diversity of *P. falciparum* in regions of low endemicity was investigated, and those studies are restricted to one geographic region, namely, Sudan.\(^7\)\(^-\)\(^9\) We undertook this study on parasite diversity in Colón, Honduras, a region where *P. falciparum* transmission is low and seasonal and most malaria cases are due to *P. vivax*, which is transmitted all year round.

Extensive allelic diversity has been demonstrated for several *P. falciparum* genes by restriction fragment length polymorphism (RFLP) and the polymerase chain reaction (PCR). In this study, we used the PCR to determine the extent of genetic diversity present in *P. falciparum* isolates from Honduras by investigating two highly polymorphic genes encoding the merozoite surface proteins-1 (MSP-1) and -2 (MSP-2). The large allelic polymorphism demonstrated in the block 2 region of the MSP-1 gene or the central repetitive domain of the MSP-2 gene, which differ both in nucleotide sequence and in number of repetitive sequences, renders these markers particularly convenient for genotyping purposes.\(^10\)\(^-\)\(^11\) In addition, the RII repeat region of the glutamate-rich protein (GLURP)\(^12\) has been shown to be a good complement for the MSP sequences in distinguishing different *P. falciparum* genotypes.\(^10\) This GLURP region exhibits size polymorphism due to the presence of different numbers of repeats.\(^12\)

Furthermore, assessment of gene polymorphism in *P. falciparum* vaccine candidate antigens is a relevant consideration in the development of a malaria vaccine. One such antigen is Pf332, which is associated with the membrane of the trophozoite- and schizont-infected red blood cells and in the very late stages is exposed on the surface of the erythrocyte,\(^13\) thus becoming an accessible target for opsonizing antibodies.\(^14\)\(^-\)\(^17\) Previous studies have demonstrated a marked diversity by RFLP in the Pf332 gene from different parasite isolates.\(^3\)\(^-\)\(^18\)\(^-\)\(^19\) Within the Pf332 locus, we examined size variability of a distinct region corresponding to a 498-basepair (bp) sequence that encodes repetitive, degenerate 11-mer amino acid sequences.\(^18\)\(^-\)\(^19\) This region, denoted EB200, has only been investigated in *P. falciparum* strains adapted to squirrel monkeys where size diversity was limited to two different bands.\(^20\)

Our results show that the *P. falciparum* strains circulating in this particular region of Central America display limited diversity in the genes analyzed and suggest a correlation between the intensity of malaria transmission and the extent of genetic polymorphism.

**MATERIALS AND METHODS**

**Study area and population.** The field study was carried out in the communities of Tocoa and Trujillo, Department of Colón, approximately 400 km from Tegucigalpa. The region is located on the northeastern coast of Honduras, near the Caribbean Sea, where most malaria cases are concentrated. The climate in this region is warm and humid with temperatures ranging from 28°C to 35°C and with a rainy season that goes from May to November. Since these communities are situated in the banana- and palm-growing region of the country, most inhabitants earn their living both as plantation workers and as subsistence farmers. Health facilities are absent in the villages studied: thus, medical treatment is normally sought in the Hospital at Tocoa or Trujillo. The predominant malaria parasite is *P. vivax*, which accounts for more than 90% of all malaria infections. The mosquito vector *Anopheles darlingi* accounts for most malaria transmission in this region. Malaria prevention programs were interrupted in 1992 and no further control measures have been implemented. As a consequence, transmission of both *P. vivax* and *P. falciparum* malaria has increased sig-
nificantly (Ministerio de Salud Publica, Tegucigalpa, Honduras, unpublished data).

**Sample collection.** Sample collection was carried out during December 1995 and January 1996. Individuals presenting one or more of the following malaria symptoms: sequential chills and fever and/or sweating, were included in the study by conducting an active search in eight villages from each community. Symptomatic patients seeking malaria treatment at the Hospital of Tocoa or Trujillo were also included. The study was reviewed and approved by the Secretary of Health Services of the Ministry of Health of Honduras and the Director General of the World Health Organization Special Program for Research and Training in Tropical Diseases. Before sample collection, all participants were informed as to the aims of the study and only those who gave their consent were included. When children were involved, consent was obtained from the head of the household. A questionnaire was completed with information from each participant regarding name, age, sex, febrile status, and head of household. Approximately 2 ml of blood was obtained by venipuncture into heparinized tubes, which were preserved in a container with ice until arrival at the Hospital of Tocoa where samples were prepared for examination and shipment to Stockholm.

**Microscopic examination and sample preparation.** Upon arrival at the hospital, blood cells were separated from plasma by centrifugation. A 1-ml aliquot from each sample was added to 1 ml of transport and stabilizing medium (Qiagen, GmbH, Hilden, Germany) and stored at −20°C until shipment to Stockholm where DNA was prepared for the PCR. Before aliquoting the blood, thin and thick blood smears were made and examined by experienced microscopists. Up to 200 microscope fields were examined before establishing a diagnosis, to which results from the PCR assay were compared.

**Amplification by PCR and analysis.** The DNA was extracted from all samples by a nonorganic procedure as previously described by Kimura and others. To detect and confirm the presence of *P. falciparum* or *P. vivax* by PCR, three oligonucleotide pairs based on the genes for the small ribosomal subunit of human plasmodes were used. The sequence and position of these primers has been previously published. Polymorphic regions from the *P. falciparum* MSP-1, MSP-2, and GLURP genes were used as genetic markers for the genotyping of parasite populations. The oligonucleotide primers used to amplify these regions are derived from conserved sequences as listed in the UNDP/World Bank/WHO-TDR Malaria Sequence database. Both the sequences and positions of these primers are described elsewhere (Snounou G, unpublished data). To analyze the polymorphic block 2 of MSP-1, which is known to be present in three allelic families (MAD20, K1, and RO33), four oligonucleotide pairs were used. The central polymorphic repeat region of MSP-2, which contains two allelic families (the Indochina and the FC27 family), were analyzed with three oligonucleotide pairs. The repeat region RII of the GLURP gene was analyzed with three primer pairs. The PCRs were performed following a two-step amplification scheme where the product of the first reaction was used as a template for the second reaction. For both MSP-1 and MSP-2, the primers used in the first amplification reaction hybridized with sequences conserved among all isolates, whereas family-specific primers were used for the second amplification reaction. For the first reaction, 0.2–0.5 μl of DNA were used as template (equivalent to approximately 10 μl of blood) while for the second reaction, 1 μl of the product from the first reaction was used. Amplification of the EB200 region was carried out in a single-step reaction, as described previously.

All amplification reactions were performed in a total volume of 20 μl in a programmable thermal controller (Perkin Elmer, Norwalk, CT) following protocols previously described. The PCR-amplified gene fragments were analyzed by electrophoresis on 2% (MSP-1 and species detection) or 1.5% (MSP-2, GLURP and Pf332) agarose gels (Gibco-BRL, Gaithersburg, MD).

**RESULTS**

**Assessment of malaria infection by microscopy and PCR.** A total of 451 donors presenting malaria symptoms were included in this study. Microscopic examination of thick blood smears detected 43 (9.5%) cases of *P. falciparum*, 170 (37.7%) cases of *P. vivax*, and nine (2%) cases of mixed species infections. Parasitemias were estimated by analyzing thin blood smears to vary from less than 500 to approximately 250,000 parasites/μl of blood. A total of 152 samples were analyzed by the PCR using *P. falciparum* species-specific primers: 52 corresponding to those diagnosed microscopically as *P. falciparum* positive (43) or as mixed infections (9) and 100 to those diagnosed microscopically as either *P. vivax* (50) or as negative (50) samples. Eight additional *P. falciparum* cases were detected by the PCR in this latter group of 100 samples. However, four samples diagnosed by microscopy as *P. falciparum* positive did not yield an amplification product with *P. falciparum* species-specific primers. Fifty-six samples were *P. falciparum* positive by the PCR, 52 by microscopy, and 48 samples were *P. falciparum* positive by both microscopy and PCR.

**Plasmodium falciparum allele distribution and size polymorphism.** All 56 samples detected as *P. falciparum* positive by PCR were further genotyped to define and differentiate parasite populations. The genetic markers used (MSP-1, MSP-2, and GLURP) exist in a single copy and should, therefore, generate after amplification, only a single gene fragment per parasite genome. Amplification products from each gene region were thus analyzed by gel electrophoresis to determine size differences, as well as multiple bands, which would indicate allelic diversity and multilocional infections, respectively.

Table 1 shows the results of the allelic analyses. The predominant MSP-1 allele was of the MAD20 family, whose frequency was almost doubled that of the K1 allelic variants. Only two length variants of approximately 150 bp or 200 bp were detected for each the MAD20-family; however, for the K-1 allelic family, the most frequent size variant in both families was the 200-bp fragment. Most isolates showed either the combination of both MSP-1 allelic families or only the MAD20 allele alone, and 13 samples could not be assigned to a specific MSP-1 allelic family (Table 1). None of the isolates showed an amplification product when analyzed with RO33-specific primers. *Plasmodium falciparum* F32,
K1, and RO33 clones, which showed exclusively the MAD20, K1, and RO33 alleles, respectively, were used as controls to ensure that the PCR method was working under optimal conditions.

The MSP-2 allelic typing showed that the predominant allele was of the FC27 family, in which only two length variants of 400 bp and 450 bp were detected. The Indochina allelic family, detected in a limited number of the samples, presented only one band of (approximately 500 bp) identical size. Comparatively less isolates had both MSP-2 alleles (FC27 and Indochina) than combined MSP-1 alleles, since most samples presented the FC27 or Indochina allele alone. Moreover, 23 samples could not be assigned to a specific MSP-2 allelic family (Table 1). The FC27 and Indochina clones were used accordingly as controls for amplification of the MSP-2 region.

When primers for the RII repeat region of the GLURP gene were used, all 56 isolates yielded an amplification product and size polymorphism that was limited to two variants (Table 1).

The presence of two size variants from the same families (MAD20, K1, FC27, or GLURP) was never detected in all 56 isolates (Table 1), but the RO33 type was present in most of the symptomatic individuals analyzed. Recently, however, RO33 parasite prevalence was found to be greater in high parasitemia samples, regardless of clinical symptoms, thereby suggesting an underestimation of RO33 representation in low-parasitemia samples. With regard to the MSP-2 locus, which also presented limited size polymorphism, the FC27 allele appears to be better represented than the Indochina family in this Honduran region. Likewise, the GLURP locus, which was detected in all 56 P. falciparum isolates examined, also displayed marked size homogeneity. In comparison, wild isolates from other areas with low transmission or high transmission present a high prevalence and a marked degree of length polymorphism for the FC27, Indochina, and GLURP alleles.

Genetic variability of the EB200 region was also examined because of our interest in the Pf332 antigen as a vaccine candidate. Size polymorphism of this gene fragment was also limited to two variants in the Honduran isolates, despite the marked RFLP displayed by the Pf332 gene in different parasite strains. Nevertheless, because the methods of analysis, RFLP and PCR amplification, are different and since the EB200 sequence has not yet been investigated in isolates

Table 1: Distribution of Plasmodium falciparum genotypes in 56 isolates from symptomatic individuals living in Colón, Honduras as determined by the polymerase chain reaction

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allelic family</th>
<th>Allelic size (bp)</th>
<th>Preva- lence (n)</th>
<th>Genotypes</th>
<th>Preva- lence (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP-1</td>
<td>MAD 20</td>
<td>150/200</td>
<td>41</td>
<td>MAD20/K1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>K1</td>
<td>150/200</td>
<td>26</td>
<td>MAD20/K1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>RO33</td>
<td>– 150/200</td>
<td>5</td>
<td>MAD20/K1</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MAD20/RO</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MAD20/RO</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K1/RO</td>
<td>13</td>
</tr>
<tr>
<td>MSP-2</td>
<td>FC27</td>
<td>400/450</td>
<td>29</td>
<td>FC27/INDO</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>INDO</td>
<td>500</td>
<td>12</td>
<td>FC27/INDO</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FC27/INDO</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FC27/INDO</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>INDO/RO</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>GLURP</td>
<td>675/700</td>
<td>56</td>
<td>GLURP/RO</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GLURP/RO</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Pf332</td>
<td>450/500</td>
<td>36</td>
<td>EB200/RO</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>EB200/RO</td>
<td>25</td>
</tr>
</tbody>
</table>

* bp = basepairs; MSP-1 = merozoite surface protein-1; – = not found; GLURP = glutamate-rich protein.

Knowledge of the nature and extent of genetic diversity within P. falciparum is essential in understanding the mechanisms underlying the pathology of malaria, the acquisition of immunity, the spread of drug resistance, and the conditions of transmission. The genetic complexity of the parasite has been well established by demonstrating the occurrence of geographic variation within the species, and of multiple infections with more than one genetically distinct parasite. 1,4,10,23–27

Wild isolates from highly endemic areas in Brazil, Senegal, Thailand, or Papua New Guinea show extensive allelic polymorphism in the MSP-1, MSP-2, or GLURP genes. Moreover, the high complexity and frequency of the infections found there are presumably associated with the transmission characteristics of those regions.

In this study, we investigated the extent of genetic diversity exhibited by P. falciparum isolates from a region in Honduras where transmission is low and seasonal. The limited number of variant MSP-1, MSP-2, or GLURP allelic forms displayed by the isolates, as well as the few parasite genotypes found in each individual, is in sharp contrast with the extensive allelic diversity found in wild isolates from highly endemic areas. 1,4,10,23–27 Interestingly, there is a marked shift in the distribution of the MAD20 and K1-like variants in the Honduran isolates compared with the distribution patterns of those alleles in isolates from other parts of the world where malaria transmission is higher. 1,4,24,27 In those studies, the K1 allele was reported to be more prevalent than the MAD20 allele, although both alleles exhibited large length polymorphism. However, the MSP-1 distribution patterns observed in the Honduran isolates are seemingly similar to those found in a mesoendemic village in Sudan and in a number of laboratory strains. 29 Also noteworthy is that the MSP-1 sequence variant RO33, whose presence has been associated with severity of the disease, 24 was not present in any of the samples investigated here. This is in sharp contrast with the findings from Brazil and Senegal, 4,23 where the RO33 type was present in most of the symptomatic individuals analyzed. Recently, however, RO33 parasite prevalence was found to be greater in high parasitemia samples, regardless of clinical symptoms, thereby suggesting an underestimation of RO33 representation in low-parasitemia samples. With regard to the MSP-2 locus, which also presented limited size polymorphism, the FC27 allele appears to be better represented than the Indochina family in this Honduran region. Likewise, the GLURP locus, which was detected in all 56 P. falciparum isolates examined, also displayed marked size homogeneity. In comparison, wild isolates from other areas with low transmission or high transmission present a high prevalence and a marked degree of length polymorphism for the FC27, Indochina, and GLURP alleles.
from regions with different levels of malaria transmission, it is unclear whether our data truly reflect limited Pf332 polymorphism in the Honduran parasites.

To conclude, recent studies suggest that the genetic structure of malaria parasite populations, the magnitude of the parasite load in infected individuals, and the rate of sexual reproduction in the mosquito gut vary according to the transmission characteristics of the region. According to this study, the low transmission of P. falciparum in Honduras may imply limited gamete cross-fertilization and consequently, infrequent emergence of variant alleles. Nevertheless, the relatively small number of samples analyzed and the fact that they were obtained from only symptomatic patients in a single cross-sectional survey, as well as the low P. falciparum parasite load in infected individuals, should also be considered as a plausible explanation for the parasite homogenicity observed. A larger number of longitudinal samples would be valuable to consider in future studies. Additional comparative field studies in other regions with similar epidemiologic factors responsible for the limited allelic diversity observed in this study.

Acknowledgments: We are grateful to the villagers of Tocoa and Trujillo for their participation in this study. We particularly appreciate the support of the Vector Control Unit of the Honduran Ministry of Health and the Section of Infectious Diseases of the Hospitals of Tocoa and Trujillo. We acknowledge the help and cooperation received from Drs. Jorge Haddad and Carlos Ponce from the Pan American Health Organization in Honduras during the planning and execution of the field study.

Financial support: This work was supported by grants from the World Health Organization Special Program for Research and Training in Tropical Diseases and the Swedish Medical Research Council.

Authors’ addresses: Diana Haddad and Klavs Berzins, Department of Immunology, Stockholm University, S-10691 Stockholm, Sweden. Georges Snounou, Department of Infection and Tropical Medicine, Imperial College of Medicine, Northwick Park Hospital, Harrow, Middlesex, HA1 3UJ, United Kingdom. Denise Mattei, Unité de Parasitologie Experimentale, Institut Pasteur, 25, Rue du Docteur Roux, 75724 Paris, Cedex 15, France. Irma Gloria Enamorado and Pedro Miguel Ramirez, Instituto de Biología, Universidad Autónoma de Honduras, Blvd. Suyapa, Tegucigalpa, Honduras. Steen Høj, Department of Biochemistry and Biotechnology, Royal Institute of Technology, Teknikringen 30 S-10044 Stockholm, Sweden.

Reprint requests: Klavs Berzins, Department of Immunology, Stockholm University, S10691 Stockholm, Sweden.

REFERENCES


