Natural polymorphism in the thrombospondin-related adhesive protein of Plasmodium falciparum

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NATURAL POLYMORPHISM IN THE THROMBOSPONDIN-RELATED ADHESIVE PROTEIN OF *PLASMODIUM FALCIPARUM*

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**Abstract.** We have developed a typing system using natural sequence variation in the thrombospordin-related adhesive protein (TRAP) gene of *Plasmodium falciparum*. This method permits a haplotype to be assigned to any particular TRAP gene. We have applied this method to a hospital-based, case-control study in Mali. Previous sequence variation and conservation in TRAP has been confirmed. Particular TRAP haplotypes can be used as geographic hallmarks. Because of the high level of conflict between characters, we have examined the phylogenetic relationships between parasites using a network approach. Having received patient samples from urban and periurban areas of Bamako, the majority of haplotypes were closely related and distinct from TRAP sequences present in other continents. This suggests that the structure of TRAP can only tolerate a limited number of sequence variations to preserve its function but that this is sufficient to allow the parasite to evade the host’s immune system until a long-lived immune response can be maintained. It may also reflect host genetics in that certain variants may escape the host immune response more efficiently than others. For vaccine design, sequences from the major regional variants may need to be considered in the production of effective subunit vaccines.

The thrombospordin-related adhesive protein (TRAP) gene of *Plasmodium falciparum* was first described in 1988. The gene derives its name from the deduced protein sequence that contains two amino acid motifs found in the adhesive host glycoprotein thrombospordin. One of these motifs is based on the amino acid sequence Glu-Trp-Ser-Pro-Cys-Ser-Val-Thr-Cys-Gly-Lys-Gly-Thr-Arg-Ser-Arg-Lys-Arg, which has been demonstrated to allow TRAP to bind to sulfatide. The protein is present in sporozoites and erythrocytic-stage parasites. Antibodies against TRAP inhibit sporozoite invasion. Recent data demonstrate that TRAP is expressed in mature, salivary gland sporozoites but not in hemocoeel sporozoites, suggesting that TRAP may be one of the unidentified accessory molecules required for infection of the vertebrate host.

Data from a longitudinal and prospective study showed that the immune response to TRAP is long-lived in adults and older children but short-lived in young children. There were seasonal fluctuations of the levels of specific antibodies, as well as age-dependent quantitative differences. These data, together with that for the TRAP homolog sporozoite surface protein 2 (SSP2) from *P. yoelii*, suggesting that adoptive transfer of a CD8+ cytotoxic T cell clone recognizing SSP2 gave complete protection against *P. yoelii* infection, suggest that TRAP would be an important component of a multi-subunit malaria vaccine. Further evidence for this has been the identification of a cytotoxic T lymphocyte response to certain conserved epitopes in TRAP.

A major problem in the design of any malaria vaccine is which antigens to choose and which variants of these antigens are relevant. We have previously shown that TRAP is polymorphic and that all the nucleotide changes occurring outside what appeared to be a conserved core, containing the sulfatide-binding motif, resulted in amino acid changes. The significance of this high degree of sequence variation is not known but it does suggest that host immune response may be a factor since the sequence of TRAP genes from parasites in culture appear to be stable. The parasites in this earlier study were from different geographic locations, apart from the three that were from children in the Gambia. The three Gambian sequences were closely related to each other, but nevertheless different. The most closely related gene sequences were from the parasite clones Dd2 (Indochina) and HB3A (Honduras). This report describes the genetic variation of the TRAP gene in Mali from samples collected during the 1992 malaria season from children (age range = 1–12 years) with mild and severe malaria.

**Patients, Materials, and Methods**

**Study area and population.** The study was carried out in Bamako, the principal city of Mali. All patients were recruited in the pediatric ward of the Gabriel Toure Hospital, which is located in the center of the city. An average of 3,200 children are admitted annually to this ward. Severe malaria cases account for 8% of all children admitted to this hospital; 23% of these cases prove fatal. The age range of the children in the study was 1–12 years. Blood samples were collected from September to November 1992. Malaria is seasonal in Mali, and the rainy season is from June to October, which corresponds to the period of maximal incidence of severe malaria in the study area. Malaria inoculation rates range one to 10 per person per year in urban areas, and from 50 and 200 in the surrounding rural villages.

*Anopheles gambiæ*, *A. arabiensis*, and *A. funestus* are the main malaria vectors in this part of Africa. The great majority of the population belongs to the Bambara ethnic group, with Fulani and Senoufu being in the minority. Parental consent was obtained for participation of all the children in the study, which was approved by the National Ethical Committee of the Ministry of Health of Mali. The modified Glasgow coma scale to be applicative to children, including those who have not learned to speak, was used in assessing severe malaria. Children having a coma score between 0 and
FIGURE 1. Schematic representation of the structure of the thrombospondin-related protein (TRAP) gene of Plasmodium falciparum and the strategy for the different polymerase chain reactions. The sequences of the oligonucleotide primers are given at the bottom of the Figure. TRAP1 and TRAP2 contain Bam HI sites at their 5' ends, TRAP1 corresponds to nucleotides (306-326), and TRAP2 to nucleotides (1979-2000) in the T9/96 sequence. The relative positions of the polymorphic restriction enzyme sites are shown. RGD = Arg-Gly-Asp.

2 were comatose. Children having a positive blood smear for P. falciparum, a body temperature greater than 37.5°C, and showing no signs of impaired consciousness were considered to have mild malaria. Children with patent bacterial infection and/or a history of repeated convulsions were excluded from the study. All children admitted to the study were treated according to World Health Organization (WHO) guidelines using drugs that were available free of charge as part of the study.

Blood samples and DNA collection. On entering the study, fingerpricks from each child were taken for thick and thin blood films and for DNA. The microscopic diagnosis of malaria was based on examination of 100 high-power fields of the thick film after staining with 3% Giemsa. Parasite counts were calculated according to the WHO method, assuming 6,000 leukocytes/µl. Every child was allocated a sheet of Whatman (Maidstone, United Kingdom) 3MM paper (13 cm × 10 cm) on which the blood spots were stored prior to DNA extraction or hemoglobin isoelectric focusing. The name, age, code number, and date of collection were recorded in pencil on the appropriate sheet of Whatman 3MM paper. After collection, the blood spots were allowed to dry before the sheets of paper were transferred into individual resealable polythene bags for shipping and long-term storage at ambient temperature.

The DNA was extracted from the paper as previously described. Briefly, a small portion of blood spot (approximately 3 mm × 3 mm) was soaked for 10–20 min at room temperature in a 1.5-ml microfuge tube containing 1 ml of phosphate-buffered saline (PBS)/1% saponin (PBS = 0.14 NaCl, 0.01 M sodium phosphate, pH 7.2). The PBS/1% saponin was discarded and the DNA was extracted from the paper by the addition of 150 µl of water and 50 µl of Chel-ex-100 (Bio-Rad Laboratories, Hercules, CA) (made up to 20% [w/v] in water and adjusted to pH 9.5 with 1 M NaOH), followed by boiling for 8 min. The Chellex was removed by centrifugation and the supernatant was transferred to another microfuge tube. The DNA was then ready to use and was stored at −20°C prior to amplification.

Polymerase chain reaction (PCR) amplification. The sequences and relative positions of the primers are shown in Figure 1. All primers were used at a concentration of 100 ng/µl. Amplification was carried out using a programmable thermocycler (Omnigene; Hybaid, Teddington, United Kingdom).

Amplification was performed in two stages. The first round, in a final volume of 25 µl, contained 5 µl DNA, 200 µM dNTPs, 1× Taq polymerase buffer containing 1.5 mM MgCl₂ and 1 unit of Ampli-taq (Cetus, Branchburg, NJ). The primers were TRAP1 and TRAP2. The cycling conditions were as follows: two cycles of 1 min at 93°C, 1 min at 37°C, and 2 min at 72°C, followed by 35 cycles of 1 min at 93°C, 1 min at 55°C, and 2 min at 72°C, with a final cycle of 1 min at 93°C, 1 min at 55°C, and 10 min at 72°C. The reaction was then diluted to 250 µl with sterile water and stored at −20°C until further use. If DNA had been purified from
liquid blood as in the Gambian study, only a single round of the PCR was found to be necessary. When using DNA purified from blood or cultured parasites, we use primers TRAP1A and TRAP2A to obtain the diagnostic fragment sizes shown in Table 1.

For restriction fragment length polymorphism (RFLP) analysis, a second round of PCR was performed using the primers TRAP1A and TRAP2A (Figure 1). Amplification was performed as before but using 2 μl of diluted DNA from the first round of amplification in a final volume of 100 μl. Cycling conditions were as for the first round except that the initial two cycles with annealing at 37°C were omitted.

For repeat analysis, the second round of the PCR was performed using primers TRAP17 and TRAP8 (Figure 1). Amplification was performed as before using 1 μl of diluted DNA from the first round of amplification in a final volume of 20 μl containing 2 μCi of 35S-dATP (Amersham, Little Chalfont, United Kingdom). Cycling conditions were as for the RFLP second round except that the annealing temperature was decreased to 45°C.

Certain DNA samples proved refractory to PCR amplification under these conditions. First and second round amplifications were repeated using Taq extender (Stratagene, Cambridge, United Kingdom) using the conditions recommended by the manufacturer. The extension times were extended to 4 min for the first round PCR and the second round RFLP PCR. This modification permitted the majority of the remaining DNA samples to be amplified.

Amplification of merozoite surface protein 1 (MSP-1) by the PCR was performed as previously described.

**Analysis by RFLP.** The product from the second round PCR amplification was digested with the restriction enzymes Bgl II, Ssp I, Taq I, and Afl II following the manufacturer’s (New England Biolabs, Beverly, MA) instructions. To aid the Afl II analysis, the enzyme Asp 718 (Boehringer Mannheim, Lewes, United Kingdom) was added to those digests. The Bgl II and Ssp I digests were resolved on 1.7% agarose gels. The Taq I and Afl II/Asp 718 digests were resolved using 1% agarose 1% NuSieve gels (FMC Bioproducts, Rockland, ME). The sizes of the various restriction fragments (Table 1) were used to define particular haplotypes. The presence of a particular restriction enzyme site is represented by + and the absence by −. Each combination of + and − was assigned a number. It should be noted that the 3′ Bgl II and Ssp I sites are mutually exclusive. Haplotype 1 is defined as Bgl II ++, Ssp I −−, Taq I +, Afl II ++ and so in abbreviated form haplotype 1 becomes (++++++). Further details can be obtained by email (krobson@hammer.imm.ox.ac.uk) or fax (44-1865-222-500) from the corresponding author (KJHR).

**Repeat polymorphism analysis.** The samples for repeat analysis were precipitated with ethanol. The DNA pellets were washed with ethanol, air-dried, and resuspended in 3 μl of formamide dyes (80% deionized formamide, 1× glycerol-tolerant gel buffer [0.089 M Tris, 0.0288 M taurine, 5.4 mM disodium EDTA], 0.1% [w/v] bromphenol blue, 0.1% xylene cyanol FF). The samples were heated at 100°C prior to loading on 5% Long Ranger® (Flowgen, Rockland, ME) gels containing 1× glycerol-tolerant gel buffer and 7 M urea. Gels (31 cm × 38.5 cm × 0.4 mm) were electrophoresed at 60 W until the xylene cyanol FF had migrated to within 10 cm of the bottom of the gel. The M13mp18 sequencing ladders were used as size markers on every gel. The PCR product (TRAP17-TRAP8) from the laboratory line T9/96 was used as an additional control on all gels, the product size being 235 basepairs (bp). Gels were allowed to cool completely before drying and exposure to x-ray film for 1–7 days.

**Sequencing of DNA.** Direct sequencing of DNA products was performed using a Sequenase PCR product sequencing kit (U.S. Biochemicals, Cleveland, OH). Primers internal to the PCR primers gave optimal sequencing results.

**Experimental design and statistical analysis.** Eighty-five children with cerebral malaria were recruited during the 1992 malaria season. Each of these children was then age matched with three children with mild malaria matched for age and place of residence (locality). A total of 337 samples were analyzed. Yates’ chi-square test (or Fisher’s exact probability test when needed) was used for the analysis of prevalence rates, and the Kruskall-Wallis test was used for the analysis of age, parasite counts, and antibody levels.

**Phylogenetic analysis.** Networks were constructed sequentially by splitting the data, restriction enzyme site by restriction enzyme site. This was performed manually. Splits are represented by branches between haplotypes, each of which represents a change in a restriction enzyme site. Each haplotype was represented as a circle containing the haplotype number. The haplotypes were simplified to the number only, with all versions with repeats being pooled.

**Hemoglobin isoelectric focusing.** Isoelectric focusing was performed using the Resolve System Hemoglobin kit (Isolab, Mechelen, Belgium). Hemoglobin (Hb) for analysis was eluted from a single dry blood spot from the same sheet of Whatman 3MM paper used for parasite DNA extraction. Samples were analyzed for the presence of Hb A, Hb S, and Hb C.

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**Table 1**

RFLP analysis of thrombospondin-related adhesive protein

<table>
<thead>
<tr>
<th>Restriction enzyme site</th>
<th>Presence/Absence</th>
<th>Restriction fragment lengths size (bp) and order</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bgl II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ +</td>
<td>129, 1,509†</td>
<td></td>
</tr>
<tr>
<td>+ +</td>
<td>129, 1,090†, 419</td>
<td></td>
</tr>
<tr>
<td>− −</td>
<td>1,219†, 419</td>
<td></td>
</tr>
<tr>
<td>− −</td>
<td>1,638†</td>
<td></td>
</tr>
<tr>
<td><strong>Afl II/Asp 718</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ +</td>
<td>351, 415, 872†</td>
<td></td>
</tr>
<tr>
<td>+ +</td>
<td>351, 33, 382, 872†</td>
<td></td>
</tr>
<tr>
<td>− +</td>
<td>384, 382, 872†</td>
<td></td>
</tr>
<tr>
<td>− −</td>
<td>766, 872</td>
<td></td>
</tr>
<tr>
<td><strong>Ssp I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ +</td>
<td>13, 248, 1,377†</td>
<td></td>
</tr>
<tr>
<td>+ +</td>
<td>13, 248, 963†, 414</td>
<td></td>
</tr>
<tr>
<td>− +</td>
<td>13, 1,211†, 414</td>
<td></td>
</tr>
<tr>
<td>− −</td>
<td>13, 1,625†</td>
<td></td>
</tr>
<tr>
<td><strong>Taq I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ +</td>
<td>926, 211†, 501</td>
<td></td>
</tr>
<tr>
<td>− −</td>
<td>1,137†, 501</td>
<td></td>
</tr>
</tbody>
</table>

* For BglII, + + indicates that the 5′ BglII site is present and that the 3′ site is absent. The same rules apply in defining the presence or absence of the other restriction enzyme sites. Details can be obtained by email or fax from the corresponding author (KJHR).
† These fragments can vary in size since they contain the region with repeats.
RESULTS

Sequence analysis of a small region (200 bp) of the TRAP gene from six isolates from Mali showed that we needed an approach that would allow us to group related parasites based on their TRAP gene sequence and that would save on the expense and workload associated with sequencing. We therefore chose to perform a haplotype analysis on the gene, examining what we believed to be four polymorphic restriction enzyme sites throughout the gene. Nucleotide changes at these polymorphic restriction enzyme sites all result in amino acid changes. The restriction enzymes chosen were Bgl II, Ssp I, Taq I, and Afl II.

Polymorphisms associated with the enzyme Bgl II. Our earlier detailed sequence analysis showed that there were two Bgl II sites in the TRAP gene (Figure 1).11 The first, at the 5′ end of the gene, appeared not to be polymorphic, whereas the second, towards the 3′ end of the gene, clearly was. Analysis of these samples from Mali showed that both 5′ and 3′ sites were polymorphic. Direct sequencing across the region containing the 5′ Bgl II site in samples that appeared to lack the site demonstrated that there was a nucleotide change resulting in the amino acid change Leu99 to Val99, which corresponds to C145G in the DNA sequence. This Bgl II site was present in 96.4% of the chromosomes analyzed.

The 3′ Bgl II site and what we describe as the 3′ Ssp I site are mutually exclusive. The presence of the 3′ Bgl II site was detected only in 0.6% of our sample chromosomes, whereas the Ssp I site was much more common (37.2%). The most common situation was the intermediate nucleotide sequence in which neither site was present (62.2%).

Polymorphisms associated with the enzyme Ssp I. In our study of the TRAP gene from different laboratory isolates and clones we found that there was a polymorphic 5′ Ssp I site associated with DNA encoding amino acids 90, 91, and 92. The original sequence for the TRAP gene was from the Thai clone T9/96 in which the DNA encodes the amino acid motif Ala90 Asn91 Ile92 and Leu90 Asn91 Ile92, which corresponds to C145G in the DNA sequence. This Ssp I site was present in 66.7% of our sample chromosomes.

Restriction enzyme analysis of the Taq I site. The Taq I site associated with DNA encoding amino acids 90, 91, and 92. The original sequence for the TRAP gene was from the Thai clone T9/96 in which the DNA encodes the amino acid motif Ala90 Asn91 Ile92 and Leu99 to Val99, which corresponds to C145G in the DNA sequence. This Bgl II site was present in 96.4% of the chromosomes analyzed.

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Polymorphisms associated with the enzyme Taq I. A different history appears to be associated with the Taq I site. If the site is absent, the amino acid motif encoded by the DNA sequence is Ser91 Ser92 Val93 Gln94; if the site is present, then the amino acid motif is Phe91 Ala92 Val93 Glu94. These sequences differ by three nucleotides. The Taq site is absent in laboratory lines T9/96 and 7G8 but is present in all the others sequenced to date. The presence of the Taq I site appears to be in linkage disequilibrium with the Phe91 Ala92 Val93 Gln94 motif since no intermediary sequences have been seen. This cannot be said for the sequence changes associated with the 5′ Ssp I site. The Taq I site was present in 66.7% of our sample chromosomes.

These data suggest that either this mutation series of mutations may have arisen independently in different continents or else is a particularly ancient mutation that has been found in samples from Thailand, Mali, and Brazil. It is interesting to note that this series of amino acid changes follows the Arg90 Gly90 Asp90 motif.

Polymorphisms associated with the enzyme Afl II. There are two Afl II sites in the gene that are 33 bp apart. Loss of the second of these is associated with a change at amino acid position 134 of Ser to Thr. The first was not known to be polymorphic until we undertook this study. Direct sequencing across the region demonstrated that loss of the 5′ site is associated with a Ser to Asn change at amino acid position 123 and corresponds to the nucleotide change G367A. All four combinations were observed in our survey. The frequencies were as follows: ++ = 6.2%, +− = 49.6%, −− = 18.1%, and −+ = 26.1%.

Haplotype analysis. Restriction enzyme analysis of the PCR-amplified TRAP gene permitted us to assign the gene to one of 96 possible haplotypes. It also allowed us to see whether mixed infections were present. It was not possible to get a complete result in all cases. Some samples (17 of 337) appeared refractory to PCR analysis regardless of whether human or parasite primers were tried. These samples were excluded from our analysis. In other cases in which more than two haplotypes were present, we could not always assign haplotypes to particular parasite chromosomes. In certain other samples, we were unable to generate data for all the restriction sites analyzed. We did not discard these data because they were still useful in assigning frequencies to particular RFLPs. We were able to assign numbered haplotypes to 319 chromosomes. Of the 96 possible numbered haplotypes, we observed 20. The results are given in Table 2.

Further analysis was possible due to our earlier observations that the TRAP gene has a small repeating three amino acid motif inserted after amino acid 352. The PCR amplification across this region permitted a more detailed analysis of the parasites in our survey. Values varying from no extra repeats up to 16 were observed (Table 2). In many instances, these could be assigned to certain haplotypes (Table 2). We never observed a chromosome with either 11 or 13 extra repeat units. It should be noted that TRAP genes with repeats of more than 10 extra units were rarely observed (Table 2). In certain cases of mixed infections, we could not assign a repeat value to a given haplotype number; these have unassigned repeat numbers (Table 2). The repeat analysis proved very useful in analyzing mixed infections. The ability to leave the gels analyzing repeat values exposed to the x-ray film for several days permitted us to see mixtures that were not apparent using staining with ethidium bromide. Using repeat analysis alone we were able to examine the proportion of parasites present in mixed infections. Applying
this approach, 180 children had single infections, 80 had two, 17 had three, three had four, two had five, and one had six infections. Combining the repeat analysis with the haplotype information, we were able to assign complete TRAP haplotypes for 242 chromosomes. We observed 74 of 1,440 of the possible combinations based on repeat and RFLP analysis (Table 2).

The degree of observed variation in the TRAP gene precluded the numbers necessary to see whether parasites present at the start of our study period were absent at the end. It should be noted that the three TRAP genes sequenced from children in the Gambia with malaria infections in 1988 used the nomenclature 42B, 42C, and 42D. Haplotype 42 was the most common in our survey. The numbers present also precluded any correlation with age, hemoglobin status, or disease severity.

The haplotypes of certain, well-known, laboratory lines that are from different geographic locations are given in Table 3. With the exception of haplotype 30, we did not observe any of the haplotypes of other known laboratory lines of non-African origin. Line FCR3, which has been thought to have been of African origin, is haplotype 1, as is K1, a well-known Thai strain. We did not observe haplotype 1 in our study. Line FCR3 differs from haplotype 42 by a minimum of three and from haplotype 3 (we observed haplotype 3 once in our study) by a minimum of three and from haplotype 3 (we observed haplotype 3 once in our study) by a minimum of one restriction enzyme site, respectively. We have previously seen haplotype 30 in Thailand (T9/96); this may be a more global haplotype. It is interesting to note that one of the ways haplotype 30 differs from haplotype 42 is by the Taq I site, which differentiates the two sequences around the Arg-Gly-Asp motif. Both T9/96 and 7G8 (Brazilian) lack the Taq I site next to the Arg-Gly-Asp motif. Changes in this motif may reflect polymorphisms in the mosquito or human host. These data suggest that such a haplotyping system reflects the geographic differences between \textit{P. falciparum} parasites.

**Phylogenetic analysis.** The network for the haplotypes in the TRAP gene is shown in Figure 2. Although networks are unrooted (having no obvious common ancestor), the neutral theory of molecular evolution implies that all else being equal, the more common haplotypes are likely to be older. With the immune pressure on this malaria gene, this may not be true.

**DISCUSSION**

Unlike other malaria vaccine candidates, TRAP has not been studied in detail in naturally occurring infections. If TRAP is to be considered seriously as a component of a subunit vaccine, we need to understand the basis of natural sequence variation so that this information is reflected in the

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**Table 2**

Summary of the observed thrombospondin-related adhesive protein haplotypes in Mali*

| Haplotype number | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | Unassigned | Total |
|------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|-------------|-------|
| 3                | 1 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 1           |
| 25               | 2 | 1 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 2           |
| 26               | 8 | 8 | 4 | 2 | 2 | 1 | 2 | 1 |   |   |   |   |   |   |   |   |   |   | 12          |
| 27               | 8 | 2 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 5           |
| 28               | 2 | 8 | 1 | 1 | 1 |   |   |   |   |   |   |   |   |   |   |   |   |   | 4           |
| 29               | 1 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 1           |
| 30               | 10| 5 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 6           |
| 31               | 1 | 1 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 2           |
| 32               | 3 | 2 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 2           |
| 41               | 3 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 1           |
| 42               | 13| 10| 10| 2 | 2 | 2 | 1 | 3 | 1 | 3 |   |   |   |   |   |   | 14          |
| 43               | 4 | 6 | 4 |   |   |   |   |   |   | 1 | 1 | 1 |   |   |   |   |   | 6           |
| 44               | 2 | 5 | 3 | 8 | 2 | 1 |   |   |   |   |   |   |   |   |   |   | 7           |
| 45               | 1 | 3 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 2           |
| 46               | 6 | 8 |   |   |   |   |   |   |   |   | 2 |   |   |   |   |   |   | 8           |
| 47               | 10| 3 | 3 | 1 |   |   |   |   |   |   |   |   |   |   |   |   | 1           |
| 48               | 1 | 3 | 2 |   |   |   |   |   |   |   |   |   |   |   |   |   | 3           |
| 74               | 1 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 1           |
| 75               | 1 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 1           |
| 90               | 1 | 1 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | 1           |
| Total            | 73| 70| 24| 6 | 38| 3 | 11| 4 | 4 | 1 | 5 | 1 |   |   |   |   | 77          |
| Frequency of extra repeat units | 118|111|42|22|67|5 | 26|8 |13|5 | 5 |0 |1 |0 |2 |3 |1 |419 |

*The haplotype number down the left hand side refers to the combination of restriction enzyme site. Haplotype 42 is +++++++. The row of letters across the top is the code applied to the number of repeating units. A being the baseline found in T9/96. Along the very bottom of the table is the frequency of the extra repeat units including those where it was not possible to assign a complete haplotype.

**Table 3**

Thrombospondin-related adhesive protein haplotypes of common laboratory clones/lines of \textit{Plasmodium falciparum}
sequences incorporated into such a vaccine. In this study, we have extended our earlier work on sequence variation, examining material from a case-control study in Mali. From our earlier work, there was a suggestion that certain regions of the molecule could tolerate point mutations and insertions whereas others could not. We have detected two new RFLPs that corresponded to amino acid changes that have increased the power of our analyses. We have applied these observations to develop a PCR-based haplotyping system for TRAP that not only examines specific amino acid changes but also detects changes in the size of the small repeat region. This combinatorial approach allowed us potentially to identify up to 1,440 TRAP haplotypes. This method could easily be extended by examining more polymorphic restriction enzyme sites, e.g., Fok I. The restriction enzyme sites detected nucleotide changes that correlated with amino acid changes. We were able to assign complete haplotypes to 242 chromosomes and in doing so detected 74 of 1,440 possible haplotypes. Limited DNA sequence analysis suggests that this is an underestimate of the sequences in circulation. Together our data suggest that the molecule is under significant immunologic pressure. This may be due to a combination of factors including sporozoite exposure and the expression of TRAP both by mature sporozoites and by parasite infected red blood cells.

Using the numbered haplotypes, number 42 predominated. This haplotype had been observed in our earlier study using samples from The Gambia taken in 1988. Clones 3D7A and 7901, parasites also of African origin, are haplotype 42 by this system, but are known to differ both from each other and the isolates in this study at other positions in TRAP. The next most common haplotypes differed by one or two restriction enzyme sites from haplotype 42. Haplotype 3, at the other end of the spectrum, was observed only once and on its own. It differs from haplotype 42 at four restriction enzyme sites. It was found in a 12-year-old child with mild malaria living in an urban area. Where mixed infections of *P. falciparum* were present and haplotypes could be completely assigned, haplotype 42 was present with closely related haplotypes, and no particular combination was seen more frequently. This is in part due to the large number of haplotypes that were present in the study area, and therefore it was difficult to obtain statistically significant results for many of the observations. No particular TRAP haplotype or polymorphism was associated with parasites from children who died of severe malaria.

The system was used to analyze mixed infections in children with mild and severe malaria. It gives a minimum estimate of the number of parasites present in any mixture. When a TRAP haplotype was used as a marker to define mixed infections of *P. falciparum*, 49.3% of the children with severe malaria and 51.1% of the children with mild malaria had mixed infections of *P. falciparum*. The number of TRAP haplotypes present varied from one to six when
the repeat polymorphism was used on its own. Studies from Thailand, 20, 21 where parasites were cloned from particular patients, found six and six clones, respectively. Using monoclonal antibodies against three polymorphic blood-stage antigens, Conway and others were able to detect up to four clones per isolate. 22 They found a mean of two P. falciparum clones in samples from patients with malaria in The Gambia. We were therefore not surprised to see this range of mixed infections of P. falciparum in children from Mali, another West African country. It is interesting to note that we were using a genetic marker that is predominantly associated with the sporozoite, whereas these other studies were looking at markers whose main biological activity was in blood-stage infections. These blood-stage proteins such as MSP-1 are much more subject to selective pressures by the host than a sporozoite protein such as TRAP.

Repeat length appears to be independent of TRAP haplotype. The significance of the small tandem repeat in TRAP is not understood. It occurs in the part of the molecule where significant repeating motifs are found in SSP2, 23, 24 P. berghei TRAP, 25 and TRAPs from P. vivax, P. knowlesi, and P. gallinaceum, 26 and is also the region where the TRAP molecules differ most from each other. Dual mixed infections based on TRAP analysis tended to be due to related parasite haplotypes differing either by repeat length or one restriction enzyme site. The high degree of sequence polymorphism precluded statistical analysis for this observation.

We have analyzed the data from the case-control study looking at levels of parasitemia, disease severity, sickle cell status, age, month of infection, and geographic location (urban, periurban, and rural) with relation to TRAP polymorphisms/haplotype. For statistical purposes, haplotypes were simplified and analyzed either for repeat letter or haplotype number. The sample size, together with the number of haplotypes and the number of comparisons, made were such that no statistically significant results were obtained. A further study would be required to increase the sample size and also to look at parasites occurring subsequently in this group of children.

The parasite samples came from a wide variety of children from one to 12 years of age living predominantly in urban and periurban regions of Bamako, Mali. Haplotype analysis revealed that 50% of the children in this study had mixed infections of P. falciparum, regardless of whether they had mild or severe disease. This suggests that if other markers are combined in a similar analysis, the vast majority of children will be shown to have mixed infections. It is difficult to say whether this is a characteristic of the children in our study or a country where malaria is hyperendemic or whether it relates to the number of infectious bites per child per year. Mixed blood feeding by mosquito vectors from more than one individual is unusual, 27 and therefore mating will mostly occur between parasites acquired from a single individual. This suggests that the children in our study who clearly had multiple infections had been bitten on more than one occasion by an infected mosquito and very possibly had failed to clear earlier infections. This result is not unexpected since the study was carried out during the main malaria transmission season in Bamako.

Based on our TRAP analysis, only two pairs of siblings had single infections, suggesting the possibility that they might have been bitten by the same mosquito. A second gene, MSP-1, was analyzed in these four children. The two children infected with parasite TRAP haplotype 47A, a haplotype that was seen 10 times, had MSP-1 genes that were clearly different. The two children with a parasite having TRAP haplotype 42I, a haplotype that was observed three times, had MSP-1 genes that could not be distinguished using the block 3 repeat polymorphism. This was the only instance observed where there was any suggestion that children might have been infected by parasites from the same mosquito.

A series of studies looking at sequence variation have been carried out for other potential malaria vaccine constituents. These include the sporozoite stage of the parasite lifecycle, the circumsporozoite protein, and the two blood-stage merozoite surface proteins MSP-1 and MSP-2. 28-33 These proteins differ from TRAP in that they all contain major repeating epitopes or blocks of repeats. The sequence variation in these antigens has been examined in a variety of ways that have included RFLP and hybridization analysis of PCR products, as well as sequencing of PCR products both cloned and uncloned.

The consequences of these studies are that for subunit malaria vaccines to be effective, the major variants of the different components for a given area may be required. This may be more important in Africa where more variants of the different protein components have been reported. This may also apply to regions of the world where there are large migrant populations such as in the Thai-Burmese border. The major influence of hemagglutinin and neuraminidase re- sorytes determines which major types are used in flu vac- cines on an annual basis. As with influenza, it must be as- sumed that host immune responses are responsible for the observed sequence variation.

The structure of TRAP has yet to be determined so it is difficult to understand the significance of these sequence changes. From sequence alignment, the extracellular portion of TRAP can be divided into three domains. The first domain shares significant sequence similarity with the I domain of the a subunit of the integrin CR3 (CD11b/CD18). 34 The majority of RFLPs that we have examined in this study are in this region of the molecule. All of these amino acid substitutions can be regarded as conservative and as such would not alter the structure of this part of the molecule signifi- cantly and probably the function, but would be sufficient to alter the antigenicity. The second domain of TRAP contains the sulfatide-binding domain and has been found both in our earlier 21 and this study to be conserved. It is interesting to note that one of the major cytotoxic T cell epitopes for a large number of Europeans is found in this conserved part of the molecule. 35 The third domain contains the polymorphism associated with the Arg-Gly-Asp motif, which can be considered to alter the hydrophobicity of the flanking se- quence. The small tandem repeat is also found in this region as is the complex Bgl II/Ssp I polymorphism.

The fact that the same point mutations are found in par- asites from different geographic locations suggests that these point mutations can arise independently and become fixed in different populations, which is suggestive of selective pressure. Each sporozoite is only exposed to the host’s im- mune system for a short time but the numbers of sporozoites
an individual is exposed to over a lifetime is large. This is clearly sufficient pressure for TRAP variants to evolve and be selected. However, it should be pointed out that since the same point mutations are found in different geographic locations, this suggests that structural constraints are important and may in fact limit the repertoire of variants found. The majority of the point mutations give rise to conservative amino acid substitutions and such changes are unlikely to give rise to major alterations in particular epitopes. It is also possible that this variation in the form of point mutations occurred at a bottleneck during evolution of the parasite before the spread to different parts of the world.

In playing a game of chance such as a slot machine or throwing four dice, the chance of getting four identical symbols or throwing four sixes occurs infrequently but at a fixed odds. Most of the time a random combination of numbers or symbols occurs and this is what appears to be the case with the polymorphic sites in TRAP. At present we do not know whether certain combinations occur more often by chance alone or whether immunologic pressure is the selective force behind this. Thrombospondin-related adhesive protein is continually evolving. This is shown by applying phylogenetic analysis to the system (Figure 2). This is the first report of this method being applied to sequence variation in malaria. A two-dimensional approach has been tried with MSP-2 to try and identify geographic clustering. This would explain why immunity develops with age after repeated exposure to parasite infections.

Acknowledgments: We thank Professor Mario Coluzzi for valued support and Professor Sir David Weatherall for continuing encouragement. We also thank Professor David Walliker and Dr. Alison Creasey for the gift of 7G8 DNA.

Financial support: This work was funded by a grant from the Commission of the European Communities, Program STD2 no. CT90-0320, and by the Italian Research Council. Amagana Dolo was supported by a TDR/WHO fellowship. The work in Mali was supported by TDR/OMS contract no. 910396.

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