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Amplification of Single-Copy Genes of *Plasmodium vivax* from Two Drops of Peripheral Blood of Infected Patients

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The Merozoite Surface Protein 1 (MSP-1) (del Portillo et al. 1991) and The Circumsporozoite Protein (CSP) (Arnot et al. 1985) of *Plasmodium vivax*, the most widely distributed human malaria parasite, have been proposed as potential components of a multivaccine against malaria. MSP-1 is a surface protein of the merozoite, the parasite stage that invades erythrocytes, while CSP is the immunodominant coat protein of the sporozoite, the infectious stage of the parasite. The genes that encode the MSP-1 and CSP proteins are single-copy and more than one allele of each gene has been characterized (Gibson et al. 1992, Rosenberg et al. 1989). At least some of the allelic variation lies within regions encoding epitopes that may be useful in vaccine development (Rosenberg et al. 1989). Clearly, further epidemiological studies are necessary to investigate the degree of antigenic variation in these *P. vivax* proteins.

Methodologies based on the polymerase chain reaction (PCR) technique have greatly facilitated the identification of coding region polymorphisms in plasmodial parasites (Rosenberg et al. 1989, Scherf et al. 1989, Kimura et al. 1990, Snewin et al. 1991, Barker et al. 1992). PCR techniques are of particular importance for research on *Plasmodium vivax* since this parasite cannot be cultured in vitro and infected individuals exhibit very low parasitemias. Here we describe a simple and reproducible way to study *P. vivax* DNA polymorphisms using PCR and only two drops of peripheral blood from infected patients.

Malaria patients in endemic areas are diagnosed with Giemsa-stained blood smears that are prepared from a drop of peripheral blood obtained by finger puncture. For our study, two extra drops of infected blood were obtained from individual patients who were diagnosed and treated at the HEMERON Blood Center in Porto-Velho, Rondônia, in the Amazon region of Brazil. The blood was collected in 1.5 ml eppendorf tubes, stored frozen at -20°C, and transported to São Paulo for further study. To lyse the red blood cells and prepare the samples for PCR analysis, each pellet was resuspended in one ml of TE (10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8.0), and centrifuged at 12,500xg for 10 seconds. The pellet was resuspended a second time in TE, and the procedure was repeated twice to remove the red cell hemoglobin from each sample. The colorless pellets were resuspended in 200 µl of PK buffer (20 mM Tris-Cl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 1% laureth 12 or 0.5% Tween 20, 10 mg/ml Proteinase K) which is PCR buffer minus gelatin with detergent and proteinase added (Kawasaki 1990). Samples were incubated at 65°C for 45 min and then at 95°C for 15 min to inactivate the Proteinase K. Samples were stored at 4°C until needed.

PCR products generated from two *P. vivax* samples prepared in this way are shown in Fig 1A. In each reaction a five µl aliquot (one-fortieth of each sample) served as the PCR substrate, and gene-specific MSP-1 (Porto et al. 1992) and CSP (Rosenberg et al. 1989) oligonucleotide primers flanking variable segments of each gene were used in the amplifications. As positive controls, PCR reactions were also performed using cloned MSP-1 (Porto et al. 1992) and CSP (Arnot et al. 1985) sequences as PCR templates. As shown in Fig 1A, fragments of similar sizes to those amplified from clones of MSP-1 (the 454 bp fragment in lane 1) and CSP (the 717 bp fragment in lane 4), were obtained. The specificity of these fragments was confirmed by Southern blot hybridization (Fig 1B) using radiolabelled fragments of the MSP-1 and CSP clones as probes. Significantly, a second MSP-1 PCR fragment of 517 bp was present in both of the Rondônia isolates (lanes 2-3). This fragment has been recently shown to correspond to a second form in this segment of the MSP-1 gene of *P. vivax* and not to a PCR artifact (Porto et al. 1992). To further demonstrate the reproducibility and applicability of this method, sixteen more samples obtained by finger puncture from individual *P. vivax* patients were amplified using the gene specific MSP-1 oligos as above. All of the samples specifically amplified MSP-1 fragments from this segment of the gene as judged by ethidium bromide-stained gels (Fig 2A) and hybridization using cloned MSP-1 as probe (Fig 2B) (Porto et al. 1992).

Due to the increased risk of HIV contamination, the use of syringe needles and the transportation of blood in glass tubes should be avoided in large epidemiological surveys. Moreover, more than 50% of the malaria patients from the region of Rondônia refused to donate venous blood. In contrast, none of them refused to cooperate with this study by donating two extra-drops of blood obtained from the same finger puncture required for their diagnoses. Lastly, we have

successfully amplified these two single-copy genes from samples stored in the PK buffer at 4°C after six months (not shown). Therefore, the method described here is a simple and efficient way to study the extent of polymorphisms in the plasmodial gene pool in endemic regions.

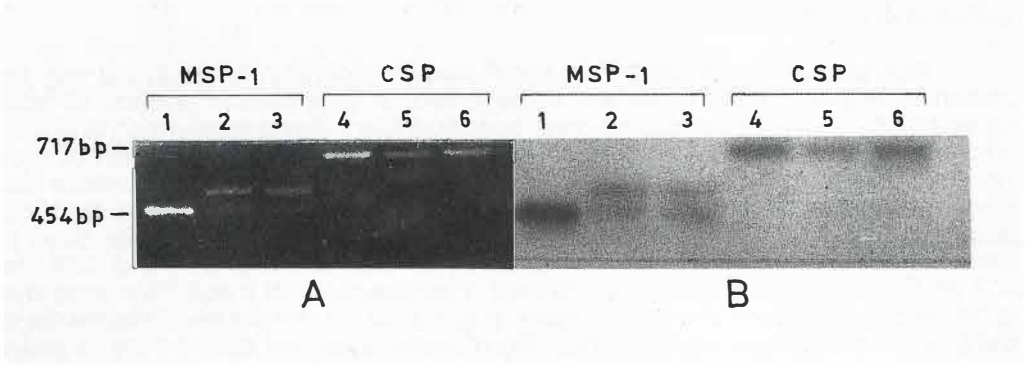

Figure 1. Amplification of MSP-1 and CSP gene segments of *P. vivax* by PCR. Five μl of genomic DNA obtained from two infected *P. vivax* patients from Rondônia (Brazil), were amplified on a Perkin Elmer Cetus thermal cycler (94°C/1min, 42°C/1min, 72°C/2min for 30 cycles) using the reagents of the PCR reagent kit (Perkin Elmer Cetus) and MSP-1 (Porto et al. 1992 and lanes 2-3) and CSP (Rosenberg et al. 1989 and lanes 5-6)-gene derived oligomers. As controls, clones containing MSP-1 (lane 1) and CSP (lane 4), sequences were amplified. After amplifications, 10 μl aliquots of each PCR reaction were electrophoresed in a 1.5% agarose gel (A), transferred to a nylon filter, and analyzed by Southern blot hybridization, using cloned MSP-1 and CSP sequences as probes (B). The molecular weights of the MSP-1 fragment (lane 1) and the CSP fragment (lane 4) are indicated on the left.

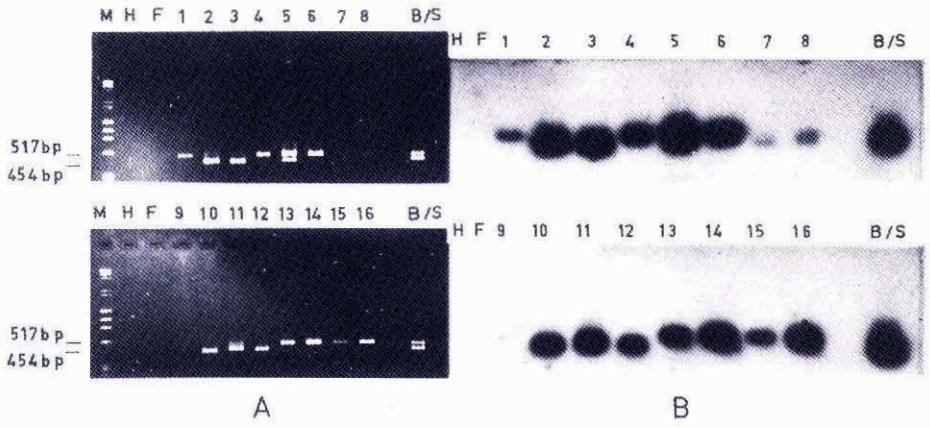

Figure 2. PCR amplification of a segment from the MSP-1 gene of *P. vivax*. Genomic DNA of 16 samples (1-16) obtained from infected patients from Rondônia (Brazil), were amplified with MSP-1-gene derived oligomers (Porto et al. 1992). As controls, DNA containing MSP-1 sequences (B/S), human DNA (H) and *P. falciparum* DNA (F) were similarly amplified with the MSP-1-gene derived oligomers. After amplification, 10 μl aliquots were electrophoresed in a 1.5 % agarose gel (A), blotted and hybridized with cloned MSP-1 sequences. The size in base pairs of the amplified fragments are indicated on the left and (M) represents lambda DRIgest molecular weight markers (Pharmacia).

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