 10.1111/j.1469-0691.2007.01815.x

Association between particular polymorphic residues on apical membrane antigen 1 (AMA-1) and platelet levels in patients with vivax malaria

P. Grynberg[1], *C. J. Fernandes Fontes*[2] *and É. Martins Braga*[1]

[1]Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte (MG) and [2]Departamento de Clínica Médica, Universidade Federal de Mato Grosso, Cuiabá (MT), Brazil

ABSTRACT

Apical membrane antigen 1 (AMA-1) is an immunogenic type 1 integral membrane protein, present in all *Plasmodium* spp., that probably has a role in the initiation of the invasion process of the erythrocyte. The DNA sequence of variable domain I of the *Plasmodium vivax ama1* gene was sequenced in Brazilian isolates obtained from thrombocytopenic patients (n = 32) and patients with normal platelet counts (n = 22). There was a significant negative correlation between parasite density and platelet counts. It was concluded that there is an additional effect of sequence on platelet counts. The presence of amino-acid residues Y^{193} and S^{210} was associated significantly with normal platelet counts in *P. vivax* malaria, independent of the level of parasitaemia ($p < 0.0001$). These data have implications for AMA-1-based vaccine design and suggest the possible use of this molecule as a marker of morbidity.

Keywords AMA-1, apical membrane antigen 1, *Plasmodium vivax*, platelet counts, polymorphism, thrombocytopenia

Original Submission: 21 November 2006; **Revised Submission:** 16 April 2007; **Accepted:** 26 June 2007

Clin Microbiol Infect 2007; **13**: 1089–1094

INTRODUCTION

Apical membrane antigen 1 (AMA-1) is an immunogenic type 1 integral membrane protein, present in all *Plasmodium* spp., with at least 16 cysteine residues incorporated into eight intra-molecular disulphide bonds that form the three domains of the protein [1]. This protein is synthesised during the last 4 h of the erythrocytic phase, when the schizonts are already mature and segmented [2]. AMA-1 may have a role in the initiation of the invasion process of the erythrocyte, and may be directly responsible for reorientation of the merozoite, as well as participating in the junctional contact between the two cells [3].

Polymorphisms occur non-randomly along the coding region inside the ectodomain, especially in domain I (the most polymorphic domain), suggesting that this region is a major target of the immune response [4]. As the gene for AMA-1 exhibits great sequence diversity, it is considered to be a useful molecular marker, with many alleles available to provide information concerning parasite populations. In addition, this gene also seems to be an important determinant of morbidity, since a strong association between clinical disease and residues Glu^{187} and Glu^{243} of the *Plasmodium falciparum ama-1* gene has been reported [5].

Plasmodium vivax, although causing less mortality than *P. falciparum*, has an enormous socioeconomic impact, particularly in South America and Asia (http://rbm.whq.int/wmr2005/html/exsummary-en.htm). According to the Brazilian Ministry of Health, *P. vivax* was responsible for *c.* 75% of the 500 000 cases of malaria reported in Brazil during 2005. Malaria caused by *P. vivax* has several clinical features, including fever, headache, myalgia, nausea, diarrhoea, vomiting, cough, liver and spleen enlargement, and

Corresponding author and reprint requests: É. Martins Braga, Av. Antônio Carlos 6627, Instituto de Ciências Biológicas, Departamento de Parasitologia, 31270-910 Belo Horizonte (MG), Brazil
E-mail: embraga@icb.ufmg.br

haematological abnormalities, e.g., anaemia, leukopenia, and thrombocytopenia [6]. Thrombocytopenia (<150 000 platelets/μL) is not usually associated with disseminated intravascular coagulation, and the platelet count typically normalises following specific treatment [7]. The mechanisms involved in *P. vivax*-related thrombocytopenia remain unknown. It has been proposed that immune and biochemical mechanisms, e.g., anti-platelet antibodies [8], oxidative stress [9] and caspase-mediated death of thrombocytes [10], may cause structural damage and loss of function of platelets. Ultrastructural studies on platelets from patients with acute *P. falciparum* malaria have revealed centralisation of granules, platelet–platelet interaction, spontaneous aggregation and degranulation [11].

The present study sequenced the *P. vivax ama-1* domain I gene (PvAMA-1) of 61 isolates of *P. vivax* from symptomatic patients, and correlated the polymorphic sites with levels of parasitaemia and platelet counts. Two particular residues at certain polymorphic sites were associated with normal platelet levels, independent of high parasitaemia, suggesting that AMA-1 is a possible marker of morbidity in *P. vivax* infections.

MATERIALS AND METHODS

Study population and blood samples

This study concerned patients (mean age 37.5 ± 13.8 years) with *P. vivax* malaria who were living in Cuiabá, the capital of Mato Grosso state, north-western Brazil, where active malaria transmission does not occur. Samples were collected between August 2004 and January 2006 from 61 patients who had been infected in regions of six Brazilian states where malaria is endemic, i.e., Acre, Amazonas, Mato Grosso, Pará, Rondônia and Roraima. The patients were unrelated, as there were no family clusters. The patients attended the Hospital Julio Müller of the Universidade Federal de Mato Grosso. Informed consent was obtained from each subject before blood collection, as specified by the Universidade Federal de Mato Grosso and Universidade Federal de Minas Gerais Ethics Committee rules.

Venous blood (5 mL) was collected in EDTA-containing tubes and was used to prepare thick smears for microscopy and to extract parasite DNA. The parasite density was quantified after examination of 200 microscopic fields at 1000× magnification under oil-immersion. Two patient groups were defined according to the level of parasitaemia: (i) 14 patients with ≤530 parasites/μL (mean age of 40.1 ± 15.9 years); and (ii) 42 patients with >530 parasites/μL (mean age of 36.7 ± 13.4 years). The threshold of 530 parasites/μL was based on the parasitaemia distribution (lower 25% percentile). Patients were also classified according to their platelet levels, as measured using a blood cell counter (ABX Pentra 90; Horiba Diagnostics, Kyoto, Japan): (i) 32 thrombocytopenic patients (mean age 35.5 ± 15.6 years) with platelet levels <150 000/μL; and (ii) 22 patients (mean age of 39.1 ± 13.3 years) with platelet levels >150 000/μL. Both sets of data were available for 49 patients, and these were analysed to evaluate the effect of parasitaemia on platelet counts. All subjects were interviewed and examined by the same physician, and all were prescribed chloroquine and primaquine in agreement with the Brazilian national policy for the treatment of malaria.

PvAMA-1 nested PCR and sequencing

Nested PCR was performed with DNA extracted from blood samples using a Purogene Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. PvAMA-1 was amplified as described previously [12], using primers PvAR11 (5′-CCTAAATTTTTACGGGGGCA) and PvAF11 (5′-AGAATTCCAGCTCCAAGATG) for the first round of amplification, and primers PvAR11 and PvAF05 (5′-TATCGTCATAGAGAATTCCG) for the second round. PvAMA-1 PCR products from each sample were purified using polyethylene glycol 8000 (PEG 8000) 20% w/v, and were then sequenced at least twice, using different AMA-1 products from different PCRs. The sequences were obtained using forward (PvAF05) and reverse (PvAR11) primers with a DYEnamic ET Dye Terminator Kit (Amersham Biosciences, Little Chalfont, UK) and a MegaBACE 1000 sequencing system (Amersham Biosciences). The quality of the sequences was verified using Phred [13], Phrap [14] and Consed [15] software. The sequences were edited using the Mega 3.1 program [16]; the same software was also used to calculate the genetic and nucleotide diversity of the sequences. The sequences were aligned using ClustalW software [17]. For haplotype generation, DNAsp 4.0 software [18] was used. As a reference standard, the sequences were compared to those for PH-84 (GenBank accession no. L27503).

Statistical analysis

STATA v.6.0 software (Stata Corp., College Station, TX, USA) was used to test the association between the PvAMA-1 polymorphisms and the morbidity parameters, i.e., level of parasitaemia and platelet levels. The two-tailed Fisher's exact test was used to compare all the frequencies of particular residues at polymorphic sites with levels of parasitaemia and platelet counts, and Bonferroni's correction was used for multiple testing when appropriate. Associations were detected by considering 26 independent tests, which were considered sufficient to confirm that any association was highly unlikely to have arisen by chance. The Mann–Whitney (Wilcoxon rank sum) test and Spearman's correlation coefficient were used to test the dependence between platelets and parasitaemia. A multiple linear regression model was created to test the independent contributions of parasitaemia and particular polymorphic amino-acid residues on the platelet blood levels. Significance was set at the 5% level.

Nucleotide sequence accession number

Nucleotide sequences described in this report have been submitted to GenBank under accession numbers EF031154–EF031216 and EF057487.