Allelic variants of the *Plasmodium falciparum* merozoite surface protein-1 gene (block 2) in Malaysian isolates

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Abstract

Allelic variation of the *Plasmodium falciparum* MSP-1 gene in Malaysian isolates was studied using a nested-PCR technique directed to block 2, one of the variable regions of the MSP-1 gene. Allele-specific primers were used for the three types of block 2: K1, MAD20, and RO33. The sizes of the amplification products were in the range of 150 to 200 bp. All three allelic types are present in Malaysian isolates. They exist either on their own or in combination with other allelic types in multiple infections. The more predominant allelic types are K1 and MAD20. Sixty three percent of the *in vitro* cultured *P. falciparum* isolates and 46% of the successfully typed isolates from Ranau, Sabah showed co-existence of parasites of both K1 and MAD20 type. The RO33 allelic type was also present in both *in vitro* cultured isolates and field specimens from Ranau, Sabah.

Key words: *Plasmodium falciparum*, MSP-1 gene, Allelic variation, Malaysian isolates

Introduction

Plasmodium falciparum merozoite surface protein-1 (MSP-1) is a prime candidate for incorporation in an asexual blood-stage malaria vaccine (Siddiqui *et al.*, 1987; Holder *et al.*, 1988) and a useful marker for genetic polymorphism of the parasite (Tanabe *etal.*, 1987). Allelic variation of the MSP-1 gene of *P. falciparum* has been recognised in parasites originating from different countries (Kaneko *et al.*, 1997; Jongwutiwes *etal.*, 1992; Scherf *et al.*, 1991; Kimura *et al.*, 1990; Peterson *et al.*, 1988). DNA sequence analysis of this gene from a number of *in vitro* cultured laboratory lines showed that it can be divided into 17 blocks that are either conserved, semi-conserved or variable at the amino acid sequence level (Tanabe *et al.*, 1987). The MSP-1 gene is largely dimorphic, represented by the Wellcome (K1) and MAD20 allelic types (Tanabe *et al.*, 1987; Miller *et al.*, 1993) except for block 2 which is found in three basic forms represented by K1, MAD20 and RO33 types (Certa *et al.*, 1987; Peterson *et al.*, 1988) and block 4. Allelic variation in the *P. falciparum* MSP-1 gene results in part from an association of the different sequence types in the variable blocks 2 and 4 and the more conserved blocks 6 to 16.

In this study we report variation in the MSP-1 gene in Malaysian isolates of *P. falciparum* by examining the block 2 allelic type in each of the isolates, using nested-PCR with primers specific to each allelic type of block 2: K1, MAD20, and RO33.

Materials and Methods

Plasmodium falciparum isolates

Blood samples were collected from Ranau, Sabah, a malaria endemic area in Malaysia. The *in vitro* laboratory isolates were originally from malaria patients around Kuala Lumpur City who seek treatment at the Hospital Kuala Lumpur. These parasites were maintained *in vitro* for several generations and stored in liquid nitrogen. They are not cloned parasite lines.

For the field specimens, 500 µl blood were taken from malaria positive patients, with a parasite infection that had been identified by microscopy. These specimens were placed in cryo-preservative (28% glycerol, 3% mannitol, 0.65% NaCl) and stored in liquid nitrogen before transporting (in ice) to Kuala Lumpur.

DNA isolation

The cryopreserved blood samples were thawed on ice and the cells washed once in washing solution (16% mannitol in 0.9% NaCl) and once in phosphate buffered saline (PBS). The red blood cells were lysed in two volumes of 0.15% saponin in PBS and allowed to stand at 37°C for 20 minutes. The cell suspension was then centrifuged and the parasite peller was collected and mixed with 500 µl of lysis buffer (10 mM Tris HCl, 150 mM Na Cl, 10 mM EDTA, 2% SDS, pH 8.0 containing 500 µg/ml Proteinase K). The suspension was incubated at 37°C overnight. The supernatant was recovered after centrifugation and a phenol chloroform extraction of DNA was carried out according to standard methods. The DNA was then precipitated with etha-

nol and 3 M Na acetate pH 4.8 and incubated for 30 minutes at -70°C. The DNA pellet was recovered after centrifugation and washed once with 70% absolute alcohol, air dried and dissolved in 50 µl of TE buffer (10 mM Tris HCl, 0.1 mM EDTA pH 8.0). DNA used for controls was prepared the same way as above from the blood of individuals negative for *P. falciparum* by microscopy.

PCR amplification

Nested-PCR was carried out using two sets of primers. The first set of primers, F1(CACAATGTGTAACACATGAAAGTTATC) and M1-OR (CTTAAATAGTATTCTAATTCAAGTGGATCA) amplifies sequence which starts from block 1 and extends to block 5 in the MSP-1 gene. PCR was carried our with an initial cycle of 95°C for 5 minutes, 58°C for 2 minutes and 72°C for 2 minures followed by 25 cycles of 94°C for 1 minute, 58°C for 2 minutes and 72°C for 2 minutes with further extension at 72°C for 5 minutes. The PCR amplification was performed in a 50 µl reaction mixture containing 100 nM each of forward and reverse primer, 200 µM each of dNTPs, 2.5 units of Taq polymerase (Perkin Elmer), 100 ng of DNA and 1.5 mM of $MgCl_2$. Controls for the assays were a reaction mixture containing DNA from a *P. falciparum* negative individual, a reaction mixture without DNA, and a reaction mixture without one of the primers.

A second round of PCR was then carried out using 2 µl of the first PCR product and using allele-specific primers based on the three allelic types of block 2 (K1, MAD20, and RO33) (Snounou *et al.*, 1999). PCR was carried out for an initial cycle with temperatures of 95°C for 5 minures, 61°C for 2 minutes and 72°C for 2 minutes, and followed by 30 cycles of 94°C for 1 minute, 61°C for 2 minutes and 72°C for 2 minutes with an extension ar 72°C for 5 minutes. The PCR amplification was performed in a 50 µl reaction mixture containing 125nM each of forward and reverse primer, 125 µM each of dNTPs, 2.5 units of Taq polymerase (Perkin Elmer) and 1.0 mM of $MgCl_2$. The PCR reactions were carried out in three separate tubes each containing one of the 3 alleles-specific primer pairs for block 2: K1F and R, MADF and R, ROF and R. The PCR products were electrophoresed on 2% agarose gel in Tris-Borate-EDTA buffer and visualised with ethidium bromide under UV illumination.

Results

Allelic typing of block 2 using each of the specific primer pairs for K1, MAD20 and RO33 gave amplified products in the expected size range: 153-279 bp (K1), 126-198 bp (MAD20) and 156 bp (RO33). Most of the K1 and MAD20 products were ~190-200 bp (Fig.1). Seventy two isolates from Ranau, Sabah and 19 specimens from *in vitro* cultured laboratory lines were examined. The amplification from the *in vitro* culrured isolares showed 68% of the samples with a mixture of K1 and MAD20 allelic types (Table. 1). Only 54 specimens from Ranau were successfully typed, 18 samples gave no product, even though the parasitaemia in each was more than 100 parasites per microlitre. Some of the specimens

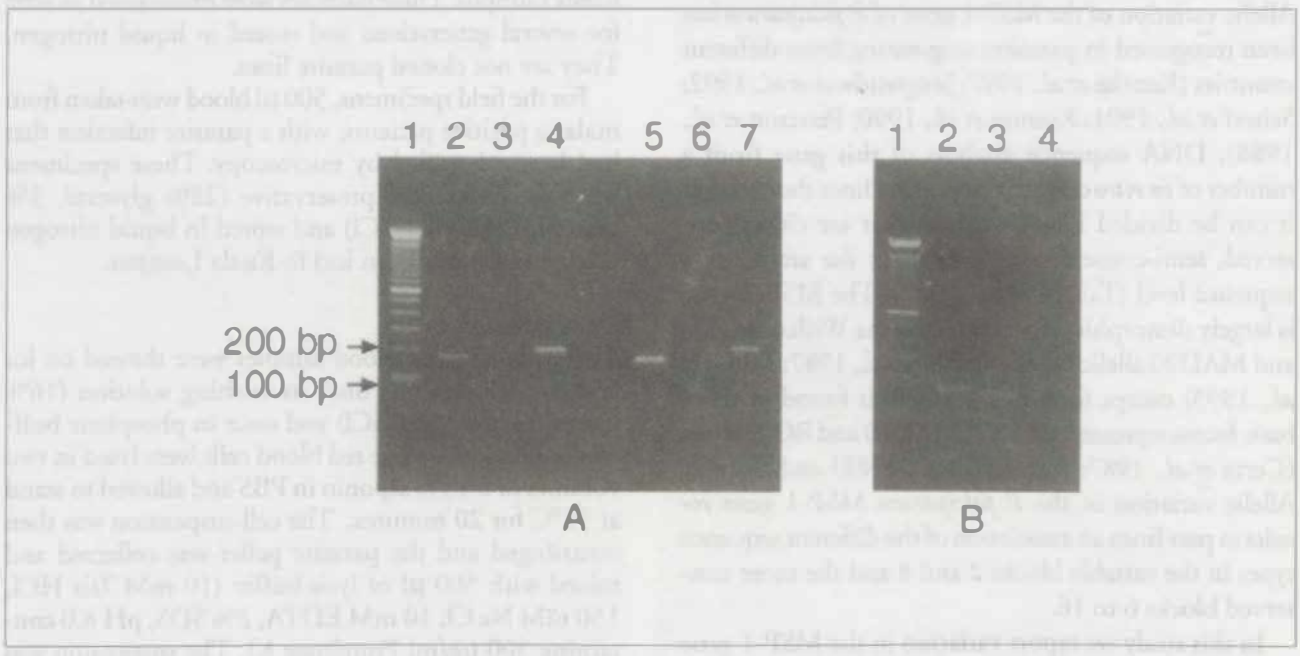

Fig. 1. Amplification of DNA coding for block 2 from the MSP-1 gene using allele specific primers. In panel A, DNA was prepared from either an *in vitro* culture (tracks 2 to 4) or a primary isolate (tracks 5 to 7). The final PCR amplification was done with primers specific for the MAD 20 (tracks 2 and 5), RO33 (tracks 3 and 6) and K1 (tracks 4 and 7) block 2 respectively. In panel B, DNA was prepared from a primary isolate, and the final PCR amplification was done with primers specific for the MAD 20 (track 2), RO33 (track 3), and K1 (track 4) block 2 types, respectively. In tracks 1 are 100 bp markers.

with a lower parasitaemia, 80 parasites per microlitre gave results. The successfully typed isolates showed 46% with a mixture of K1 and MAD20. The presence of the RO33 allelic type in the Sabah specimens was also noted. The most commonly found allelic types in Malaysia are K1 and MAD20, occurring together in a single patient. Fifteen percent of the *in vitro* cultured laboratory isolates and 22% of the successfully typed isolates from Ranau, Sabah contained the RO33 type. Some patients harbored parasites with all three allelic types at one time (Table1).

Discussion

This study gives information on variation of the MSP-1 gene among *P. falciparum* isolates in Malaysia and a minimum estimate of the proportion of infections that contain more than one parasite clone. To determine the MSP-1 types we carried out nested-PCR directed to sequences corresponding to block 2. The MSP-1 gene is mostly dimorphic but in block 2 can consists of three quite distinct sequences defined as the K1, MAD20, and RO33 types. This study showed that all three allelic types are present in the Malaysian isolates of *P. falciparum*. They exist either alone or in combination with other allelic types in individual patients, indicating multiple infections. The more predominant allelic types are K1 and MAD20. Some patients harbored all three allelic types at one time, indicating that several populations of *P. falciparum* may be present at a time in a patient.

Previous work had shown that the RO33 allelic type is rarely present in *in vitro* cultured laboratory isolates, and it has been suggested that a host factor absent in *in vitro* culture could play a role in the asexual reproduction of the RO33 type parasite (Scherf *et al.*, 1991). The RO33 type was originally identified in samples from Brazilian patients (Kimura *et al.*, 1990) but then a study carried out in West Africa also showed a high prevalence of *P. falciparum* with RO33 allelic type MSP-1 in this malaria endemic region (Scherf *et al.*, 1991). These two previous findings indicated that the RO33 allelic type is not geographically restricted, an observation that is consistent with the finding of this type at low frequency in Malaysia.

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Table 1. Allelic typing of the isolates on MSP-1 block 2

Specimens	Total no.	K1	MAD20	RO33	K1 & MAD20	RO33 & K1	RO33 & MAD20	RO33, MAD20 & K1
In vitro cultured isolates	19	2	1	1	13	-	1	1
Ranau, Sabah	54	4	13	1	25	1	2	8

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