GENETICS

Use of Nested PCR for Differential Diagnosis of *Falciparum* Malaria Reinfection and Relapse in Drug-Resistant Patients

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Microscopic examination does not allow differentiation of drug-resistant *P. falciparum* infection relapse from reinfection. However, this differential diagnosis is essential for adequate therapy. Three highly polymorphic *P. falciparum* genes (*msp1*, *msp2*, and *glurp*) and their alleles reflecting the structural state of these genes were used as genetic markers for differential diagnosis by PCR with internal primers. In 27 patients the characteristics of these alleles were identical before treatment with artersunate and during repeated manifestation of symptoms 14-28 days after the end of therapy, which attested to malaria relapses. In 24 patients the structure of these allele before mefloquine therapy and during repeated manifestation of the symptoms after 2-3 months was different, which attested to reinfection.

Key Words: *falciparum malaria; PCR with internal primers; genetic markers; relapse; reinfection*

Differential diagnosis of drug resistance and reinfection (repeated infection) is essential for the choice of proper therapy of *falciparum* malaria. The efficiency of specific treatment for *falciparum* malaria is usually evaluated by microscopic detection of the parasite in the peripheral blood. However, in highly endemic regions reappearance of *Plasmodium falciparum* in the blood and development of the corresponding symptoms can be associated with not only a relapse because of drug resistance after specific therapy, but also with reinfection. In order to rule out hyperdiagnosis of drug resistance (which requires modification of treatment) and carry out adequate specific therapy for reinfection, these two conditions should be differentiated, which is impossible to do by microscopy, but can be done by the analysis of differences in parasite genome.

P. falciparum population in endemic regions is characterized by high genetic polymorphism. High-polymeric genes encoding merozoite surface proteins 1 and 2 (MSP1 and MSP2) and glutamate-rich protein (GLURP) are widely used as genetic markers. Allele polymorphism is now effectively studied by PCR. Identity of alleles of *P. falciparum* before the treatment and during a relapse and differences in these alleles before therapy and at various periods after the end of treatment allow differentiation between the relapse and reinfection. However the possibility of multiple infection in endemic areas can impede this analysis [2,3].

We investigated the possibility of differentiation between malaria relapse associated with drug resistance and reinfection with *P. falciparum* by comparing alleles of three genes (*msp1*, *msp2*, and *glurp*) before specific therapy and after reappearance of clinical symptoms at different periods after the end of specific treatment.

MATERIALS AND METHODS

Capillary blood from 51 patients with microscopically confirmed *P. falciparum* infection was analyzed. All

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patients were treated with specific antimalaria drugs artersunate (group 1, *n*=27) or mefloquine (group 2, *n*=24). After the treatment the patients were discharged from hospital if no parasites were detected by microscopic examination.

In group 1 patients symptoms of malaria appeared again within 3 weeks after the end of treatment, while in group 2 patients this occurred 2-3 months after treatment, which suggests that group 1 patients had a relapse of artersunate-resistant infection, while group 2 patients were infected repeatedly.

In order to differentiate relapses from reinfection, PCR with internal primers was carried out on the day of the initial and repeated consultation.

Blood was collected in medical centers where the patients were consulted. A thick-drop preparation for microscopic analysis and blood drops on filters for PCR with internal primers were prepared. The samples were analyzed at the Institute of Malariology, Parasitology, and Entomology (Hanoi), additional treatment of the material was carried out at E. V. Martsinovskii Institute of Medical Parasitology and Tropical Medicine.

Isolation of DNA and PCR with internal primers were carried out using standard equipment and reagents [1]. Forward and reverse primers specific for *msp1*, *msp2*, and *glurp* genes and their alleles *MAD20*, *K1*, RO33, FC27, *and IC* were used.

DNA was isolated using routine methods [1], PCR was carried out in 2 stages. Stage 1 consisted in amplification of DNA fragments corresponding to *msp1*, *msp2*, and *glurp* genes; the purpose of amplification was accumulation of PCR products of each of the three genes in order to increase the efficiency of stage 2. The reaction mixture and optimal protocol of amplification were modified in comparison with the standard method [1].

The reaction mixture (total volume 25 μl) contained: 2.5 μl 10x PCR buffer, 2.5 μl 2.0 mM deoxynucleotide triphosphate, 1.25 μM forward and reverse primers (1 μl each), 1 μl Taq DNA polymerase (1 U), 3 μl DNA matrix, 14 μl deionized H_2O, 3 drops mineral oil.

The following primers were used: for *msp1*: 5'-CTA GAA GCT TTA GAA GAT GCA GTA TTG-3' (forward) and 5'-CTT AAA TAG TAT TCT AAT TCA AGT GGA TCA-3' (reverse); for *msp2*: 5'-ATG AGG GTA ATT AAA ACA TTA TCT ATT ATA-3' (forward) and 5'-CTT TGT TAG CAT AGG TAC ATT CTT-3' (reverse); for *glurp*: 5'-TGA ATT TGA AGA TGT TCA CAC TGA AC-3' (forward) and 5'-GTG GAA TTG CTT TTT CTT CAA CAC TAA-3' (reverse).

The optimal amplification protocol: denaturation at 95°C for 5 min, 25 cycles (annealing 2 min at 58°C, elongation 2 min at 72°C, and denaturation 1 min at 94°C), the final annealing of primers 2 min at 58°C, and final elongation 10 min at 72°C. The mixture was cooled to 20-25°C and stored at 4°C.

Stage 2 included amplification of DNA fragments corresponding to *msp1* gene *MAD20*, *K1* and *RO33* alleles, *msp2* gene *FC27* and *IC* alleles, and *glurp* gene *GN* allele. The reaction mixture for stage 2 was the same as for stage 1, except DNA matrix (amplification product obtained at stage 1: *msp*1 product for *MAD20*, *K1*, *RO33* fragments, *msp*2 product for *FC27* and *IC* fragments, and *glurp* product for *GN* fragment).

The following primers were used at stage 2: M1-2M (*MAD20* fragment): 5'-AAA TGA AGG AAC AAG TGG AAC GAC TGT TAC-3' (forward) and 5'-ATC TGA AGG ATT TGT ACG TCT TGA ATT ACC-3' (reverse), 100-150 b. p. each [7]; M1-2K (*K1* fragment): 5'-AAA TGA AGA AGA AAT TAC TAC

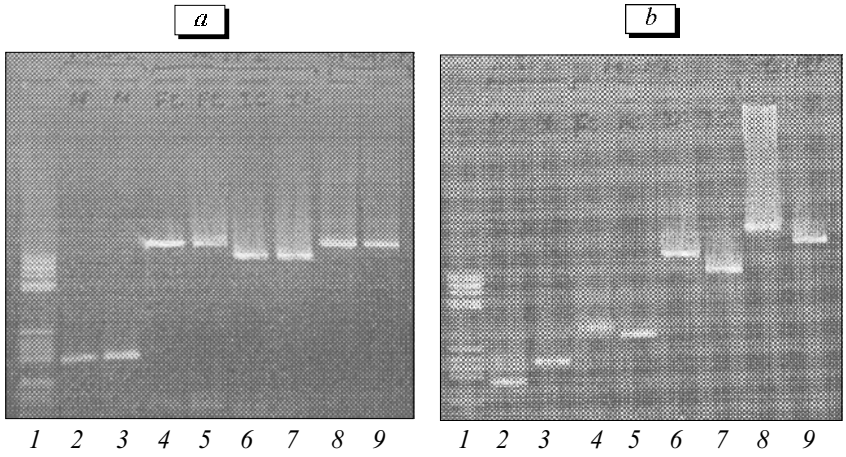

Fig. 1. Electrophoregrams of products of PCR with internal primers in relapses of drug-resistant malaria (*a*) and reinfection (*b*). *1*) pB 322 Hae III DNA marker; DNA fragments and: *2* and *3*) *msp1* gene *MAD20* allele; *4* and *5*) *msp2* gene *FC* allele; *6* and *7*) *msp2* gene *IC* allele; *8* and *9*) *glurp* gene *GN* allele. Even rows: before treatment (day 0); odd rows: after treatment (*a*: day 21; *b*: day 60).

AAA AGG TGC-3' (forward) and 5'-GCT TGC ATC AGC TGG AGG GCT TGC ACC AGA-3' (reverse), 150-200 b. p. each; M1-2R (*RO33* fragment: 5'-TAA AGG ATG GAG CAA ATA CTC AAG TTG TTG-3' (forward) and 5'-CAT COG AAA GATT TAG CAGE CACAO COG GCS ACT-3' (reverse), 160 b. p.; G-N (*GN* fragment): 5'-TAT TEA CACAO TAG ACA TAT AGE TAT AGE TEA-3' (forward) and 5'-GT. GAO TAG CAT TAT CAT CAA CACAO AT-3' (reverse), 600-1200 b. p. Primers to *FC27* and *IC* alleles were given by Dr. G. Snounou [4-6] ready, and their structure was not deciphered.

The amplification protocol and number of cycles at stage 2 were in general the same as during stage 1, but annealing during amplification of *msp1* and *msp2* fragments (*MAD20*, *K1*, *RO33*, FC27 *and* IC) was carried out at 65°C and during *GN* fragment amplification at 60°C; a total of 30 annealing-elongation-denaturation cycles were carried out. The last elongation of primers was performed for 8 min.

The size of PCR product was evaluated by the position of specific electrophoretic band in 2% agarose gel containing ethidium bromide (0.5 μg/ml TBE 1x buffer). The size of the resultant product was evaluated using pB 322 Hae III DNA size marker (Advanced Biotechnologies Ltd.) containing 22 DNA fragments (8, 11, 18, 21,51, 57, 64, 80, 89, 104, 123, 124, 184, 192, 213, 234, 267, 434, 458, 504, 540, 587 b. p.) [1].

RESULTS

Electrophoresis of PCR products with internal primers allowed differentiation between relapses and reinfection by the analysis of parasite genotypes for each patient of both groups. Electrophoregrams of 2 patients from different groups are presented. Pairs of PCR products characterizing the respective alleles did not differ before specific therapy and during a relapse 2-3 weeks later (group 1) (Fig. 1, *a*). This suggests that primary manifestations of *falciparum* malaria and the relapse were caused by parasites of the same genotype. In group 2 pairs of stage 2 PCR products characterizing the respective alleles before treatment (at first consultation) and during manifestation of malaria symptoms 2-3 months after treatment differed (Fig. 1, *b*). These differences indicate that the symptoms of *falciparum* malaria appeared 2-3 months after the treatment were caused by parasites with different genotypes (reinfection).

Hence, PCR with internal primers specific for highly polymorphic *P. falciparum* genes can differentiate the relapse of drug-resistant *falciparum* malaria from malaria reinfection.

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