PLASMODIUM FALCIPARUM: DETECTION AND STRAIN IDENTIFICATION OF INDIAN ISOLATES BY POLYMERASE CHAIN REACTION

Amar Bir Singh Sidhu and R Madhubala

School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067, India

Abstract. The polymerase chain reaction (PCR) was employed for detection and strain identification of *P. falciparum* in a comparative field study of Indian isolates. The primers were selected from highly conserved regions flanking the variable, tandemly repeated regions of highly polymorphic cell surface antigens, major merozoite surface antigen-1 (MSP-1), major surface antigen-2 (MSP-2), circumsporozoite surface antigen (CSP) and ring-infected erythrocyte surface antigen (RESA). Out of the 52 microscopically positive *P. falciparum* infected field samples, 47 samples were positive by PCR. Variation in the size of the amplified products was observed using MSP-1, MSP-2 specific primers respectively in different field isolates of *P. falciparum*, but CSP and RESA did not exhibit any variation in size of the amplified product. The multiplex PCR results demonstrated that amplified products from these surface antigens vary in size and there is a specific pattern for each strain and this could be utilized to identify a particular field isolate. One *P. falciparum* infected field sample detected by the above PCR method was found to be a mixed infection by two different strains. Five microscopically positive *P. vivax* infeced samples were also analyzed by PCR method using *P. falciparum* cell surface antigen (MSP-2) specific primers. PCR results showed one *P. vivax* infected sample was positive when *P. falciparum* specific primers were used, this could be due to inaccurate and reduced limit of detection of *Plasmodial* species by microscopic examination.

INTRODUCTION

Malaria is a major cause of morbidity and mortality in many tropical and subtropical countries, affecting more than 300 million people each year (WHO, 1992). The major problem in recent years has been development of drug resistant parasites and appearance of various new strains within pre-existing *Plasmodium* species. Diagnosis and epidemiological surveillance are essential elements in the management of the malaria infection both at the level of treatment of malaria infected patient and to control the spread of the drug resistance strains. Traditional diagnosis of malaria, which is based on microscopic examination of Giemsa-stained thick and thin blood smears, has drawbacks due to its labor intensive nature, inability to identify drug resistance strains, strain heterogeneity and to monitor the efficacy of chemotherapeutic treatment of malaria (Makler *et al*, 1998a). Hence the development of new diagnostic methods for identification of low level of infection and of different strains is of utmost importance.

Correspondence: Dr R Madhubala, School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067, India.
Fax: (91 11) 6165886; E-mail:rentala@hotmail.com

The polymerase chain reaction (PCR) has been widely used to monitor treatment response in human immunodeficiency virus infection (Hamed *et al*, 1993), *P. falciparum* and *P. vivax* infection (Kain *et al*,1993; Irion *et al*,1998) and other antimicrobial chemotherapeutic treatments (Aurelius *et al*, 1990, Degrave *et al*, 1994). The PCR amplification of highly polymorphic cell surface antigens (Edoh *et al*,1997; Wooden *et al*, 1992), a small-subunit rRNA gene (Ciceron *et al*, 1999; Das *et al*, 1995), highly repeated subtelomeric sequence (rep20) (Urdaneta *et al*, 1998), the DHFR-TS (dihydrofolate reductase-thymidylate synthase) gene (Wataya *et al*, 1991) and p126 gene of *P. falciparum* (Zalis *et al*, 1996), is another alternative highly sensitive and non-radioactive method for diagnosis of the malaria parasite. Due to its high specificity and sensitivity PCR can detect parasite level which is below the microscopic threshold and also is very useful in genotying of different *P. falciparum* strains from different geographic origins.

Genes that encode cell surface antigens, *eg* major merozoite surface antigen-1 (MSP-1; Mackay *et al*,1985), major surface antigen-2 (MSP-2; Fenton *et al*, 1991; Smythe *et al*, 1991), circumsporozoite surface antigen (CSP: Dame *et al*, 1984) and ring-infected erythrocyte surface antigen (RESA; Favalaro *et al*, 1986) which are considered potential vaccine

candidates, are generally used to identify different strains of the *P. falciparum*. The genes have conserved 5′ and 3′ ends but contain a region with blocks off tandemly repeated sequences which vary in size and DNA sequence from strain to strain of *P. falciparum* (Kemp *et al*, 1987). The highly conserved DNA sequences in the 5′ and 3′ regions flanking the variable repeats have earlier been used to design primers that amplified the products spanning the repeats (Wooden *et al*, 1993). These primer pairs for four different surface antigens were used in this study to identify polymorphisms and rapid sensitive typing of different field isolates from India.

MATERIALS AND METHODS

Materials

Thermus aquaticus (Taq) DNA polymerase and dNTPs mixture was purchased from Promega (Madison, USA) and all other materials and reagents were of highest quality procured locally. Chelex-100 was purchased from Sigma Chemical Company (St Louis MO, USA).

Patient isolates

A total of 52 Giemsa stained blood smears were collected from Malaria Research Center, New Delhi, India. These smears were detected positive for *P. falciparum* infection based on smear examination. Blood smears from 7 normal individuals were also collected from the same area. Five Giemsa stained blood smears that were detected positive microscopically for *P. vivax* were obtained from the All India Institute of Medical Sciences (AIIMS) New Delhi, India.

DNA Isolation

For detection and strain identification of *P. falciparum* Giemsa stained thin blood smears from infected patients were used for DNA extraction by chelex-100 method as described by Masatsugu *et al* (1995) and Wooden *et al* (1993).

Designing of PCR primers

Based upon the conserved regions in the *P. falciparum* surface antigens, MSP-1, MSP-2, CSP and RESA, four different sets of highly specific primers were used (Wooden *et al*, 1993). The primer sequences used for MSP-1 were 5′ Primer (5′ GAAGATGCAGTATTGACAGG 3′) and 3′ Primer (GAGTTCTTTAATAGTGAACAAG 3′), for MSP-2 the 5′ Primer (5′ GAGTATAAGGAGAAGTATGG 3′) and 3′ Primer (5′CCTGTACCTTTATTCTCTGG 3′), for CSP the 5′ Primer (5′ ATAGTAGATCACT TGGAGA 3′) and 3′ Primer (5′ GCATATTGTG ACCTTGTCCA 3′) and for RESA the 5′ Primer (5′GATCAAGGAGGAGAGAGAACC 3′) and 3′ Primer (5′CAGCATTAACACCAACACC 3′) were used.

Polymerase chain reaction

PCR was performed with parasite DNA from different Giemsa-stained thin and thick smears with Taq DNA polymerase (Promega), 200 μM of each dNTPs, 1x PCR buffer and 0.25 μM of each primer. The reaction mixture was incubated in a thermocycler (PTC-100™, MJ Research Inc, USA) at 95ºC, 52ºC and 72ºC for 1 minute, 30 seconds and 1 minute respectively for 30 cycles respectively. PCR product was analysed on the 1.8% agarose (Seakem Me FMC Bioproducts, USA) gel.

RESULTS

The *P. falciparum* strain specific polymorphic genetic markers merozoite surface protein 1 (MSP-1), merozoite surface protein 2 (MSP-2), circumsporozoite protein (CSP) and ring-infected erythrocyte surface antigen (RESA) were used in field genotyping of *P. falciparum* strains. PCR was performed on 52 samples which were reported positive by microscopic examination for *P. falciparum* and out of these 47 samples were observed positive for *P. falciparum* infection by PCR using different set of primers representing the surface antigens.

PCR was performed on 16 samples collected from patients that were reported positive for *P. falciparum* infection by microscopic examination, using MSP-1 specific sense and antisense primers (Fig 1 A). Lanes 1-13 and 21-23 show variation in the size of the MSP-1 amplified PCR product when DNA from different field isolates were used as templates. The size of the PCR product varied from 200- 300 bp. Lanes 14-20 represent samples from normal individuals and no PCR product was observed when MSP-1 specific primers were used.

Fig 1B shows PCR products from a total of 17 samples of infected blood smears using the primers for MSP-2. As with MSP-1 primers, MSP-2 specific primers yielded products of different sizes ranging from 400-500 bp when the DNA from these isolates was used as templates. Interestingly

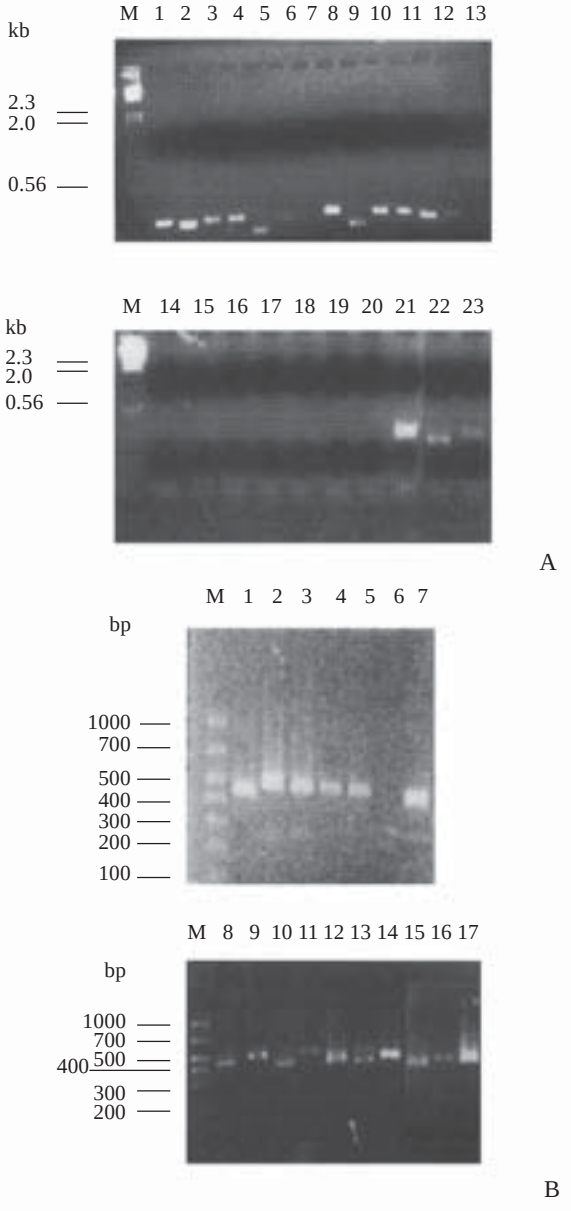

Fig 1–Gel photographs showing PCR amplified products of MSP-1 (A) and MSP-2 (B) from different *P. falciparum* infected isolates. The DNA size markers are *Hind*III digested lambda DNA and 100 bp ladder, shown on the left side.

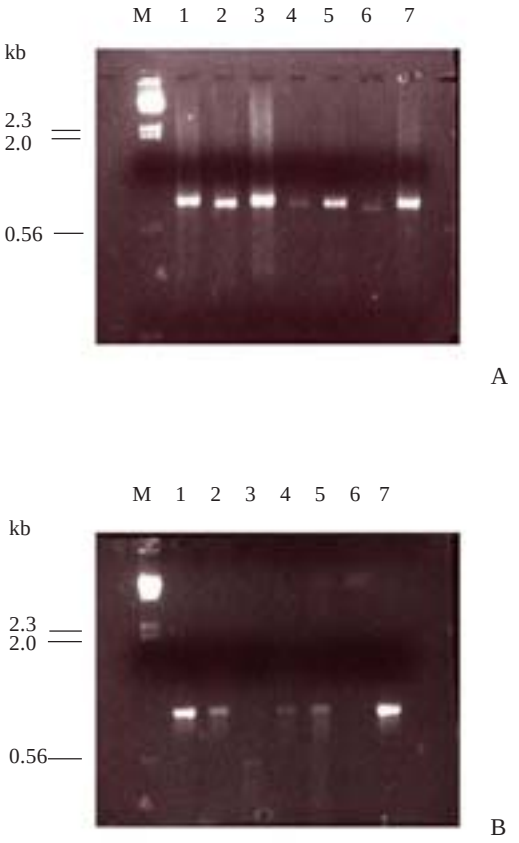

Fig 2–PCR amplified CSP (a) and RESA (b) products from different patient isolates of *P. falciparum*. The DNA size marker is a *Hind*III digested lambda DNA.

lane 13 showed two PCR products ranging from 400- 500 bp. This may signify infection with two different isolates of *P. falciparum*. Thus variation in size of MSP-1 and MSP-2 PCR amplified product from different *P. falciparum* patient isolates shows that these two *P. falciparum* antigens, located on the merozoite surface exist in numerous alleles in these field isolates of *P. falciparum* exhibiting length polymorphism in the tandemly repeated domain.

The amplified PCR products using CSP and RESA cell surface antigens specific primers and DNA template from different patient isolates are shown in Fig 2 A and B respectively. The expected size of the PCR product using CSP and RESA specific primers is approximately 600 and 900 bp, respectively. When run on 1.8 % agarose gel both CSP and RESA products were indistinguishable in all the strains assayed.

The multiplex PCR using the primer pairs for MSP-1 and MSP-2 and DNA from 6 different Giemsa stained *P. falciparum* patient isolates was performed. Each of these strains has a unique pattern of bands and this pattern can be used to identify easily the origin of the targeted DNA (Fig 3). The specific

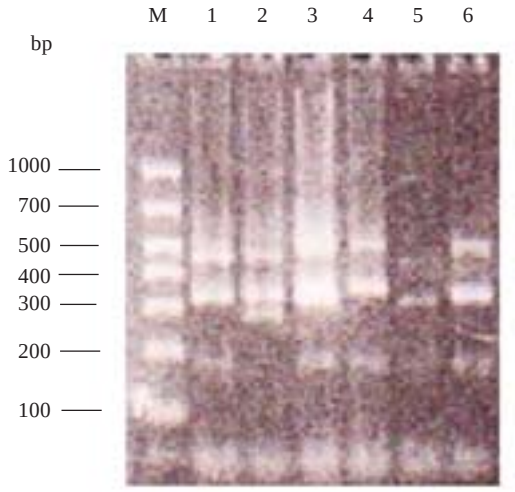

Fig 3–The amplified products from MSP-1 and MSP-2 genes of *P. falciparum* isolates by using both primer pairs together in the same multiplex PCR reaction. The DNA size marker is a 100 bp ladder.

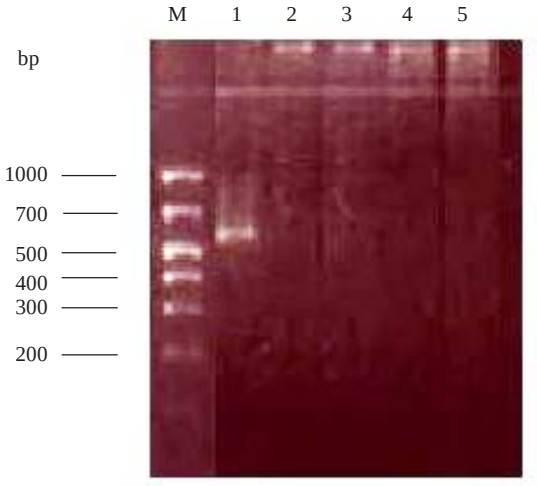

Fig 4–Gel photographs showing PCR amplified products of MSP-2 from different *P. vivax* infected isolates. The DNA size marker is 100 bp ladder, shown on the left side.

banding pattern obtained in multiplex PCR could be used to identify different strains.

Fig 4 represents 5 samples from infected individuals reported to be infected with *P. vivax* as identified by microscopic examination of the smears. The DNA from these samples was amplified using MSP-2 specific primers. All but one sample showed no product formation with MSP-2 specific primers. However lane 1 showed a PCR product of 500 bp. This could be due to the infection of the patient with both *P. falciparum* and *P. vivax*.

DISCUSSION

The different isolates of *P. falciparum* and of other plasmodial species exhibit a high degree of biological diversity in cell surface antigens. The allelic diversity that exits in certain genes of *P. falciparum* is due to an extensive degree of recombination in the parasite population which generates different strains with variable characters (Walliker *et al*, 1994). Due to limitations of microscopic diagnosis of malaria, now more advanced malaria diagnostic methods have been developed, which are based on the amplification of polymerase chain reaction (Edoh *et al*,1997, Tirasophon *et al*, 1994), detection of trophozoite derived histidine-rich protein-2 antigen (ParaSight F antigen capture assay; Dipstick Test) (Genton *et al*,1998; Humar *et al*, 1997; Schiff *et al*, 1993) and parasite-specific lactate dehydrogenase (pLDH, OptiMAL® assay) (Makler and Hinrichs, 1993, Makler *et al*, 1998b) by monoclonal antibodies have become available for sensitive detection, speciation, and quantitation of all human *Plasmodium* infections.

The PCR genotyping of the genes encoding the highly polymorphic loci MSP-1, MSP-2, CSP and RESA of *P. falciparum* provides a highly sensitive and specific method for the detection of malaria infection and strain identification of *P. falciparum*. The variations observed in various surface antigens in the present study, illustrate extensive allelic diversity in natural *P. falciparum* populations, however no size polymorphism was detected in these isolates in relation to CSP and RESA antigens. One *P. falciparum* infected blood sample which was identified by microscopic examination as a single strain was diagnosed by the PCR method as mixed infection of two different strains of *P. falciparum*.

A multiplex PCR approach using primer pairs for MSP-1 and MSP-2 simultaneously with different *P. falciparum* infected isolates showed different banding patterns for MSP-1 and MSP-2 due to genetic polymorphism in different alleles of these surface antigens. This difference in banding pattern obtained could be used to identify different strains of *P. falciparum*.

The primer pairs used in this study are highly specific for surface antigens MSP-1, MSP-2, CSP and RESA of *P. falciparum*, still they diagnosed

one *P. vivax* infected blood sample when amplified using MSP-2 specific primers. This difference in results from microscopy and PCR method is due to incorrect speciation. By microscopic examination of Giemsa-stained blood smears it is difficult to distinguish *P. falciparum* infection from *P. vivax* if only ring stage is present.

Out of a total of 52 isolates, 47 were identified positive by using different primer pairs for surface antigens MSP-1, MSP-2, CSP and RESA of *P. falciparum*. The five microscopically positive but PCR negative samples may be due to degradation of DNA during extraction or storage, inhibition of the PCR amplification by sample, insufficient amount of target DNA due to inadequate cellular lysis and most importantly incorrect species identification by microscopic examination.

It is conceivable from these studies that PCR method, using specific primers for four surface antigens of *P. falciparum* is an important diagnostic tool for identifying different strains of *P. falciparum* in the laboratory and in different field isolates, or where the possibility of mixed infections in the host needs to be identified or for epidemiological studies and heterogenity studies of *P. falciparum* and also to identify the geographical distribution of different strains.

REFERENCES

Aurelius E, Johansson B, Skoldenberg B, Staland A, Forsgen M. Rapid diagnosis of herpes simplex encephalitis by nested polymerase chain reaction assay of cerebrospinal fluid. *Lancet* 1990; 337: 189-92.

Ciceron L, Jaureguiberry G, Gay F, Danis M. Development of a *Plasmodium* PCR for monitoring efficacy of antimalarial treatment. *J Clin Microbiol* 1999; 37: 35-8.

Dame JB, Williams JL, McCutchan TF, Weber JL, Wirtz RA. Structure of the gene encoding the immnuodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. *Science* 1984; 225: 593-9.

Das A, Holloway B, Collins WE, *et al*. Species-specific 18S rRNA gene amplification for the detection of *P. falciparum* and *P. vivax* malaria parasites. *Mol Cell Probes* 1995; 9: 161-5.

Degrave W, Fernandes O, Campbell D, Bozza M, Lopes U. Use of molecular probes and PCR for detection and typing of *Leishmania*—a mini-review. *Mem Inst Oswaldo Cruz* 1994; 89: 463-9.

Edoh D, Steiger S, Genton B, Beck HP. PCR amplification of DNA from malaria parasites on fixed and stained thick and thin blood films. *Trans R Soc Trop Med Hyg* 1997; 91: 361-3.

Favalaro JM, Coppel RL, Corcoran LM, *et al*. Structure of the RESA gene of *Plasmodium falciparum*. *Nucleic Acids Res* 1986; 14: 8265-77.

Fenton B, Clark JT, Khan CMA, *et al*. Structural and antigenic polymorphism of the 35- to 48-kDa merozoite surface antigen (MSA-2) of the malaria parasite, *Plasmodium falciparum*. *Mol Cell Biol* 1991; 11: 963-71.

Genton B, Paget S, Beck HP, Gibson N, Alpers MP, Hii J. Diagnosis of *Plasmodium falciparum* infection using Parasight(R)-F test in blood and urine of Papua New Guineans. *Southeast Asian J Trop Med Public Health* 1998; 29: 35-40.

Hamed KA, Winters MA, Holodmy M, Katzenstein DA, Merigan TC. Detection of human immunodeficiency virus type I in semen: effects of disease stage and nucleoside therapy. *J Infect Dis* 1993; 167: 798-802.

Humar A, Ohrt C, Harrington MA, Pillai D, Kain KC. Parasight F test compared with the polymerase chain reaction and microcscopy for the diagnosis of *Plasmodium falciparum* malarias in travelers. *Am J Trop Med Hyg* 1997; 56: 44-8.

Irion A, Felger I, Abdulla S, *et al*. Distinction of recrudescences from new infections by PCR-RLFP analysis in a comparative trial of CGP56697 and chloroquine in Tanzanian chlidern. *Trop Med Int Health* 1998; 3: 490-7.

Kain KC, Brown AE, Lanar DE, Ballou WR, Webster HK. Response of *Plasmodium vivax* variants to chloroquine as determined by quantitative polymerase chain reaction of VK210 and VK247 circumsporozoite DNA. *Am J Trop Med Hyg* 1993; 49: 478-84.

Kemp DJ, Coppel RL, Anders RF. Repetitive proteins and genes of malaria. *Ann Rev Microbiol* 1987; 41: 181-208.

Mackay M, Goman M, Bone N, *et al*. Polymorphism of the precursor for the major surface antigens of *Plasmodium falciparum* merozoite: Studies at the genetic level. *Eur Mol Biol Org J* 1985; 4: 3832-29.

Makler MT, Hinrichs DJ Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. *Am J Trop Med Hyg* 1993; 48: 205-10.

Makler MT, Palmer CJ, Ager AL. A review of practical techniques for the diagnosis of malaria. *Ann Trop Med Parasitol* 1998a; 92: 419-33.

Makler MT, Piper RC, Milhous WK. Lactate dehydrogenase and the diagnosis of malaria. *Parasitol Today* 1998b; 14: 376-7.

Masatsugu K, Osamu K, Akira I, Akira IS, Kazuyuki T.

Amplification by polymerase chain reaction of *Plasmodium falciparum* DNA from Giemsa-stained thin blood smears. *Mol Biochem Parasitol* 1995; 70: 193-7.

Schiff CJ, Premji Z, Minjas JN. The rapid manual Parasight® F test. A new diagnostic tool for *Plasmodium falciparum* infection. *Trans R Soc Trop Med Hyg* 1993; 87: 646-8.

Smythe JA, Coppel RL, Day KP, *et al*. Structural diversity in the *Plasmodium falciparum* merozoite surface antigen-2. *Proc Natl Acad Sci USA* 1991; 88: 1751-5.

Tirasophon W, Rajkulchai P, Ponglikitmongkol M, Wilairat P, Boonsaeng V, Panyim S. A highly sensitive, rapid and simple polymerase chain reaction method to detect malaria (*Plasmodium falciparum* and *Plasmodium vivax*) in blood samples. *Am J Trop Med Hyg* 1994; 51: 308-13.

Urdaneta L, Guevara P, Ramirez JL. Evaluation of DNA recombinant methodologies for the diagnosis of *Plasmodium falciparum* and their comparison with the microscopy assay. *Mem Inst Oswaldo Cruz* 1998; 93: 639-46.

Walliker D. The role of molecular genetics in field studies on malaria parasites. *Int J Parasitol* 1994; 24: 799-808.

Wataya Y, Kubochi F, Mizukoshi C, *et al*. DNA diagnosis of falciparum malaria. *Nucleic Acids Symp Ser* 1991; 2: 155-6.

Wooden J, Gould EE, Paull AT, Sibley CH. *Plasmodium falciparum*: a simple polymerase chain reaction method for differentiating strains. *Exp Parasitol* 1992; 75: 207-12.

Wooden J, Kyes S, Sibley CH. PCR and strain identification in *Plasmodium falciparum*. *Parasitol Today* 1993; 9: 303-5.

World malaria situation 1990. Division of control of Tropical Diseases. World Health Organization, Geneva. *World Health Stat Q* 1992; 45: 257-66.

Zalis MG, Ferreira-da-Cruz MF, balthazar-Guedes HC, *et al*. Malaria diagnosis:standardization of a polymerase chain reaction for the detection of *Plasmodium falciparum* parasites in individuals with low-grade parasitemia. *Parasitol Res* 1996; 82: 612-6.