# PCR - BASED DIAGNOSIS TO EVALUATE THE PERFORMANCE OF MALARIA REFERENCE CENTERS

Silvia Maria DI SANTI(1), Karin KIRCHGATTER(1), Karen Cristina Sant'Anna BRUNIALTI(1), Alessandra Mota OLIVEIRA(1),
Sergio Roberto Santos FERREIRA(1) & Marcos BOULOS(2)

## SUMMARY

Although the Giemsa-stained thick blood smear (GTS) remains the gold standard for the diagnosis of malaria, molecular methods are more sensitive and specific to detect parasites and can be used at reference centers to evaluate the performance of microscopy. The description of the *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale* ssrRNA gene sequences allowed the development of a polymerase chain reaction (PCR) that had been used to differentiate the four species. The objective of this study was to determine *Plasmodium* species through PCR in 190 positive smears from patients in order to verify the quality of diagnosis at SUCEN's Malaria Laboratory. Considering only the 131 positive results in both techniques, GTS detected 4.6% of mixed and 3.1% of *P. malariae* infections whereas PCR identified 19.1% and 13.8%, respectively.

**KEYWORDS:** Malaria; Diagnosis; *Plasmodium*; PCR; ssrRNA; Thick blood smear.

## INTRODUCTION

One of the strategies to control malaria is the precise laboratorial diagnosis in order to treat positive cases appropriately. The gold standard to detect parasites in the blood is still the Giemsa-stained thick blood smear (GTS)[32]. When performed by qualified personnel, this technique presents some advantages, such as: differentiation among species/stages, observation of morphological changes caused by recent treatment, quantification and storage for long periods permitting posterior quality control[44]. Although easy to apply and cost-effective, this technique assumes that laboratories will have a certain infrastructure in place and it requires highly qualified professionals. Detecting from 10 to 50 parasites/μl, it has a reduced sensitivity[4,14,29,43] depending on the qualification of the technician. Even in reference centers qualified to perform hemoscopic diagnosis, the GTS may not allow to differentiate plasmodia, whose morphology is similar to two or more species, as occurs with *P. vivax*, *P. malariae* and *P. ovale*. This differentiation is possible using the thin blood smear that permits to correlate shape and size of parasites and erythrocytes. However, this technique cannot be used alone for malaria diagnosis, because its sensibility is from 10 to 30 times lower than GTS[27,39].

Alternative methods have been studied in order to replace or complement the diagnosis through GTS. Rapid immuno-chromatographic tests were developed based on detection of antigens in the blood of malaria patients and can be used in specific situations. Some of them[3,10] detect the *P. falciparum* Histidine Rich Protein II (PfHRPII)[15], and others[37] detect the PfHRPII and a genus-specific pan-malarial antigen.

Another test[30] employs two isoforms of a *Plasmodium* enzyme, the lactate dehydrogenase (pLDH), where one is specific to *P. falciparum* and the other occurs in the four human plasmodia species. Although appropriate to the diagnosis in remote areas, these tests show a variation in sensitivity and specificity[11,16,24,37]. Furthermore, false-positive results can occur due to circulating antigens that are detected even after the parasitological clearance[1] and to the presence of the rheumatoid factor in the plasma[17].

The molecular diagnosis of malaria was created as a more sensitive and specific methodology to detect parasites. In 1989, the use of four species-specific oligonucleotides for the small sub-unit ribosomal RNA (ssrRNA) genes was reported; they were used in hybridization studies as probes for detection of all *Plasmodium* species in humans[40]. However, this methodology has disadvantages, such as presenting low sensitivity and using radioactive material for which special laboratory conditions are necessary[42]. Based on the ssrRNA genes description of *P. falciparum*[26], *P. vivax*[41], *P. malariae*[13] and *P. ovale*[40], a polymerase chain reaction (PCR) was developed to differentiate the four *Plasmodium* species in humans[35]. In order to improve the sensitivity of this methodology, a nested PCR was developed. This technique utilizes two genus-specific primers in a first amplification reaction and species-specific primers in a second reaction[36].

In Brazil, the technique used to perform laboratorial diagnosis is the GTS, established as the gold standard. In 2002, in this country 350,000 cases were reported, 99.5% of which in the Amazon Basin, mainly due to *P. vivax*. Outside the Amazon, 1,635 cases were detected, 223 in São Paulo alone, where 74% of infections were caused by *P. vivax*, 23.3%

(1) Núcleo de Estudos em Malária, Superintendência de Controle de Endemias (SUCEN)/Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, 05403-000 São Paulo, SP, Brazil

(2) Departamento de Doenças Infecciosas e Parasitárias, Faculdade de Medicina, Universidade de São Paulo, 01246-903 São Paulo, SP, Brazil

**Correspondence to:** Silvia Maria Di Santi. Av. Dr. Enéas de Carvalho Aguiar 470, 05403-000 São Paulo, SP, Brazil. E-mail: santi@usp.br

by *P. falciparum* and 2.7% by *P. vivax* and *P. falciparum*. The Malaria Laboratory at SUCEN, the reference on diagnosis in the São Paulo State, detected 154 cases in 1998; 79.23% of the infections were caused by *P. vivax*, 18.83% by *P. falciparum* and 1.94% by both. In 1999, 122 cases were notified: 70.5% of *P. vivax*, 28.7% of *P. falciparum* and 0.8% of mixed infections. In 2000, of 105 cases, *P. vivax* was responsible for 65.7%, *P. falciparum* for 28.6%, *P. malariae* for 2.85% and *P. vivax* plus *P. falciparum* for 2.85% (Table 1). The objective of this study was to evaluate the diagnosis performed by the Malaria Laboratory of SUCEN that is responsible for the quality control of the exams carried out in the São Paulo State. Here, we used a semi-nested PCR with genus and species-specific primers for *Plasmodium* ssrRNA genes[20], producing fragments of about 110 bp and individually identifying the four species of parasites. Such methodology is suitable to perform PCR with DNA template extracted by techniques that include boiling, which leads to DNA fragmentation, as the ones used on samples obtained from smears[2,9,19].

**Table 1**
GTS diagnosis performed by the Malaria Laboratory at SUCEN

| Year | # of cases notified | Species detected (%) |
| --- | --- | --- |
| 1998 | 154 | V (79.23) |
|  |  | F (18.83) |
|  |  | V+F (1.94) |
| 1999 | 122 | V (70.5) |
|  |  | F (28.7) |
|  |  | V+F (0.8) |
| 2000 | 105 | V (65.7) |
|  |  | F (28.6) |
|  |  | M (2.85) |
|  |  | V+F (2.85) |

GTS = Giemsa-stained thick blood smear; N = negative; V = *P. vivax*; F = *P. falciparum* and M = *P. malariae*.

## MATERIAL AND METHODS

**Study population -** We considered GTS from the Malaria Laboratory archives collected between 1998 and 2000 (n = 381). For each year, 50% of the elements were selected by simple random sampling[6]. All selected smears (n = 190) were re-examined in an independent blind study by a qualified professional. The parasitemias were calculated considering the number of parasites/mm³ according to the Pan-American Health Organization[28]. Five GTS with more than 10,000 parasites/mm³ were used for the standardization of the PCR in our laboratory. These slides were selected to avoid the misconception that negative results could be due to failure in the technique instead of being consequence of low parasitemias. Seven smears collected in 1994, 1995 and 1996 were processed to verify the technique's efficiency years after its preparation. The patients were treated according to the GTS diagnosis, as proposed by SUCEN's Malaria Therapy Manual[8].

The Ethics Commission for Review of Research Projects of the "Hospital das Clínicas", Medicine Faculty of the University of São Paulo (Research protocol # 555/00) approved this study.

**DNA extraction -** DNA was obtained as described[2]. Concisely, the material was scrapped from the GTS, and then re-suspended in 500 μl of 1% Saponin (Sigma, St. Louis, MO) in water. Samples were incubated on ice for 60 min, vortexed and centrifuged. The pellets were washed in PBS (pH 7.2) and 100 μl of a 5% (wt/vol) solution of Chelex®100 (Bio-Rad, Hercules, CA) were added. After incubation at 56 °C for 15 min, the samples were vortexed and incubated at 100 °C for 10 min. After centrifugation, the supernatant was collected and stored at -20 °C until performing the PCR.

**PCR amplification -** The amplification of ssrRNA gene fragments of DNA extracted from smears was performed as described[20]. Briefly, 5 μl of DNA were used to amplify the genus-specific fragments with primers P1 and P2. The reactions occurred in 20 μl, under the following conditions: 0.4 μM of P1 and P2, 125 μM of each dNTP, buffer (50 mM KCl, 10 mM Tris-HCl and pH 8.3), 1.5 mM MgCl$_2$ and 0.75 U of *Taq* polymerase. The amplification program consisted of 92 °C for 2 minutes, 35 cycles at 92 °C for 30 seconds and 60 °C for 90 seconds, and a final step of 5 minutes at 60 °C. For the amplification of the species-specific fragments, 2 μl of the product from the first reaction (1:50) were used with 1 μM of P1 and 1 μM F2, V1 or M1, 312.5 μM of each dNTP, buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1.5 mM MgCl$_2$ and 0.75 U of *Taq* polymerase. The amplification program consisted of 92 °C for 2 minutes, 18 cycles at 92 °C for 30 seconds and 60 °C for 60 seconds, and a final step of 5 minutes at 60 °C. The PCR fragments were separated by 2%-agarose gel electrophoresis and stained with ethidium bromide. The PCR experiments, whose results were not in agreement with those from GTS, were re-assayed. All experiments were carried out in an isolated area, using filter tips to avoid cross-contamination.

## RESULTS

Five smears collected in 1999 were used in the PCR standardization, three of which were positive for *P. vivax* and two for *P. falciparum* by the GTS method. The PCR results showed amplification in all samples, detecting a *P. vivax* and *P. falciparum* mixed infection and an infection by *P. malariae*, while GTS detected *P. vivax*. In order to verify the condition of the material years after the smears had been collected, seven samples collected between 1994 and 1996 were used, with amplification in all samples.

To analyze the performance of the diagnosis carried out by SUCEN's Malaria Laboratory, a total of 190 smears was used, whose diagnosis by GTS detected 142 patients with *P. vivax*, 42 with *P. falciparum*, 02 with *P. malariae*, and 04 with *P. vivax* and *P. falciparum* mixed infections. Using PCR on material extracted from these smears, 38 samples had a negative result, 95 were positive for *P. vivax*, 26 for *P. falciparum*, 02 for *P. malariae*, 10 for *P. vivax* plus *P. falciparum*, 13 for *P. vivax* plus *P. malariae*, 01 for *P. falciparum* plus *P. malariae*, and 05 for *P. falciparum*, *P. vivax* plus *P. malariae*. Considering the results obtained after the GTS re-examination, of samples with negative PCR, 15 out of 38 were also negative in the second diagnosis by GTS and one was not re-evaluated by microscopy. From the remaining, 18 showed parasitemia below 2,000/mm³, three showed parasitemia between 2,000 and 4,000/mm³ and one showed parasitemia of 10,000/mm³. PCR and GTS results were compared taking into account only the 131 positive samples for both techniques. PCR identified 86 (65.6%) samples with *P. vivax*, 18 (13.7%) with *P. falciparum*, 02 (1.5%) with *P. malariae*, 9 (6.9%) with *P. vivax* plus *P.*

**Table 2**

Comparison of *Plasmodium* species results obtained by GTS and PCR in 131 positive smears for both techniques, considering the GTS re-examination

| GTS (# of positive samples) | PCR (# of samples) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | V | F | M | V+F | V+M | F+M | F+V+M | Total |
| V | 82 | - | 02 | 07 | 09 | 01 | 03 | 104 |
| F | 01 | 18 | - | 02 | - | - | - | 21 |
| V+M | 03 | - | - | - | 01 | - | - | 04 |
| V+F | - | - | - | - | - | - | 02 | 02 |
| Total | 86 | 18 | 02 | 09 | 10 | 01 | 05 | 131 |

GTS = Giemsa-stained thick blood smear; N = negative; V = *P. vivax*; F = *P. falciparum* and M = *P. malariae*.

*falciparum*, 10 (7.7%) with *P. vivax* plus *P. malariae*, 01 (0.8%) with *P. falciparum* plus *P. malariae*, and 05 (3.8%) with *P. falciparum*, *P. vivax* plus *P. malariae*, while GTS detected 104 (79.4%) samples with *P. vivax*, 21 (16%) with *P. falciparum*, 02 (1.5%) with *P. vivax* plus *P. falciparum* and 04 (3.1%) with *P. vivax* plus *P. malariae* (Table 2).

**DISCUSSION**

Besides the diagnosis as such, malaria reference centers have an important role in controlling the quality of the exams performed by other agencies. In the São Paulo State, the Malaria Laboratory at SUCEN reviews all positive smears and 10% of the negative ones. Due to the morphological changes that are enhanced by the staining and the similarities between several parasites, even highly qualified professionals can make an incorrect or incomplete assessment of the *Plasmodium* species[12]. In these cases, the PCR can be used from GTS-extracted material, which would allow a precise evaluation of these reference centers' performance. The methodology used here permitted the retrospective evaluation of slides stored for several years, as observed in another study[22].

The discrepancy observed between the first and second GTS examination could be explained by the fact that the smears were used in the routine of training in the Malaria Laboratory leading to the lost of some material. Also, four GTS diagnosed originally as *P. vivax*, showed *P. malariae* plus *P. vivax* in the re-examination, what could be explained by the presence of few band forms in the smear. Moreover, some smears were not re-examined.

Considering the number of samples with positive GTS after re-examination, 85.6% of the smears were positive by PCR in our study. In another work, PCR was positive only in 71% of the smears with low parasitemias[9], suggesting that the quantity of parasite DNA, proportionally to the GTS blood volume, could be below the PCR detection limit. When the same PCR protocol was used in GTS positive samples with 200 μl of blood, DNA amplification was observed in all reactions[5]. Therefore, unlike other authors[18], we believe that the quality of the GTS and the blood volume directly influence the PCR results. Furthermore, the DNA extraction using Chelex®100 and boiling could be responsible for the low sensitivity of PCR[7,36].

The rate of mixed infections detected in our study through PCR was 19.2%, higher than that found (13%) when isolates from different regions (western, eastern and Central Africa, Asia and Central America) were used[34], but lower than the 29% obtained in Africa[33], 23.1% in Asia[38],

30% in Brazil[5] and 29% in Venezuela[31]. These variations are probably due to different transmission intensities in each area or to different volumes of blood used for DNA extraction.

Infections caused by *P. malariae* were detected in 13.8% of the smears, comparable to the results obtained in another study with Brazilian samples, using primers to ssrRNA genes[5]. Although *P. malariae* is present in all Brazilian endemic regions, the widespread use of GTS makes it difficult to identify this parasite. *Plasmodium malariae* can be identified as *P. vivax* by using GTS, causing an unnecessary administration of primaquine. Several cases of diagnosis of *P. vivax* relapses could be a recrudescence due to *P. malariae*, since the reduced sensitivity to chloroquine of this parasite was already identified by our group (unpublished data) and also in Indonesia[23]. The high incidence of *P. malariae* found could indicate that these parasites have remained in the individual for a long period, as it has been already suggested[25], instead of being the product of a recent infection.

Regarding the discrepancy found between the GTS and PCR diagnosis involving other species, we can presume that the mistaken identification of *P. falciparum* instead of *P. vivax* probably occurs due to the presence of young forms only, without evident Schüffner's dots, leading to treatments without administration of primaquine, which consequently increases the relapse rate and sources of infection. On the other hand, cases where GTS did not detect the presence of *P. falciparum* had been mainly reported in patients taking chloroquine for the past 30 days[31].

The high cost of diagnosis by PCR, linked to the required laboratorial infrastructure, makes its routine use difficult in laboratories. However, reference centers that are responsible for the quality control and frequently are asked to clarify specific situations, as the ones related to nosocomial infections[21], blood banks and organ transplant units, can use this methodology.

**RESUMO**

**Diagnóstico baseado em PCR para avaliar o desempenho de centros de referência em malária**

Embora a gota espessa corada por Giemsa (GTS) permaneça o padrão ouro para o diagnóstico de malária, métodos moleculares são mais sensíveis e específicos para detectar parasitas e podem ser utilizados em centros de referência para avaliar o desempenho da microscopia. A descrição das seqüências dos genes ssrRNA de *Plasmodium falciparum*,

*P. vivax*, *P. malariae* e *P. ovale* permitiu o desenvolvimento de uma reação em cadeia da polimerase (PCR) que tem sido utilizada para diferenciar as quatro espécies. O objetivo deste estudo foi determinar as espécies de *Plasmodium* através de PCR em 190 lâminas positivas de pacientes para verificar a qualidade do diagnóstico realizado no Laboratório de Malária da SUCEN. Considerando somente os 131 resultados positivos em ambas as técnicas, GTS detectou 4,6% de infecções mistas e 3,1% de *P. malariae* enquanto o PCR identificou 19,1% e 13,8%, respectivamente.

## ACKNOWLEDGEMENTS

## REFERENCES

1. ABUL FAIZ, M.; RASHID, R.; PALIT, R. *et al.* - *Para*Sight-F test results in cerebral malaria patients before and after treatment in Chittagong Medical College Hospital, Bangladesh. **Trans. roy. Soc. trop. Med. Hyg., 94:** 56-57, 2000.

2. ALGER, J.; ACOSTA, M.C.; LOZANO, C.; VELASQUEZ, C. & LABRADA, L.A. - Stained smears as a source of DNA. **Mem. Inst. Oswaldo Cruz, 91:** 589-591, 1996.

3. AVILA, P.E.; KIRCHGATTER, K.; BRUNIALTI, K.C.S. *et al.* - Evaluation of a rapid dipstick test, Malar-Check™, for the diagnosis of *Plasmodium falciparum* malaria in Brazil. **Rev. Inst. Med. trop. S. Paulo, 44:** 293-296, 2002.

4. BRUCE-CHWATT, L.J. - Diagnostic methods in Malaria. In: BRUCE-CHWATT, L.J., ed. **Essential malariology**. 2. ed. London, William Heinemann, 1985. p. 103-126.

5. CAVASINI, M.T.; RIBEIRO, W.L.; KAWAMOTO, F. & FERREIRA, M.U. - How prevalent is *Plasmodium malariae* in Rondonia, western Brazilian Amazon? **Rev. Soc. bras. Med. trop., 33:** 489-492, 2000.

6. COCHRAN, W.G. - **Sampling techniques**. 3. ed. New York, John Wiley, 1977.

7. COX-SINGH, J.; MAHAYET, S.; ABDULLAH, M.S. & SINGH, B. - Increased sensitivity of malaria detection by nested polymerase chain reaction using simple sampling and DNA extraction. **Int. J. Parasit., 27:** 1575-1577, 1997.

8. DUTRA, A.P.; BARATA, L.C.B. & BOULOS, M. - **Manual de terapêutica de Malária**. São Paulo, Superintendência de Controle de Endemias, Secretaria de Estado da Saúde, 1998.

9. EDOH, D.; STEIGER, S.; GENTON, B. & BECK, H.P. - PCR amplification of DNA from malaria parasites on fixed and stained thick and thin blood films. **Trans. roy. Soc. trop. Med. Hyg., 91:** 361-363, 1997.

10. GARCIA, M.; KIRIMOAMA, S.; MARLBOROUGH, D.; LEAFASIA, J. & RIECKMANN, K.H. - Immunochromatographic test for malaria diagnosis. **Lancet, 347:** 1549, 1996.

11. GATTI, S.; BERNUZZI, A.M.; BISOFFI, Z. *et al.* - Multicentre study, in patients with imported malaria, on the sensitivity and specificity of a dipstick test (ICT Malaria *P.f./P.v.*) compared with expert microscopy. **Ann. trop. Med. Parasit., 96:** 15-18, 2002.

12. GAUTRET, P.; LEGROS, F.; KOULMANN, P.; RODIER, M.H. & JACQUEMIN, J.L. - Imported *Plasmodium vivax* malaria in France: geographical origin and report of an atypical case acquired in Central or Western Africa. **Acta trop. (Basel), 78:** 177-181, 2001.

13. GOMAN, M.; MONS, B. & SCAIFE, J. - The complete sequence of a *Plasmodium malariae* SSUrRNA gene and its comparison to other plasmodial SSUrRNA genes. **Molec. Biochem. Parasit., 45:** 281-288, 1991.

14. GUERIN, P.J.; OLLIARO, P.; NOSTEN, F. *et al.* - Malaria: current status of control, diagnosis, treatment, and a proposed agenda for research and development. **Lancet infect. Dis., 2:** 564-573, 2002.

15. HOWARD, R.J.; UNI, S.; AIKAWA, M. *et al.* - Secretion of a malarial histidine-rich protein (*Pf* HRP II) from *Plasmodium falciparum*-infected erythrocytes. **J. Cell Biol., 103:** 1269-1277, 1986.

16. HUONG, N.M.; DAVIS, T.M.; HEWITT, S. *et al.* - Comparison of three antigen detection methods for diagnosis and therapeutic monitoring of malaria: a field study from southern Vietnam. **Trop. Med. Int. Hlth., 7:** 304-308, 2002.

17. IQBAL, J.; SHER, A. & RAB, A. - *Plasmodium falciparum* histidine-rich protein 2-based immunocapture diagnostic assay for malaria: cross-reactivity with rheumatoid factors. **J. clin. Microbiol., 38:** 1184-1186, 2000.

18. IVANOVA, N.V.; MOROZOV, E.H.; KUKINA, I.V. *et al.* - ITS1, 5.8S and A-type ITS2 rDNA sequences from *Plasmodium vivax* and development of a method for retrospective PCR diagnosis of malaria by stained thick blood smears. **Molec. Biol. (Moskva), 35:** 515-525, 2001.

19. KIMURA, M.; KANEKO, O.; INOUE, A.; ISHII, A. & TANABE, K. - Amplification by polymerase chain reaction of *Plasmodium falciparum* DNA from Giemsa-stained thin blood smears. **Molec. Biochem. Parasit., 70:** 193-197, 1995.

20. KIMURA, M.; KANEKO, O.; QING, L. *et al.* - Identification of the four species of human malaria parasites by nested PCR that targets variant sequences in the small subunit rRNA gene. **Parasit. Int., 46:** 91-95, 1997.

21. KIRCHGATTER, K.; WUNDERLICH, G.; BRANQUINHO, M.S. *et al.* - Molecular typing of *Plasmodium falciparum* from Giemsa-stained blood smears confirms nosocomial malaria transmission. **Acta trop. (Basel), 84:** 199-203, 2002.

22. LI, J.; WIRTZ, R.A. & McCUTCHAN, T.F. - Analysis of malaria parasite RNA from decade-old Giemsa-stained blood smears and dried mosquitoes. **Amer. J. trop. Med. Hyg., 57:** 727-731, 1997.

23. MAGUIRE, J.D.; SUMAWINATA, I.W.; MASBAR, S. *et al.* - Chloroquine-resistant *Plasmodium malariae* in south Sumatra, Indonesia. **Lancet, 360:** 58-60, 2002.

24. MASON, D.P.; KAWAMOTO, F.; LIN, K.; LAOBOONCHAI, A. & WONGSRICHANALAI, C. - A comparison of two rapid field immunochromatographic tests to expert microscopy in the diagnosis of malaria. **Acta trop. (Basel), 82:** 51-59, 2002.

25. MAY, J.; MOCKENHAUPT, F.P.; ADEMOWO, O.G. *et al.* - High rate of mixed and subpatent malarial infections in southwest Nigeria. **Amer. J. trop. Med. Hyg., 61:** 339-343, 1999.

26. McCUTCHAN, T.F.; DE LA CRUZ, V.F.; LAL, A.A. *et al.* - Primary sequences of two small subunit ribosomal RNA genes from *Plasmodium falciparum*. **Molec. Biochem. Parasit., 28:** 63-68, 1988.

27. MOODY, A.H. & CHIODINI, P.L. - Methods for the detection of blood parasites. **Clin. Lab. Haemat., 22***:* 189-201, 2000.

28. ORGANIZACIÓN PANAMERICANA DE LA SALUD - **Manual para el diagnóstico de la malaria.** 4. ed. Washington, OPS, 1975. (Publicación Científica nº 276)

29. PAYNE, D. - Use and limitations of light microscopy for diagnosing malaria at the primary health care level. **Bull. Wld. Hlth. Org., 66:** 621-626, 1988.

30. PIPER, R.; LEBRAS, J.; WENTWORTH, L. *et al.* - Immunocapture diagnostic assays for malaria using *Plasmodium* lactate dehydrogenase (pLDH). **Amer. J. trop. Med. Hyg., 60:** 109-118, 1999.

31. POSTIGO, M.; MENDOZA-LEON, A. & PEREZ, H.A. - Malaria diagnosis by the polymerase chain reaction: a field study in south-eastern Venezuela. **Trans. roy. Soc. trop. Med. Hyg., 92:** 509-511, 1998.

32. ROSS, R. - An improved method for microscopical diagnosis of intermittent fevers. **Lancet, 1:** 186-187, 1903.

33. RUBIO, J.M.; BENITO, A.; ROCHE, J. *et al.* - Semi-nested, multiplex polymerase chain reaction for detection of human malaria parasites and evidence of *Plasmodium vivax* infection in Equatorial Guinea. **Amer. J. trop. Med. Hyg., 60:** 183-187, 1999a.

34. RUBIO, J.M.; BENITO, A.; BERZOSA, P.J. *et al.* - Usefulness of seminested multiplex PCR in surveillance of imported malaria in Spain. **J. clin. Microbiol., 37:** 3260-3264, 1999b.

35. SNOUNOU, G.; VIRIYAKOSOL, S.; JARRA, W.; THAITHONG, S. & BROWN, K.N. - Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. **Molec. Biochem. Parasit., 58:** 283-292, 1993a.

36. SNOUNOU, G.; VIRIYAKOSOL, S.; ZHU, X.P. *et al.* - High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. **Molec. Biochem. Parasit., 61:** 315-320, 1993b.

37. TJITRA, E.; SUPRIANTO, S.; DYER, M.; CURRIE, B.J. & ANSTEY, N.M. - Field evaluation of the ICT malaria *P.f/P.v* immunochromatographic test for detection of *Plasmodium falciparum* and *Plasmodium vivax* in patients with a presumptive clinical diagnosis of malaria in eastern Indonesia. **J. clin. Microbiol., 37:** 2412-2417, 1999.

38. TOMA, H.; KOBAYASHI, J.; VANNACHONE, B. *et al* - A field study on malaria prevalence in southeastern Laos by polymerase chain reaction assay. **Amer. J. trop. Med. Hyg., 64:** 257-261, 2001.

39. TRAPE, J.F. - Rapid evaluation of malaria parasite density and standardization of thick smear examination for epidemiological investigations. **Trans. roy. Soc. trop. Med. Hyg., 79:** 181-184, 1985.

40. WATERS, A.P. & McCUTCHAN, T.F. - Rapid, sensitive diagnosis of malaria based on ribosomal RNA. **Lancet, 1:** 1343-1346, 1989a.

41. WATERS, A.P. & McCUTCHAN, T.F. - Partial sequence of the asexually expressed SU rRNA gene of *Plasmodium vivax*. **Nucleic Acids Res., 17:** 2135, 1989b.

42. WATERS, A.P. - The ribosomal RNA genes of *Plasmodium*. **Advanc. Parasit., 34:** 33-79, 1994.

43. WORLD HEALTH ORGANIZATION - Severe and complicated malaria. World Health Organization, Division of Control of Tropical Diseases. **Trans. roy. Soc. trop. Med. Hyg., 84 (suppl. 2):** 1-65, 1990.

44. WORLD HEALTH ORGANIZATION - New Perspectives: Malaria diagnosis. In: **Report of a joint WHO/USAID informal consultation.** Geneva, WHO, 2000. p. 1-29.