High Specificity of Semi-Nested Multiplex PCR Using Dried Blood Spots on DNA Banking Card in Comparison With Frozen Liquid Blood for Detection of *Plasmodium falciparum* and *Plasmodium vivax*

S. Ataei,¹ M. Nateghpour,^{1*} H. Hajjaran,¹ G.H. Edrissian,¹ and A. Rahimi Foroushani²

¹Department of Medical and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

²Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

Background: Venipuncture sampling in test tubes for detecting malaria parasites using PCR assays possesses a number of limitations such as reluctance of patients, some difficulties in transportation of blood samples and freezing them for long time. To overcome the mentioned limitations, some approaches have been employed by a number of authors. This study was proposed to compare between DNA Banking Card (DBC) filter papers containing dried finger-prick blood and venipunctured frozen liquid blood. **Methods:** A total of 75 specimens was prepared from the equal enrolled individuals using three blood storage approaches; making Geimsa-stained thin and thick smears from each individual to determine the malaria-positive or -negative specimens, spotting two to three drops of finger-prick blood onto the DBC filter paper, and collecting a 2-ml venous blood sample into EDTA-contained test tube from each individual. A semi-nested Multiplex PCR

technique with DNA extracted from the two latter sets of specimens was used for plasmodia diagnosis. **Results:** DNA samples isolated from dried blood spotted on the DBC filter papers resulted in 32 (42.7%) positive and 43 (57.3%) negative cases comparable with the results outcome of frozen liquid blood with 35 (46.7%) positive and 40 (53.3%) negative cases. Statistical analysis revealed higher sensitivity for SnM-PCR using DNA from liquid blood with 100% vs. dried blood spotted on DBC with 97% but higher specificity for the DBC with 100% vs. liquid blood with 95.2%. **Conclusions:** Based on the results obtained from this study to overcome the problems of venipuncture frozen liquid blood sampling, replacement of a reliable filter paper for preserving finger-prick blood samples is a trustable and useful facilitator particularly in remote malaria-endemic areas. *J. Clin. Lab. Anal.* 25:185–190, 2011. © 2011 Wiley-Liss, Inc.

Key words: SnM-PCR; DBC filter paper; liquid blood; *Plasmodium falciparum*; *Plasmodium vivax*

INTRODUCTION

Malaria is a major parasitic cause of morbidity and mortality in many tropical and sub tropical countries affecting more than 300 million people each year (1). At present, more than 90 countries are in regions where malaria is endemic. One of the most important problems in controlling malaria is the limited access to effective and accurate diagnosis of malaria parasites (2). Prompt and accurate diagnoses are essential elements for achievement to the effective treatment and control of malaria, particularly in areas with intensive transmission of malaria (1,3). Since 1880 that Charles Laveran detected

the malaria parasites in the blood of a patient by using microscope, the mainstay of malaria diagnosis has been the microscopic examination of blood smears (4). Despite many advantages of microscopic examination method,

Grant sponsor: School of Public Health and National Institute of Health Research, Tehran University of Medical Sciences.

*Correspondence to: M. Nateghpour, Department of Medical and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. E-mail: nateghpourm@sina.tums.ac.ir

Received 9 June 2010; Accepted 29 January 2011

DOI 10.1002/jcla.20454

Published online in Wiley Online Library (wileyonlinelibrary.com).

it is well known that this technique is not the definite choice for cases with low-level parasitemia and mixed infections (5). Misdiagnosis of *Plasmodium vivax* or *Plasmodium falciparum* in mixed infections may cause relapse or recrudescence, respectively, and consequently malaria complications (6). PCR methods have been proved to be more specific and sensitive than conventional microscopy. Some authors reported that such technique can detect malaria parasites even with low level of parasitemia (7–11). Venipuncture sampling in test tubes for PCR assays possesses a number of limitations such as reluctance of patients, particularly young children, some difficulties in transportation of blood samples and freezing them for long time.

To overcome the mentioned limitations, some approaches have been employed by a number of authors, for instance spotting few drops of finger-prick blood onto filter papers and preparing fixed and stained thick and thin blood smears (12,13).

This study was proposed to compare between two transporting procedures of malaria parasite infected blood namely, DNA Banking Card (DBC) filter papers (Kawsar Genomics & Biotech Centers, Iran) containing dried finger-prick blood and venipunctured frozen liquid blood.

MATERIALS AND METHODS

Blood Specimens Collection

The study protocol was approved by the Research Ethical Committee of Tehran University of Medical Science.

Before sampling, an informed consent was obtained from each participant. Blood specimens were collected from those individuals who were living in Iranshahr, Sarbaz, and Jask, three malaria-endemic districts located in southeastern Iran.

A total of 75 specimens was prepared from the equal enrolled individuals using three blood storage approaches; in the first approach, about 40 µl of finger-prick blood was collected from each individual to make thin and thick blood smears to determine the malaria-positive or -negative specimens. In the second approach, two to three drops of the finger-prick blood were spotted onto the DBC filter paper (Kawsar Genomics & Biotech Center). In the third approach, a 2-ml venous blood sample of each individual was collected into EDTA-contained test tube (EDTA K3, Shandong Weigao Group Medical Polymer Co., Ltd, China).

The two latter sets of specimens were obtained for PCR processing intention.

Forty-five of the samples were obtained from malaria-suspected patients who were referred to health centers of the studied districts and two equal 15 specimens were

prepared from microscopically confirmed Plasmodium-positive and Plasmodium-negative cases as positive and negative controls. Plasmodium-positive control cases were collected from individuals living in the malaria-endemic studied districts and Plasmodium-negative control cases were collected from individuals without any history of malaria infections, living in nonendemic areas.

All the obtained samples, including Giemsa-stained thick and thin blood smears, dried blood spotted on the DBC, and frozen liquid blood specimens, were transferred to the malaria research laboratory in Tehran University of Medical Sciences under suitable condition.

Microscopical Examination

Thick and thin blood smears were examined by an expert microscopist at the Iranshahr and Jask districts. For more confirmation, the slides were re-examined in the malaria research laboratory, School of Public Health, Tehran. The parasitemia of positive smears was estimated per microliter of blood in which the number of parasites per 500 white blood cells was multiplied by 8,000 and the sum divided by 500 (14).

DNA Preparation From Blood Spotted on DBC

Blood-spotted DBC filter paper was air dried and sealed into a plastic bag and stored at room temperature until use.

DNA was extracted from blood spots using Kawsar Genomics & Biotech Center kit.

Briefly, each dried blood spots was cut into small pieces (1.2–2 mm) using a micropunch instrument and transferred into 0.5-ml microtube. Two hundred microliter of purifying reagent was added to the test tube and kept at the room temperature for 5 min. The used DBC purifying reagent was discarded and the procedure was repeated two or four times. Two hundred microliter of dH₂O was added to the test tube and kept at the room temperature for 5 min. The used dH₂O was discarded and the procedure was repeated two or four times. The piece of washed DBC was dried completely for 15 min at the room temperature, before to be used as DNA template in PCR assay.

DNA Preparation From Liquid Blood

Isolation of DNA from liquid blood was carried out using Bioneer Accuprep[®] Genomic DNA extraction kit (Korea).

Proteinase K was poured into the test tube containing 200 µl of liquid blood. Then the binding buffer (GC) was added to the tube and incubated at 60°C for 10 min. After incubating period, the isopropanol was poured and

then the lysate was transferred into the upper reservoir of binding column and centrifuged at 8,000 rpm for 1 min, then washed with washing buffer1 (W1) and washing buffer2 (W2) and centrifuged again at 8,000 rpm for 1 min. At the final stage, the Elution buffer was poured and centrifuged at 8,000 rpm for 1 min.

PCR Analysis of Samples

In this study, semi-nested multiplex PCR (SnM-PCR) amplification of 18srRNA was performed to detect the human malaria parasites using liquid blood and DBC filter paper for preparation of DNA template.

The used protocols and primers were described by Rubio et al. (15). The primers are shown in Table 1.

The PCR mix for the first reaction consisted of 2.5 µl PCR buffer 10 × (Roche, Germany), 1.25 mM MgCl₂ (Roche), 0.5 µl dNTP (10 mM, Roche), 25 Pmol of each primer: UNR and PLF, 5 unit of Taq polymerase (GenetBio, Korea) and 10 ng of genomic DNA extracted from liquid blood or 1 punch (1.2–2 mm) of DBC filter paper respectively in liquid blood and DBC PCR assay, for a final volume of 25 µl.

The PCR mix for the second reaction consisted of 2.5 µl PCR buffer 10 × (Roche), 1.5 mM MgCl₂ (Roche), 1 µl dNTP (10 mM, Roche), 25 Pmol of PLF and 10 Pmol of each VIR and FAR primers, and 5 unit of Taq polymerase (GenetBio, Korea). The used DNA template was 2 µl of the amplicon from the first PCR product diluted to 500 µl with sterile distilled water, 1 µl of which were added to the mixture, for a final volume of 25 µl.

For both PCR, 25 µl mineral oil was added before the amplification to prevent from evaporation of PCR mix.

In the first amplification, the reaction mixture was initially denaturated at 94°C for 4 min, followed by 35 cycles of amplification (94°C for 45 sec, 50°C for 1 min and 30 sec, and 72°C for 2 min). At the end of 35 cycles, the extension was completed by incubation at 72°C for 8 min, in order to reach final extension.

In the second amplification, the reaction mixture was initially denaturated at 94°C for 3 min, followed by 35 cycles of amplification (94°C for 30 sec, 48°C for 30 sec, and 72°C for 60 sec). At the end of 35 cycles, the

extension was completed by incubation at 72°C for 5 min, in order to reach final extension.

Ten microliter of each PCR product was fractionated electrophoretically on a 1% agarose gel, and the amplified products were visualized under ultraviolet (UV) light after staining with ethidium bromide.

The first PCR incorporates primers PLF and UNR, which was expected to yield a band of 787 bp (Fig. 1). In the second PCR, a band of 499 bp indicates *P. vivax* infection and a band of 395 bp indicates *P. falciparum* infection (Fig. 2).

The second PCR reaction includes a Plasmodium-specific forward primer plus species-specific reverse primers for *P. vivax* and *P. falciparum*.

The obtained data were statistically analyzed using SPSS 11.5 for windows (LEAD technologies, Inc., Charlotte, NC).

RESULTS

From a total of 75 studied cases, 59 (78.7%) and 16 (21.3%) cases were male and female, respectively. The result of microscopical examination revealed 27 (36%) cases *P. vivax*, 6 (8%) cases *P. falciparum*, and 42 (56%)

Fig. 1. Agarose gel electrophoresis, showing results with PLF and UNR primers. Lane 1: positive control; Lane 2: negative control; Lane 3: plasmodium (patient); Lane 4: molecular weight marker (100 bp).

TABLE 1. List of Primers Used in the Study

Primer name	Sequence (5'–3')	Specificity	Size of PCR product (bp).
UNR	GACGGTATCTGATCGTCTTTC	Universal	^a
PLF	AGTGTGTATCAATCGAGTTTC	Plasmodium	787
FAR	AGTCCCCTAGAATAGTTACA	<i>Plasmodium falciparum</i>	395
VIR	AGGACTTCCAAGCCGAAGC	<i>Plasmodium vivax</i>	499

^aPosition 1229 on the published sequence X03205 for human 18srRNA.

cases negative. The parasite density which was calculated from 100 field of each thick smear, ranged from 100 to 24,571 parasites per microliter of blood. Four (5.3%) of the positive samples contained ≤ 500 parasites/ μ l of blood that were detected by SnM-PCR method in liquid blood, whereas the least parasitemia (100 parasites per microliter of blood) was diagnosed negative in blood-spotted DBC using the same method. The liquid blood SnM-PCR analysis indicated 35 (46.7%) positive and 40 (53.3%) negative cases. Analysis of samples spotted on the DBC filter papers using SnM-PCR method indicated 32 (42.7%) positive and 43 (57.3%) negative cases.

Fig. 2. Agarose gel electrophoresis, showing liquid blood and DNA Banking Card results. Lane 1: *Plasmodium vivax* 18s rRNA amplicon from liquid blood; Lane 2: *P. vivax* 18s rRNA amplicon from DNA Banking Card; Lane 3: *Plasmodium falciparum* 18s rRNA amplicon from liquid blood; Lane 4: *P. falciparum* 18s rRNA amplicon from DNA Banking Card; Lane 5 and 6: positive controls from liquid blood and DNA Banking Card; Lane 7: negative control from liquid blood and DNA Banking Card; M, molecular weight marker (100 bp).

For more confirmation, four samples including three samples of liquid blood source and one sample of DBC source were randomly selected and sent to Bioneer Co. Korea, for sequencing. The results of sequencing were registered as accession numbers of GU979521, HM234009, HM234010, from liquid blood and GU937821 from DBC, in GENE BANK.

Sensitivity and specificity of SnM-PCR method using isolated DNA from frozen liquid blood in comparison with microscopical examination, as a gold standard test in diagnosis of malaria parasites, were 100 and 95.2%, respectively. *P* value of chi-square McNemar Test and the rate of agreement (κ value) were calculated to be 0.5 and 0.946, respectively. Sensitivity and specificity of the same technique for detecting plasmodia using isolated DNA from blood spotted on DBC in comparison with microscopical examination method were 97 and 100%, respectively. *P* value of chi-square McNemar Test and the rate of agreement (κ value) were calculated to be 1 and 0.973, respectively (Table 2).

Two of 42 samples were diagnosed as negative, using microscopical method, while the samples became positive as *P. vivax*, when were examined by means of SnM-PCR technique in the liquid blood. On the other hand, one of 33 samples, which was microscopically detected as *P. vivax*, was distinguished as negative sample using SnM-PCR method in blood-spotted DBC.

Two frozen liquid blood samples were detected positive using SnM-PCR method, which were negative by microscopical examination and also one sample, which had been spotted on DBC, was diagnosed negative by the same technique comparing with the reference standard method.

DISCUSSION

Although microscopy of malaria is the reference method for diagnosis of human Plasmodia, owing to same considerable advantages for PCR techniques, such as detecting Plasmodium at lower density of parasitemia (16)

TABLE 2. Comparative Results Prepared From Liquid Blood and DNA Banking Card Sampling Methods Based on SnM-PCR Technique

		Methods		
		SnM-PCR		Microscopical examination
Status		Frozen liquid blood	DNA banking card	
Positive	[*] P.v	29 (38.6%)	26 (34.6%)	27 (36%)
	⁺ P.f	6 (8%)	6 (8%)	6 (8%)
Negative		40 (53%)	43 (57.3%)	42 (56%)
Sensitivity		100%	97%	
Specificity		95.2%	100%	
Statistical analysis		Mcneemar <i>P</i> = 0.5 Kappa = 0.946	Mcneemar <i>P</i> = 1 Kappa = 0.973	

^{*}P.v = *Plasmodium vivax*; ⁺P.f = *Plasmodium falciparum*.

and more sensitive than microscopy in diagnosis of mixed infections (17), the techniques become increasingly preferable diagnostic method in many malaria laboratories. Venipuncture blood sampling and transfer of the samples under frozen situation to the laboratories are the main accounted problems for using the PCR techniques. Utilization of different filter papers as blood preservative tools in some studies prepared a convenient situation for transferring the samples (18) but venipuncture blood sampling still remains unsolved problem in a number of the studies. In this study, we have tried to compare the results obtained from venipuncture liquid blood sampling and finger-prick blood samples spotted on the DBC filter paper for diagnosis of *P. vivax* and *P. falciparum* by using SnM-PCR technique. The DBC filter paper bears some advantages including preservation of patients parasitic samples for long-term in room temperature, comfortable transporting, and ability to protect the deleterious contamination due to its specific buffer (Kawsar Genomics & Biotech Center kit, Iran). Results of this study indicated that the both Venipuncture blood sampling and finger-prick blood samples spotted on the DBC filter paper methods for detection of *P. vivax* and *P. falciparum* by using SnM-PCR technique ended to more or less the same results with a significant agreement between them. Rubio et al. gained a better result with dried blood spots on filter paper than frozen pellets of centrifuged blood samples for detection of *P. vivax*, *P. falciparum*, *Plasmodium malarie*, and *Plasmodium ovale* by using SnM-PCR method (15). Results obtained from a molecular epidemiologic study based on nested-PCR technique indicated that air-dried spots onto Whatman chromatography filter paper resulted in a better outcome for detection of *P. falciparum* than thick Geimsa-stained blood films in microscopical examination (19). Maeno et al. in a molecular-based *P. falciparum* gametocyte detection study suggested that blood sample spotted on the filter paper is a useful sampling for some fields of malaria studies (20). In another study, some authors reported that mRNA is a stable target for amplification by RT-PCR technique using 3-month stored dried blood at ambient temperature (18). The results of this study were obtained from at least 2-month stored dried blood onto DBC filter papers in room temperature. Chaorattanakawee et al. in a study proposed for long-term storage of sample filters on the sensitivity of PCR detection of *P. falciparum* showed that although length of storage is not too critical issue in PCR sensitivity for detection of *P. falciparum*, older storage of blood spots may address the better results (3). Statistical analysis in this study indicated that the sensitivity of SnM-PCR technique using DNA from blood samples spotted onto the DBC filter paper for detection of plasmodia is

comparable with the same technique when the frozen venipuncture blood samples were used with 97 and 100%, respectively. Our results about liquid blood sample collected on filter paper are close to those obtained by Long et al. using different kind of filter paper (21). Result obtained from dried blood samples spotted on Whatman chromatography filter paper that were processed by nested PCR method showed sensitivities from 92 to 100% for the method depending on the parasite density per microliter of blood (12). A 100 parasites per microliter blood infection was not detected in our study when DBC blood sample was used as DNA source. In a method setting, study for extracting DNA from dried blood spots on filter paper, capability of nested PCR for detecting one *P. falciparum* and two *P. vivax* from the blood-spotted filter paper was reported (22). In another study, sensitivity of nested PCR using filter paper with dried blood for detection of Plasmodium was accounted as 73% (23). Indeed, discrepancies between the results acquired from the different studies depend on the many involvements, such as quality and brand of filter papers, skill of workers, employed PCR techniques, and quality of reagents. The specificity of SnM-PCR for detection of plasmodia species isolated from blood samples on DBC filter paper and liquid blood was 100 and 95.2%, respectively, in this study. In a comparative study, for the detection of asymptomatic malaria using PCR and microscopical methods in Thailand, the sensitivity and specificity of nested PCR for detection of plasmodia from blood samples spotted on filter paper (Isocode stix and Whatman) were 75.1 and 91.9%, respectively (14). In an epidemiological retrospective study conducted by Scople et al., the specificity of nested PCR for detecting Plasmodium from blood samples burdened on filter paper was reported as 61% (23). Our study showed that the specificity of SnM-PCR using DNA from blood contained DBC filter paper is higher than frozen liquid blood samples.

In conclusion, according to the results obtained from this study to overcome the problems of venipunctured frozen liquid blood sampling, replacement of a reliable filter paper for preserving finger-prick blood samples is a trustable and useful facilitator particularly in remote malaria-endemic areas.

ACKNOWLEDGMENTS

We thank Dr. M. Mohebbali, Mrs. A. Motavalli, Mrs. L. Farivar, and Mr. A. Amiri for their contribution in this study and special thanks to Mr. Gh Mohseni, Mr. Parehkar, Mr. A. Shahbakhsh, and Mrs. Shahrokhi for their useful technical cooperation.

REFERENCES

1. Sidhu AB, Madhubala R. *Plasmodium falciparum*: detection and strain identification of Indian isolates by polymerase chain reaction. Southeast Asian J Trop Med Public Health 2000;31:213–218.
2. Zakeri S, Naimi P, Zare M, Zand Haghighi M, Dinparast-Djadid N. Molecular evidence on changing pattern of mixed *Plasmodium falciparum* and *P. vivax* infections during year-round transmission of malaria in Cahbahar, Iran. Iranian Biomed J 2004;8:89–93.
3. Chaorattanakawee S, Natalang O, Hananantachai H, et al. Storage duration and polymerase chain reaction detection of *Plasmodium falciparum* from blood spots on filter paper. Am J Trop Med Hyg 2003;69:42–44.
4. Sutherland CJ, Hallett R. Detecting malaria parasites outside the blood. J Infect Dis 2009;199:1561–1563.
5. Gama BE, Silva-Pires FdES, Lopes MNKR, et al. Real-time PCR versus conventional PCR for malaria parasite detection in low-grade parasitemia. Exp Parasitol 2007;116:427–432.
6. Ebrahimzadeh A, Fouladi B, Fazaeli A. High rate of detection of mixed infections of *Plasmodium vivax* and *Plasmodium falciparum* in South-East of Iran, using nested PCR. Parasitol Int 2007;56:61–64.
7. Seesod N, Nopparat P, Hedrum A, et al. An integrated system using immunomagnetic separation, polymerase chain reaction, and colorimetric detection for diagnosis of *Plasmodium falciparum*. Am J Trop Med Hyg 1997;56:322–328.
8. Snounou G, Viriyakosol S, Zhu XP, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol 1993;61:315–320.
9. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. 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. Mol Biochem Parasitol 1993;58:283–292.
10. Tirasophon W, Rajkulchai P, Ponglikitmongkol M, Wilairat P, Boonsaeng V, Panyim S. A highly sensitive, rapid, and simple polymerase chain reaction-based method to detect human malaria (*Plasmodium falciparum* and *Plasmodium vivax*) in blood samples. Am J Trop Med Hyg 1994;51:308–313.
11. Oliveira D, Holloway B, Durigon E, Collins W, Lai A. field evaluation of a polymerase chain reaction-based nonisotopic liquid hybridization assay for malaria diagnosis. J Infect Dis 1995;173:1284–1287.
12. Singh B, Cox-Singh J, Miller AO, Abdullah MS, Snounou G, Rahman HA. Detection of malaria in Malaysia by nested polymerase chain reaction amplification of dried blood spots on filter papers. Trans R Soc Trop Med Hyg 1996;90:519–521.
13. 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;91:361–363.
14. Coleman RE, Sattabongkot J, Promstaporm S, et al. Comparison of PCR and microscopy for the detection of asymptomatic malaria in a *Plasmodium falciparum/vivax* endemic area in Thailand. Malaria J 2006;5:121.
15. Rubio JM, Post RJ, van Leeuwen WM, Henry MC, Lindergerd G, Hommel M. Alternative polymerase chain reaction method to identify *Plasmodium* species in human blood samples: the semi-nested multiplex malaria PCR (SnM-PCR). Trans R Soc Trop Med Hyg 2002;96:S199–S204.
16. Greenwood B. The molecular epidemiology of malaria. Trop Med Int Health 2002;7:1012–1021.
17. Zakeri S, Najafabadi ST, Zare A, Djadid ND. Detection of malaria parasites by nested PCR in south-eastern, Iran: evidence of highly mixed infections in Chahbahar district. Malaria J 2002;1:2.
18. Maeno Y, Nakazawa S, Nagashima S, Sasaki J, Higo KM, Taniguchi K. Utility of the dried blood on filter paper as a source of cytokine mRNA for the analysis of immunoreactions in *Plasmodium yoelii* infection. Acta Trop 2003;87:295–300.
19. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg 1999;60:687–692.
20. Maeno Y, Nakazawa S, Dao le D, et al. A dried blood sample on filter paper is suitable for detecting *Plasmodium falciparum* gametocytes by reverse transcription polymerase chain reaction. Acta Trop 2008;107:121–127.
21. Long GW, Fries L, Watt GH, Hoffman SL. Polymerase chain reaction amplification from *Plasmodium falciparum* on dried blood spots. Am J Trop Med Hyg 1995;52:344–346.
22. Cox-Singh J, Mahayet S, Abdullah MS, Singh B. Increased sensitivity of malaria detection by nested polymerase chain reaction using simple sampling and DNA extraction. Int J Parasitol 1997;27:1575–1577.
23. Scopel KK, Fontes CJ, Nunes AC, Horta MF, Braga EM. Low sensitivity of nested PCR using *Plasmodium* DNA extracted from stained thick blood smears: an epidemiological retrospective study among subjects with low parasitaemia in an endemic area of the Brazilian Amazon region. Malaria J 2004;3:8.