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TECHNICAL REPORT

EVALUATION OF THREE DIFFERENT DNA EXTRACTION METHODS FROM BLOOD SAMPLES COLLECTED IN DRIED FILTER PAPER IN *Plasmodium* SUBPATENT INFECTIONS FROM THE AMAZON REGION IN BRAZIL

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SUMMARY

Asymptomatic *Plasmodium* infection is a new challenge for public health in the American region. The polymerase chain reaction (PCR) is the best method for diagnosing subpatent parasitemias. In endemic areas, blood collection is hampered by geographical distances and deficient transport and storage conditions of the samples. Because DNA extraction from blood collected on filter paper is an efficient method for molecular studies in high parasitemic individuals, we investigated whether the technique could be an alternative for *Plasmodium* diagnosis among asymptomatic and pauciparasitemic subjects. In this report we compared three different methods (Chelex®-saponin, methanol and TRIS-EDTA) of DNA extraction from blood collected on filter paper from asymptomatic *Plasmodium*-infected individuals. Polymerase chain reaction assays for detection of *Plasmodium* species showed the best results when the Chelex®-saponin method was used. Even though the sensitivity of detection was approximately 66% and 31% for *P. falciparum* and *P. vivax,* respectively, this method did not show the effectiveness in DNA extraction required for molecular diagnosis of *Plasmodium*. The development of better methods for extracting DNA from blood collected on filter paper is important for the diagnosis of subpatent malarial infections in remote areas and would contribute to establishing the epidemiology of this form of infection.

KEYWORDS: Malaria; Asymptomatic *Plasmodium* infection; Molecular diagnostic; DNA extraction methods.

INTRODUCTION

Malaria is one of the most important parasitic diseases in tropical areas of the world. A high percentage of cases occur in dispersed areas far from cities where health systems infrastructure is often deficient or absent. In recent years asymptomatic *Plasmodium* infections have been reported in the Amazon region[2,15]. Although a thick smear is the gold standard for malaria diagnosis, this method is not efficient for diagnosing sub patent parasites in asymptomatic *Plasmodium* infections[5,14]. Molecular methods such as the Polymerase Chain Reaction (PCR) are efficient for increasing the sensitivity and specificity of *Plasmodium* diagnosis[3,13] especially in pauciparasitemics (individuals with few parasites detected in the blood)[13]. Efficient DNA extraction is critical for a good PCR performance[6]. Whole blood samples must be adequately collected and immediately freeze-stored to guarantee high-quality PCR results[7]. This process requires regular health service structures and electricity, which are not always available in remote areas of the Amazon region[11]. Collection of blood samples on filter paper is a suitable alternative for malaria diagnostic screening, drug resistance monitoring, genetic analysis and other molecular studies in patients in distant areas[4,6]. Here we investigated whether the blood samples collected on filter paper as a source for DNA extraction for malaria diagnosis by PCR could be an alternative for *Plasmodium* detection among asymptomatic and pauciparasitemic subjects living in remote areas. To this end, we tested the three most frequently used protocols of DNA extraction from blood spotted on filter paper.

MATERIALS AND METHODS

Sample collection: Whole blood samples (5 mL) were collected from each of 21 well characterized individuals (18 asymptomatic infected with *P. vivax,* two with *P. falciparum* and one with mixed infection *P. vivax* + *P. falciparum*) using Vacutainer® tubes (Bekson Dickenson, USA) with ethylenediaminetetraacetic acid (EDTA) and stored at -20 ºC for DNA extraction for PCR diagnosis of malaria. From the same patients, four samples of 25 µL of blood were collected on 3MM Whatman® (Brentford, United Kingdom) filter papers. Each sample was dried at room temperature and stored in separate plastic packets to avoid cross contamination. The whole blood samples and dried filter paper were colleted simultaneously. Asymptomatic individuals were defined as those

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who had been infected with the *Plasmodium* species (with positive thick smear and/or PCR) for at least 30 days around the day of sample collection and presented none of the clinical symptoms attributable to malaria. Out of the 19 individuals infected with *P. vivax*, only four had a positive thick smear (Mean: 225 parasites/μL, range: 5-1000) but all were positive in the PCR; all individuals infected with *P. falciparum* were positive in the thick smear (Mean 367 parasites/μL, range 5-100) and in the PCR.

DNA extraction. Three different protocols were used for DNA extraction from the blood samples dried on filter paper, based on the method of BERECZKY *et al.* (2005):

Chelex®-saponin protocol: for the Chelex®-saponin extraction, each filter paper punch was incubated overnight at 4 °C in one mL of 0.5% saponin in phosphate buffered saline (PBS). The punches were washed for 30 minutes in PBS at 4 °C, transferred into new tubes containing 25 μL of 20% Chelex® and vortexed for 30 seconds. Then the tubes were heated at 99 °C for 15 minutes to elute the DNA, vortexed, and centrifuged at 10,000 × *g* for two minutes. The supernatants (± 65 μL) were transferred into new tubes[4]. The DNA extract was kept at 4 °C for use within a few hours or at -20 °C for long time storage.

Methanol protocol: for methanol extraction, each filter paper punch was soaked in 125 μL of methanol and incubated at room temperature for 15 minutes. Then the methanol was removed and the samples were dried before adding 65 μL of distilled water. Each punch was mashed using a new pipette tip and heated at 97 °C for 15 minutes to elute the DNA[4]. The DNA extract was kept at 4 °C for use within a few hours or at −20 °C for long time storage.

Tris-EDTA protocol: for Tris-EDTA extraction, each filter paper punch was soaked in 65 μL of TE buffer (10 mM Tris base plus Tris-HCl (pH 8.0) and 0.1 mM EDTA in distilled water, conserved at room temperature) and incubated at 50 °C for 15 minutes. The punches were pressed gently at the bottom of the tube several times, using a new pipette tip for each punch and heated at 97 °C for 15 minutes to elute the DNA. The liquid condensing on the lid and the wall of the tubes was removed by a short centrifugation (2-3 seconds). The DNA extract was kept at 4 °C for use within a few hours or at -20 °C for long time storage[4].

DNA extraction control: DNA was extracted from whole blood samples collected in Vacutainer® tubes using the Promega Wizard® Genomic Purification kit (Madison, USA). This extraction was used as gold standard. In short, 900 μL of red blood cell (RBC) lysis solution was added to a tube with 300 μL of whole blood sample. After 10 minutes incubation at room temperature the sample was centrifuged for 20 seconds at 13,000-16,000 g. The supernatant was discarded without disturbing the white blood cells (WBC) pellet. The visible pellet was vortexed for 10-15 seconds and 300 μL of nuclear lysis solution was added, mixing the contents by pipetting several times to lyse white cells; 100 μL of protein precipitation solution was added and vortexed for 10 to 20 seconds and then centrifuged for three minutes. The supernatant was transferred to a new microtube with 300 μL isopropanol, then gently mixed and centrifuged for one minute. The DNA visible as a little white pellet was washed with 300 μL of ethanol 70%. After being dried at room temperature the pellet was re-hydrated with 100 μL of re-hydrating solution. Aliquots were stored at -20 °C.

Polymerase chain reactions: PCRs were performed according to SNOUNOU *et al.*[13] nested-protocol with some minor modifications. A species-specific region of the 18 rDNA of *Plasmodium* was amplified. In brief, all PCR amplifications (Termocycler GeneAmp PCR Systems 9600, Applied, USA) were performed in 25 μL volume containing 1.5 mM MgCl2, 0.12 mM dNTPs, 240 nM of each oligonucleotide and 0.5 U of *Taq* polymerase (Invitrogen®, USA). In the first reaction, 3 μL of extracted DNA was added, using pairs of primers targeting an outer region specific to the *Plasmodium* genus. The primers used were: rPLU5: 5'-CTT GTT GTT GCC TTA AAC TTC-3', and rPLU6: 5'-TTA AAA TTG TTG CAG TTA AAA CG-3'. One μL of the first reaction product was used as a template in a second nested reaction to yield specific *P. falciparum* and *P. vivax* products. The temperature profile for the PCR was: five minutes at 95 °C; 25 cycles of one minute at 94 °C, two minutes at 58 °C, two minutes at 72 °C; followed by the second reaction where the annealing temperature was modified to 65 °C and the cycles repeated 30 times. Primers used for *P. falciparum* detection were: rFAL1:5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3' and rFAL2: 5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3' and for *P. vivax* detection: rVIV1: 5'-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3' and rVIV2: 5´ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3'. The PCR products were visualized under UV light after 2% agarose gel electrophoresis in 0.5X Tris borate EDTA buffer and ethidium bromide staining. A sample was considered positive if a 205 and/or 120 base-pair product (for *P. falciparum and P. vivax*, respectively) was detected. In every set of reactions, negative and positive controls were used (DNA extracted from patients presenting clinical malaria and patent parasitemia with *P. falciparum* and *P. vivax*). All the experiments were repeated twice.

Ethics: This study is part of a larger research project of malaria in the Amazon region. The protocol was submitted and approved by the Ethics Committee of Fundação Oswaldo Cruz/Fiocruz (protocol number 157-02).

RESULTS

PCR of DNA extracted from blood with the gold standard method showed that out of 21 samples, 18 (85.7%) were negative and three (14.3%) were positive for *P. falciparum*; for *P. vivax*, two (9.5%) were negative and 19 (90.5%) were positive. PCR reactions performed with DNA extracted from samples dried on filter paper using the Chelex®-saponin protocol showed that two (9.5%) samples were positive and 19 (90.5%) negative for *P. falciparum* and seven (33.3%) were positive and 14 (66.7%) negative for *P. vivax*; using the methanol protocol, 100% of samples were negative for *P. falciparum* and one (4.8%) was positive for *P. vivax*; and using the TRIS-EDTA protocol, 100% of samples were negative for both species. The sensitivity and specificity varied among the different DNA extraction methods and the best results were obtained when the Chelex®-saponin protocol was used. In this case, the sensitivity was 66.7% and 31.6% and specificity was 100% and 50% for *P. falciparum* and *P. vivax*, respectively; the kappa index for *P. falciparum* was 0.77 (IC 95% 0.35-1.00) but low for *P. vivax* (Table 1).

DISCUSSION

For individuals with a positive thick smear for *Plasmodium* sp., the collection of dried blood samples on filter paper is an alternative for

Table 1
Validity and confiability measures between three protocols of DNA extraction using gold standard as a control for the identification of *Plasmodium* species by PCR

P. falciparum									
Gold standard (Whole Blood)	Chelex®-saponin			Methanol			Tris-EDTA		
	Pos	Neg	Total	Pos	Neg	Total	Pos	Neg	Total
Positive	2	1	3	0	3	3	0	3	3
Negative	0	18	18	0	18	18	0	18	18
Total	2	19	21	0	21	21	0	21	21
Sensitivity		66.7			0			0	
Specificity		100			100			100	
Kappa		0.77 (CI 95%: 0.35-1.00) $p = 0.000$			0			0	
P. vivax									
Gold standard (Whole Blood)	Chelex®-saponin			Methanol			Tris-EDTA		
	Pos	Neg	Total	Pos	Neg	Total	Pos	Neg	Total
Positive	6	13	19	1	18	19	0	19	19
Negative	1	1	2	0	2	2	0	8	8
Total	7	14	21	1	20	21	0	21	21
Sensitivity		31.6			5.3			0	
Specificity		50.0			100			100	
Kappa		-0.05 (CI 95%: - 0.26-0.16) $p = 0.700$			0.01 (CI 95%:-0.01-0.04) $p = 0.370$			0	

Pos: positive; Neg: negative. CI: confidence interval.

molecular research in remote areas where collection, storage and transport are a problem[6,11,12,16,17]. However, this method has not shown consistent results in hypo and mesoendemic areas in the American[9] or Easter Mediterranean[1] region where subpatent *Plasmodium* infections occur. In the Amazon region, *Plasmodium*-infected asymptomatic individuals are characterized as having low parasite densities which are at times even undetected by microscopy[2,11,14,15].

In this technical communication, to evaluate the usefulness of dried blood collected on filter paper as a source of DNA for detecting *Plasmodium* by PCR in pauciparasitemic individuals, we tested three different DNA extraction methods. We obtained the best sensitivity for detection of *P. falciparum* and *P. vivax* when the Chelex®-saponin protocol was used. However, PCR of DNA extracted with this and the other two methods did not show high-quality results because of its low sensitivity and specificity compared to the PCR of DNA extracted from whole blood (used as gold standard). Sensitivity and specificity higher than 95% are considered adequate for laboratory diagnostic tests. Other studies evaluating the performance of blood collected on filter paper in subpatent *Plasmodium* infections are not available.

FÄRNET *et al.*[7] suggested that the low sensitivity of PCR from dried blood on filter paper may be the consequence of less purity, stability or integrity of DNA when this kind of blood collection is used. An important question to be considered in subpatent *Plasmodium* infections is the limited blood volume collected (+/- 25 µL) on filter papers. However, it should not interfere with PCR performance since this amplification method can detect numbers as small as 2-10 parasites/µL of blood[6]. In conclusion, none of the methods of DNA extraction from dried blood samples tested in this study provided good results. Further work is necessary to 1) develop more efficient DNA extraction methods and/or 2) to improve the sensitivity of PCR amplification from blood samples collected on filter paper. The development of better methods for extracting DNA from blood collected on filter paper for the study of asymptomatic *Plasmodium* infections would contribute to establishing the epidemiology of this form of infection, important for malaria control programs, especially in remote areas.

RESUMO

Avaliação de três métodos de extração de DNA obtidos de amostras de sangue coletadas em papel de filtro em infecções subpatentes de *Plasmodium* da região Amazônica no Brasil

Infecção assintomática por *Plasmodium* é um novo desafio para a saúde pública no Brasil. A reação em cadeia da polimerase (PCR) é o melhor método para detectar baixas parasitemias presentes em pacientes com infecção assintomática. Nas áreas endêmicas, a coleta de sangue

total é dificultada pela distancia geográfica, transporte e adequada armazenagem das amostras. A coleta de sangue em papel de filtro pode ser uma alternativa nessas áreas de difícil acesso. Neste estudo foram comparados três diferentes métodos de extração de ADN a partir de papel de filtro usando como controle extração a partir de sangue total. O protocolo Chelex®-Saponina foi o que obteve o melhor resultado quando comparado com os outros três protocolos. No entanto a sensibilidade foi de 66,7% para o *P. falciparum* e 31,6% para o *P. vivax.* Conclui-se que em caso de infecção assintomática o papel de filtro não é ainda uma boa alternativa para coleta de amostras.

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