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Comparison of two DNA obtainment methods as alternative protocols for the detection of human malaria parasites by nested PCR

Comparação entre dois métodos de obtenção de DNA a serem usados como protocolos alternativos para a detecção de parasitas humanos causadores de malária por nested PCR

Comparación entre dos métodos de obtención de DNA a ser usados como protocolos alternativos para la detección de parásitos humanos causadores de malaria por nested PCR

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ABSTRACT

The correct and precise laboratory diagnosis of human malaria is still a challenge because the reference method, the Giemsa-stained thick blood smear (TS), has limitations that present problems for malaria control. Because of these problems, several studies have attempted to develop alternative methods for malaria diagnosis. Many of these studies focus on molecular diagnosis methods and have led to the development of some alternatives to TS. However, their limitations include high cost, protocol complexity and variable quality of DNA sources and reagents. Nested PCR has been shown to be a good method in this respect and it can be improved by using a high-quality source of DNA. In this study we evaluated two methods for the obtainment of DNA from dried blood samples on filter paper: 1) washing and 2) saponin/chelex-100. The second method showed higher sensitivity and specificity compared to the first, as it detected more infections, whether single or mixed, as well as *Plasmodium malariae* infections. Based on these results, we present this method as the protocol of choice for DNA obtainment. Nested PCR using saponin/chelex-100 for DNA extraction could be an alternative or complementary diagnosis method for human malaria parasites, but it is not appropriate for routine use.

Keywords: Malaria; DNA; Polymerase Chain Reaction.

INTRODUCTION

Malaria is one of the main public health problems in Brazil, affecting mainly poor populations in the Amazon region[16,18]. The precise identification of the human-infecting *Plasmodium* species is very important to ensure adequate and early treatment. The reference method for malaria diagnosis is the Giemsa-stained thick blood smear (TS); it is inexpensive, has good sensitivity and specificity and allows for the identification of all developing forms of the *Plasmodium* species as well as the quantification of parasite stages[8,21]. Generally, the sensitivity range for detection by microscopists is 10-30 parasites/μL of blood; however, sensitivity can be limited when there is a high volume of samples to be examined in a very short time. It is possible to obtain false-negative results due to low levels of parasitemia, mixed infections or inexperience on the part of the microscopist. This fact can compromise one of the main strategies for malaria control: early and precise diagnosis for the implementation of the correct treatment[2,3,7,20].

In order to overcome some of the limitations of TS, methods based on the Polymerase Chain Reaction (PCR),

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such as the nested PCR protocol developed by Kimura et al[11], have been used to detect and identify malaria parasites. These methods are more sensitive and specific than TS and can detect even a single parasite per microliter of blood[5,10,21,23]. However, the success of a PCR technique depends on various factors, such as the quality of the DNA obtained from different sources and of the reagents. In addition, it has been demonstrated that DNA amplification from dried blood samples on filter paper is a reliable source of DNA when the transportation conditions, manipulation and packing are adequate to prevent DNA contamination or degradation[1,4,6,12]. For this reason, it is important to test different DNA obtainment methods as alternative protocols for the detection of human malaria parasites by nested PCR. This study aimed to compare the two following techniques: 1) DNA extraction by washing[26] and 2) DNA extraction by saponin/chelex-100 treatment of dried blood samples on filter paper[27].

MATERIALS AND METHODS

In this study we used 75 positive (range of parasitemia – 0.001% to 2%) and 78 negative dried blood samples on 2.5-cm-diameter Whatman® filter paper discs (Titertek, ICN Biomedicals – England). Blood samples were collected from people living in the municipalities of Novo Repartimento (04°14' 55" N; 50° 07' 25" W), Parauapebas (06° 04' 03" N; 49° 54' 08" W), Tucuruí (03° 45' 03" N; 49° 40' 03" W) and Belém (01° 27' 20" N; 48° 30' 15" W), in the State of Pará. Controls for each human malaria parasite included well characterized positive samples and ultrapure water and human DNA for the negative controls. All samples were examined by Giemsa-stained thick blood smear (TS) for diagnosis. Quantification of parasites was carried out for the positive slides[14].

DNA OBTAINED BY THE WASHING METHOD

The filter paper samples were prepared with five drops of 20 μL of each sample on Whatman® filter paper discs (2.5-cm diameter) (Titertek, ICN Biomedicals – England) and dried at room temperature (RT, 25-27° C). The samples were then packed into pre-labeled plastic bags (BHL Limited, Poole – England) that were kept at -20° C until needed. They were washed with sterile distilled water and 0.9% saline solution using a Millipore® filter system to remove all PCR inhibitors[26].

DNA EXTRACTION BY THE SAPONIN/CHELEX-100 METHOD

One-quarter of each dried blood spot on filter paper was cut into small pieces and incubated with 0.5% saponin solution in PBS in an ice bath for one hour. Afterwards, the solution was mixed using a vortex and the supernatant was discarded. This was followed by a wash with PBS, the addition of 20% chelex-100 solution in water and incubation in a dry bath for one hour. After the incubation, the supernatant was transferred to sterile Eppendorf® tubes and kept at -20° C[27].

NESTED PCR REACTION

The reaction for the amplification of the 18S gene subunit of the *Plasmodium* rRNA (ssu-rRNA) was conducted according to the protocol of Kimura et al[11]. Primers P1 and P2 were used as universal primers in the first round and primers F2, M1 and V1 were used as specific primers for *P. falciparum*, *P. malariae* and *P. vivax*, respectively, in the second round. The nested PCR products obtained were fractionated electrophoretically in 2% agarose gel (Ultra pure agarose, BRL 155517-014) at 100 V for one hour and stained with ethidium bromide (5 μL/mL). The expected PCR product sizes were 130 bp for the first step and 100-110 bp for the second step. Technicians conducting the nested PCR procedure were blinded to any microscopy results.

ETHICS

The protocol of this study was approved by the Research Ethics Committee of the Instituto Evandro Chagas (004/2003-CEP/IEC/SVS/MS). The purpose and procedures of the study were explained to all participants, signature on a written informed consent form was obtained from all patients and socio-demographic and clinical data were collected.

RESULTS

Out of 153 samples, 49.01% (75/153) were positive by TS (parasitemia range 0.001% to 2.0%) and 50.98% (78/153) were negative. With nested PCR, 43.79% (67/153) of samples were positive and 56.20% (86/153) were found to be negative with the washing method for DNA extraction, compared to 51.63% (79/153) positive and 48.36% (74/153) negative with the saponin-chelex-100 method (Table 1).

Table 1 – *Plasmodium* species identification by Giemsa-stained thick blood smear (TS) and nested PCR using the washing and saponin/chelex-100 methods to extract DNA

TS	Negative		PF		PV		PM		Mixed*		Mixed†		Total	
Nested PCR	W	S	W	S	W	S	W	S	W	S	W	S	W	S
Negative	78	74	2	–	6	–	–	–	–	–	–	–	86	74
P. falciparum	–	1	25	27	–	–	–	–	–	–	–	–	25	28
P. vivax	–	–	–	–	37	42	–	–	–	–	–	–	37	42
P. malariae	–	3	–	–	–	–	–	–	–	–	–	–	–	3
Mixed infection*	–	–	–	–	4	3	–	–	1	1	–	–	5	4
Mixed infection†	–	–	–	–	–	2	–	–	–	–	–	–	–	2
TOTAL	78	78	27	27	47	47	–	–	1	1	–	–	153	153

W: Washing method; S: Saponin/chelex-100 method; PF: *P. falciparum*; PV: *P. vivax*; PM: *P. malariae*; *: *P. falciparum* + *P. vivax*; †: *P. vivax* + *P. malariae*; –: not detected.

The performance of nested PCR compared to TS was as follows: 1) washing method - sensitivity = 89.33%, specificity = 100%, accuracy = 94.77%; and 2) saponin/chelex-100 method - sensitivity = 100%, specificity = 94.87%, accuracy = 97.39%. The agreement between these methods and microscopic examination was almost perfect [*Kappa* = 0.8952 and 0.9477, respectively; 95% CI range: 0.80 to 1.00]. The saponin/chelex-100 method provides a higher quality DNA sample than is obtained by the washing method.

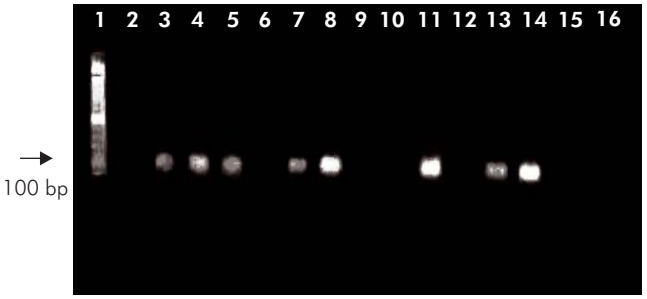
The frequency of *Plasmodium* species and mixed infections detected by nested PCR using DNA extracted by the washing and saponin/chelex-100 methods is shown in table 2. The saponin/chelex-100 method provided better DNA samples, as demonstrated by the fact that, with this method, nested PCR could detect *P. malariae* infections either alone or in mixed infection and detected a greater number of other infections compared to the washing method.

Table 2 – Frequency of *Plasmodium* species and mixed infections detected by nested PCR using the washing and saponin/chelex-100 methods for DNA extraction

Results	Frequency	
	Washing method	Saponin/chelex-100 method
Negative	56.20% (86/153)	48.36% (74/153)
P. falciparum	13.33% (25/153)	18.30% (28/153)
P. vivax	24.18% (37/153)	27.45% (42/153)
P. malariae	–	1.96% (3/153)
Mixed (*P. falciparum*+*P. vivax*)	3.26% (5/153)	2.61% (4/153)
Mixed (*P. vivax*+*P. malariae*)	–	1.30% (2/153)
TOTAL	100% (153/153)	100% (153/153)

–: not detected.

Figure 1 shows the results of a nested PCR analysis using DNA obtained by the saponin-chelex-100 method. Lanes 2, 3 and 4 contain samples from a single patient; each well corresponds to reactions for *P. falciparum*, *P. malariae* and *P. vivax* respectively, showing that the patient has a mixed infection with *P. malariae* and *P. vivax*.



Lanes: 1 = 50 bp marker; 3 and 4 = samples positive for *P. malariae* and *P. vivax*; 5 and 7 = samples positive for *P. falciparum* and *P. vivax*; 8 = sample positive for *P. falciparum*; 11 = positive control for *P. falciparum*; 12 = negative control (human DNA); 13 = positive control for *P. malariae*; 14 = positive control for *P. vivax*; 15 and 16 = negative controls (ultrapure water).

Figure 1 – Agarose gel electrophoresis analysis of lanes 1 to 16

DISCUSSION

Although the Giemsa-stained thick blood smear (TS) remains the reference method for malaria diagnosis, molecular techniques are more sensitive and specific in detecting parasites and they can be used to evaluate the performance of microscopy[20,28].

Dried blood samples on filter paper provide a simple and feasible method for collection and storage and they have be widely adopted in diagnostic screening, genetic analysis and molecular epidemiologic studies in remote areas with tropical climates where transport and storage conditions are often not optimal. Loss of sensitivity of PCR-based methods due to field conditions has already been reported and it is probably a consequence of lower purity, stability and integrity of the DNA obtained from blood samples on filter paper. A problem with sample collection on filter paper is the limitation in the sample volume that can be used for DNA extraction. Therefore, an optimal DNA isolation process is a prerequisite to ensuring the quality and efficiency of molecular methods[7,20].

Several studies have been aimed at decreasing the costs of PCR-based protocols and making them rapid and accurate. One part of the protocol that is crucial for getting good results is the obtainment of a high-quality DNA sample[1,5,6,7].

In this study, we carried out a comparative analysis of two protocols for DNA obtainment, taking into account the time requirement, the cost and the quality of the extracted DNA[7]. The results showed that the nested PCR/washing method failed to detect eight positive samples (six *P. vivax* and two *P. falciparum*) identified by TS whereas the nested PCR/saponin/chelex-100 method detected all positive samples detected by TS, as well as an additional five samples (three *P. malariae* single infections and two mixed infections with *P. vivax* and *P. malariae*). One possible reason for the failure to detect some positive samples is that the washing method did not properly eliminate nested PCR inhibitors such as hemoglobin. Results showed that the nested PCR/saponin/chelex-100 method was more sensitive and specific than the washing method, especially for the detection of *P. malariae*.

Analyses of DNA extraction by washing as described by Warhurst et al[26] and DNA extraction with saponin/chelex-100 by Wooden et al[27] from 153 dried blood samples on filter paper demonstrated that both methods are sensitive and have high specificity, in addition to being easy and quick to perform. Furthermore, because these methods consist of "in house" protocols, the cost was lower than that of available commercial kits. However, the saponin/chelex-100 method was found to be more sensitive and thus should be the protocol of choice for the DNA-based detection of malaria parasites by nested PCR.

The nested PCR protocol developed by Kimura et al[11] for human-infecting *Plasmodium* species proved to be useful, as it is highly sensitive and specific as well as reproducible and there was no evidence of cross-contamination in the study. Using this protocol, we detected mixed infections that were identified by TS as single infections, which is in accordance with the data from Ebrahimzadeh et al[5], Scopel et al[21], Singh et al[22], Snounou et al[24] and Toma et al[25].

In mixed infections there is a predominance of one *Plasmodium* species that generally is the one detected by both TS and nested PCR, whereas the non-dominant species is identified only by PCR-based methods. Moreover, low-level *P. malariae* parasitemia is especially frequent in mixed infections, probably because of the characteristics of this species and the density-dependant regulation mechanism. Thus, this species could easily be overlooked by microscopy when *P. falciparum* or *P. vivax* is the major species. This fact also helps to explain the greater number of mixed infections detected with molecular biology methods compared to conventional microscopic tools[27].

PCR-based malaria diagnosis methods are very specific and sensitive but they cannot replace conventional microscopy in routine use[9], as they do not allow for the quantification of parasite forms; moreover, they are complex techniques with several steps and high costs. Thus, they can be useful as alternative and complementary tools for the diagnosis of human malaria in developing countries[4,13,19,22].

CONCLUSION

The results suggest that there were mixed infections (*P. falciparum* + *P. vivax*; *P. vivax* + *P. malariae*) in the study area that were not identified by the conventional microscopy method (TS). Furthermore, the two DNA obtainment methods evaluated, washing[26] and extraction with saponin/chelex-100[27], showed sensitivity and high specificity, as well as lower cost compared to available commercial kits; they were also easy and quick to perform. However, the second method was more sensitive; it provides a simple protocol for DNA extraction from dried blood spots and could be the protocol of choice for the DNA extraction step.

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Comparação entre dois métodos de obtenção de DNA a serem usados como protocolos alternativos para a detecção de parasitas humanos causadores de malária por nested PCR

RESUMO

Um diagnóstico laboratorial correto e preciso de malária humana ainda é considerado um desafio, pois o método de referência, o da gota espessa com colocação pelo Giemsa, apresenta limitações que dificultam o controle da malária. Devido a esses problemas, várias pesquisas têm objetivado desenvolver métodos alternativos para o diagnóstico da malária. Grande parte desses estudos aborda métodos de diagnóstico molecular, que têm acarretado o desenvolvimento de algumas alternativas ao método de coloração pelo Giemsa. No entanto, esses métodos, por sua vez, apresentam suas limitações, que incluem seu alto custo, a complexidade do protocolo e a variação da qualidade das fontes de DNA e de reagentes. Neste aspecto, a técnica de nested PCR tem demonstrado ser um bom método e pode ser melhorado usando uma fonte de DNA de alta qualidade. Neste estudo foram avaliados dois métodos para a obtenção de DNA de amostras de sangue seco colhidas em papel de filtro: 1) lavagem e 2) saponina/Chelex-100. O segundo método apresentou sensibilidade e especificidade mais altas em relação ao primeiro, pois detectou mais infecções, tanto simples como mistas, bem como infecções por *Plasmodium malariae*. Com base nesses resultados, apresentamos o segundo como o protocolo de escolha para a obtenção de DNA. A técnica de nested PCR usando saponina/Chelex-100 para extração de DNA pode ser um método alternativo ou complementar de diagnóstico de parasitas da malária humana, mas não é considerado adequado para o uso de rotina.

Palavras-chave: Malária; DNA; Reação em Cadeia da Polimerase.

Comparación entre dos métodos de obtención de DNA a ser usados como protocolos alternativos para la detección de parásitos humanos causadores de malaria por nested PCR

RESUMEN

Un diagnóstico de laboratorio correcto y preciso de malaria humana todavía se considera un desafío, pues el método de referencia, el de la gota espesa con colocación en Giemsa, presenta limitaciones que dificultan el control de la malaria. Debido a esos problemas, varias investigaciones han tenido como objetivo desarrollar métodos alternativos para el diagnóstico de la malaria. Gran parte de esos estudios aborda métodos de diagnóstico molecular, que han resultado en el desarrollo de algunas alternativas al método de teñido por Giemsa. Sin embargo, esos métodos, por su vez, presentan limitaciones, que incluyen su alto costo, la complejidad del protocolo y la variación de la calidad de las fuentes de DNA y de reactivos. En este aspecto, la técnica de nested PCR ha demostrado ser un buen método y puede ser mejorado usando una fuente de DNA de alta calidad. En este estudio se evaluaron dos métodos para la obtención de DNA de muestras de sangre seca recogidas en papel de filtro: 1) lavado y 2) saponina/Chelex-100. El segundo método presentó sensibilidad y especificidad más altas en relación al primero, pues detectó más infecciones, tanto simples como mixtas, bien como infecciones por *Plasmodium malariae*. Con base en estos resultados, presentamos el segundo método como el protocolo de elección para la obtención de DNA. La técnica de nested PCR usando saponina/Chelex-100 para extracción de DNA puede ser un método alternativo o complementario de diagnóstico de parásitos de la malaria humana, pero no se considera adecuado para uso de rutina.

Palabras clave: Malaria; ADN; Reacción en Cadena de la Polimerasa.

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