Rev. Inst. Med. trop. S. Paulo
40 (5): 333-334, September-October, 1998.

SIMPLIFIED DIAGNOSIS OF MALARIA INFECTION: GFM/PCR/ELISA A SIMPLIFIED NUCLEIC ACID AMPLIFICATION TECHNIQUE BY PCR/ELISA.

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SUMMARY

We report an adaptation of a technique for the blood sample collection (GFM) as well as for the extraction and amplification of *Plasmodium* DNA for the diagnosis of malaria infection by the PCR/ELISA. The method of blood sample collection requires less expertise and saves both time and money, thus reducing the cost by more than half. The material is also suitable for genetic analysis in either fresh or stored specimens prepared by this method.

KEYWORDS: Malaria; Diagnosis; Glass fibre membrane; PCR/ELISA

Rapid and prompt diagnosis of malaria infections followed by adequate chemotherapy is a major tool in malaria control. Traditionally, laboratory diagnosis of malaria parasites is based on Giemsa-stained blood smear. The limitations of this technique demanded more sensitive and accurate methods. The Quantitative Buffy Coat (QBC®)[4] and serological tests such as Indirect Immunofluorescence Antibody Test (IFAT)[2] were subsequently introduced. Although these techniques are useful, they present restrictions for the diagnosis of malaria infections and alternative methods are needed to overcome these problems. Recently, OLIVEIRA et al.[5] reported a polymerase chain reaction/enzyme-linked immunosorbent assay (PCR/ELISA) technique for the detection of plasmodia. In our hands, the QBC®, IFAT and PCR/ELISA techniques have worked well. The ability of the PCR/ELISA to detect all the human malaria parasites (*P. falciparum, P. vivax, P. malariae* and *P. ovale*) coupled with its sensitivity (parasitemia = 0.0001%) made it an attractive technique. This was adopted in our laboratory in Belém, Pará, Brazil for routine diagnosis and epidemiological surveys. The technique however is rather expensive and the DNA extraction step is relatively time consuming. MERCIER et al.[3] in 1990, described a more simple and efficient method to amplify DNA directly from whole blood samples without purification. Aliquots of amplified fragments from PCR, performed either on purified DNA or on non purified whole blood, showed no difference when analyzed directly on agarose gels by ethidium bromide staining. This method proved to be efficient not only on fresh blood samples but also on frozen blood samples stored for several months at -20 °C. WARHURST et al.[6] applied the glass fiber membrane (GFM) technique for malaria parasites aiming genetic studies. In the present study we report adaptations of the use of the GFM in the PCR/ELISA technique (GFM/PCR/ELISA) which allows a simple and rapid DNA preparation and cost-effective PCR amplification. Because funding is often limited in countries combatting malaria and other tropical diseases, simple and cost-effective diagnostic techniques are necessary to better serve for public service. This is important for methods applicable to epidemiological studies in which large numbers of samples accumulate for laboratory examinations.

For performing GFM/PCR/ELISA, blood samples obtained directly from patients were spotted on glass fibre membrane (GFM), and prepared for PCR using the method of WARHURST et al.[6] PCR amplification was carried out in 50 µl volume utilizing GFM prepared DNA. Primer sequences/concentrations and reaction conditions were described previously[4]. In the standardization step, parallel PCR amplifications were set up. One-hundred blood samples were used in each set of reactions (n=100 X 2). The first set of reactions used, a freshly prepared master-mix {33.25µl of ddH_2O, 0.25µl (1.25U) of Taq polymerase (Bioline - London, England), 1µl of each dNTP to provide 200 µM final concentration, and 5µl of 10 X reaction buffer (10mM tris-HCL pH8.3; 0.01% (w/v) gelatin; 1.5 mM $MgCl_2$; 50 mM KCl)}. The PCR Master®, a commercial master-mix kit (Boehringer Mannheim - Frankfurt, Germany) was used in the second set of reactions.

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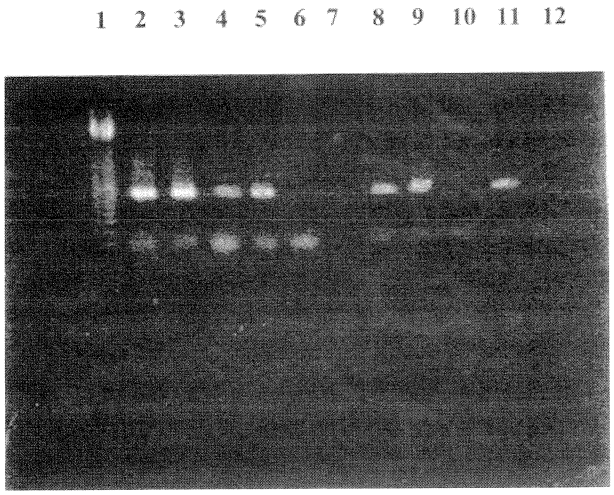

Fig. 1 - PCR amplified fragments eletrophoresed on a 1% agarose gel of the 678 bp from a *Plasmodium* genus - specific region of the 18 S rRNA [5]. Lane 1 shows 100 bp marker; lane 2-5 shows products using freshly prepared master mix; lane 7 is empty; lane 8-11 shows products obtained from using PCR Master® kit and lane 6,12 contain negative control. Lane 2 and 8 contain *Plasmodium vivax*; lane 3 and 9: *P. falciparum*; lane 4 and 10: *P. malariae* and lane 5 and 11: *P. ovale*.

The figure 1 presents gel electrophoresis of the 678pb products. Lanes 2-6 shows products obtained using freshly prepared master-mix while lanes 8-11 contain products obtained from using the PCR Master®. Interestingly, all the four human *Plasmodium* species were detected using the freshly prepared master-mix, while only *P. falciparum, P. vivax* and *P. ovale* were detected using the PCR Master®. Absence of a band however does not suggest amplification failure; perhaps the amount of PCR product electrophoresed is less than 25 ng. If so, it is unlikely that it will show up with ethidium bromide staining. However, it appears that using the home-made master-mix and GFM yielded better result (Figure 1). This suggests that the commercial master-mix kit in the PCR amplification may be substituted. The GFM/PCR technique was observed to be sensitive, detecting 0.001% parasitemia[1]. This is an added advantage to our already highly sensitive ELISA test. The PCR products were reacted with respective species-specific digoxigenin-labelled probes (see OLIVEIRA et al.[5]). The hybridization assay was positive for all products. Thus GFM/PCR/ELISA technique is highly sensitive. It is not surprising that the assay was positive for *P. malariae,* despite negative by agarose gel electrophoresis, due to the high sensitivity of the PCR/ELISA technique.

Although the QIAmp® Blood Kit (Boehring Mannheim - Frankfurt, Germany)/PCR has worked very well in our hands, the GFM/PCR procedure is simpler, rapid and less expensive. In the GFM/PCR/ELISA technique, we have been able to reduce cost greatly (about 1/3 of the cost) by using GFM prepared DNA and freshly prepared master-mix rather than QIAmp® Blood kit and PCR Master® as recommended in the original protocol. This new method is now routinely used in our laboratory for the diagnosis of malaria infection in Belém, Pará-State, Brazil. We are currently using the modified technique in an epidemiological survey of malaria infection in Belém and Macapá/Serra do Navio (Amapá State-Brazil). Field samples are spotted on GFM, allowed to air dry (avoiding direct sunlight), and kept in self-sealing bags which are subsequently mailed to our laboratory in Belém for analysis. The present method of blood sample collection requires less expertise and saves time, reducing the cost by more than half. Specific malarial genes sequences can also be analyzed from fresh and stored material.

RESUMO

Diagnóstico simplificado da infecção malárica: GFM/PCR/ELISA uma técnica simplificada de amplificação de ácido nucleico por PCR/ELISA

Relatamos a adaptação de uma técnica para coleta de amostras (MFV) e outra para extração, amplificação de DNA de parasitas da malária para diagnóstico por PCR/ELISA. O método de coleta de amostras requer menos habilidade e economisa tempo e dinheiro, assim reduzindo a mais da metade o custo. O material é também adequado para análise genética em especimens frescos ou estocados, preparados por este método.

ACKNOWLEDGEMENTS

We acknowledge the financial support of British Council (BRAS/881/161)/CNPq (Process 910119/95-3), CDC Atlanta, Welcome Trust and Instituto Evandro Chagas/FNS.

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Received: 19 February 1998
Accepted: 24 September 1998