Communication/Comunicação

Improvement of a PCR test to diagnose infection by *Mansonella ozzardi*

Adequação da técnica da PCR para diagnóstico de infecção de *Mansonella ozzardi*

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ABSTRACT

Introduction: Mansonelliasis is caused by *Mansonella ozzardi.* It is widespread in the Amazon region, with a high prevalence. The common exam of thick blood smears stained with Giemsa shows low efficacy levels and has been an obstacle to diagnosing individuals with low blood parasitemia. **Methods:** In order to increase diagnosis efficacy, the PCR technique was improved. **Results and Conclusions:** PCR demonstrated the best performance, with sensitivity and negative predictive values (NPV) of 100%, followed by blood filtration through membrane filters, which showed a sensitivity of 88.9% and a NPV of 84.6%, when compared to thick blood smears.

Keywords: *Mansonella ozzardi*. Diagnosis. PCR.

RESUMO

Introdução: A mansonelose é uma filariose causada pela *Mansonella ozzardi*, ocorrendo na Amazônia com prevalências de até 60%. A técnica de diagnóstico habitual (hemoscopia através da gota espessa) tem baixa eficácia o para o diagnóstico de pacientes com baixa parasitemia. **Métodos:** Neste contexto foi aperfeiçoada a técnica da PCR para seu diagnóstico. **Resultados e Conclusões:** Quando comparada à gota espessa, a PCR apresenta sensibilidade de 100%, e valor preditivo negativo (VPN) de 100% mostrando eficácia bastante superior à técnica da filtração em membrana que apresenta sensibilidade de 88,9% e VPN de 84,6%, quando também comparada à gota espessa de sangue.

Palavras-chaves: *Mansonella ozzardi*. Diagnóstico. PCR.

The microfilariae of *Mansonella ozzardi* (Nematode, Onchocercidae) is the etiological agent of the mansonelliasis. The parasite has a geographic distribution limited to the Americas and it is found from Mexico through Argentina, except Chile, Uruguay, and Paraguay[1]. This filariasis was first reported in Brazil in the City of Manaus in the State of Amazonas[2] and people infected by *M. ozzardi* were later identified along the Solimoes, Purus and Negro Rivers[3]. These findings reinforce many studies that have warned that *M. ozzardi* was widely distributed in the State of Amazonas[4], Brazil; however studies concerning this filariasis remain scarce[5-7].

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Mansonella ozzardi is transmitted by insects of the families Ceratopogonidae and Simuliidae (Diptera). Up to now, only the Simuliidaes have been identified as vectors in Brazil[8,9]. Infection symptomatology has been widely discussed and according to Batista et al[10], people suffering from mansonelliasis present moderate fever, leg coldness, joint aches, dizziness and headaches. Recently, new symptomatology has been attributed to this filariasis, which is characterized by ocular lesions and white rings on the cornea that may eventually cause blindness[11].

Typically, microscopic examination of a thick blood smear (TBS) stained with Giemsa, has been the main method used in epidemiological assays. Despite the low efficacy of TBS, particularly for patients with low microfilaremia, it is more economically feasible, promptly and easily obtained, and also permits identification of the parasite species even in locations where intermingled infections by other filariidaes occur[12]. More laborious and effective methods are feasible, such as Knott's concentration method (blood lysis by 2% formalin)[12,13], and by filtering the blood through a polycarbonate membrane filter (PMF)[12]. The PMF is considered the gold-standard. Morales-Rojas et al first suggested polymerase chain reaction (PCR) as an effective method to detect *M. ozzardi*[14].

This study aimed to standardize the PCR for diagnosing *M. ozzardi* and compare its effectiveness in relation to the PMF, which is considered the gold-standard for diagnosing the disease compared to the traditional TBS method.

Forty-seven patients infected by *M. ozzardi* were randomly selected from 232 individuals diagnosed by PMF (Poretics Corporation, Livermore, USA). These individuals lived alongside the rivers in the municipality of Labrea, State of Amazonas, Brazil (07°15'S 64°51'W), an area with high prevalence of mansonelliasis. The TBS method was used according to the following procedure: blood was collected from the digital pulp (equivalent to 0.06mL) using disposable lancets and then prepared on microscope slides and left to dry at room temperature. Next, the slides were washed with distilled water, fixed with methanol, stained with Giemsa and dried off. The slides were then subjected to microscopic examination using optical objectives of 10x and 40x by two professionals in a blind controlled trial. PMF was conducted by filtering 1mL of venous blood previously diluted with saline 0.9% through a polycarbonate membrane filter of 3-micrometer pore diameter size (Poretics Corporation - Livermore-USA)[12].

The PCR method was based on the detection of the GenBank AF228564 DNA fragment from *M. ozzardi* which comprises the partial sequence of the 18S ribosomal RNA gene, the internal transcribed spacer 1, the complete sequence of the 5.8S ribosomal

RNA gene and internal transcribed spacer 2 (ITS2) and the partial sequence of the 28S ribosomal RNA gene[13]. The forward primer used was 5'GAAAGAAGAAGGATTTTACT3' on the ITS2 and the reverse primer used was 5'CTTTTCCTCCGCTTAATTATA3' on the 28S ribosomal DNA. These genes are commonly used for species identification on molecular bases since they comprise highly conserved sequences next to highly variable ones. The primers were verified using the NCBI primer design on-line tool based on the Primer3[15] software and showed no unintended DNA products.

Genomic DNA was extracted from the samples according to the instructions of the Quiamp DNA Blood Kit (Qiagen®). Blood samples of newborns from the city of Porto Velho were taken as negative control. The eluted DNA were maintained in microtubules and stored at -20ºC. The following procedure was used to amplify the DNA sequence: the samples were maintained A) at a temperature of 94°C for 4min (1 cycle); B) at 94°C for 45sec; at 55°C for 30sec; 72°C for 45sec (35 cycles); C) 72°C for 5min (1 cycle), and D) 4°C for 1min (final time). The amplification product up to 312bp was viewed on 10% polyacrylamide gel with bromide staining (**Figure 1**).

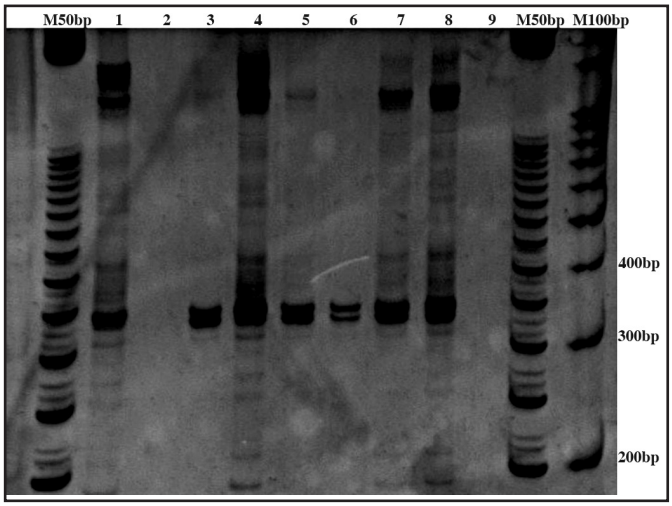

FIGURE 1 - Electrophoresis in 10% polyacrylamide gel showing the PCR results for DNA extracted from blood.

M50pb stands for the 50 base pairs size marker, M100bp for the 100 base pairs size marker and the numbered lanes are: 1: sample 709, positive control; 2: newborn 90, negative control; 3-8: samples 704, 708, 711, 712, 714 and 715; 9: sterile water negative control.

Both the PMF and PCR methods were compared to the common method for *M. ozzardi* diagnosis (TBS). In order to compare the effectiveness of the methods, the following parameters were considered: sensitivity, specificity, negative predictive value (NPV), and Cohen's kappa coefficient (**Tables 1** and **2**). The OpenEpi® software was used for statistical analysis, with a confidence interval (CI) of 95%. The project was submitted to the Research Ethics Committee of the São Lucas College, Porto Velho and was approved under the registry number 344/09.

Of the 29 individuals who presented negative by TBS, 62.1% were identified as positive by PMF, while among the 18 who were positive by the TBS method, 88.9% showed the same results by PMF. On the other hand, TBS diagnosed 2 cases that were not diagnosed by PMF (false-negatives), despite its greater sensitivity. Regarding the PCR technique, 89.6% of those who presented negative by TBS were positive by PCR, while 100% of the positive cases showed the same results by PCR. Thus, the PCR technique showed the greatest

TABLE 1 - Effectiveness parameters of the polycarbonate membrane filtering method and the thick blood smear exam.

Laboratory method	Positive TBS	Negative TBS	Total
Positive PMF	16	18	34
Negative PMF	2	11	13
Total	**18**	**29**	**47**
Sensitivity	88.9%	(67.2 - 96.9)*	
Specificity	37.9%	(22.69 - 56.0)*	
Negative predictive value	84.6%	(57.76 - 95.67)*	
Kappa coefficient	0.2	(0.004 - 0.40)*	

TBS: thick blood smear exam, PMF: polycarbonate membrane filtering method. *confidence interval.

TABLE 2 - Effectiveness parameters of the polymerase chain reaction and the thick blood smear exam.

Laboratory method	Positive TBS	Negative TBS	Total
Positive PCR	18	26	44
Negative PCR	0	3	3
Total	**18**	**29**	**47**
Sensitivity	100.0%	(82.41 - 100.0)*	
Specificity	10.3%	(3.6 - 26.4)*	
Negative predictive value	100.0%	(43.8 - 100.0)*	
Kappa coefficient	0.08	(-0.03 - 0.19)*	

TBS: thick blood smear exam, PCR: polymerase chain reaction, *confidence interval.

sensitivity and NPV, followed by PMF, when compared to TBS. However, both these methods showed low specificity and low kappa coefficient compared to TBS.

Despite the encouraging results, the cost of PCR is approximately US$8.60 per test, while PMF has a significantly lower cost, US$0.80 (author's personal information). The difficulties of performing PCR under field conditions must also be taken into account, since this is an important limiting factor in its application. Traditionally, studies concerning the prevalence of the *M. ozzardi* indicate its low prevalence rate in young individuals[6-8], which is probably due to their low microfilaria concentration. The advent of PCR for mansonelliasis diagnosis, which shows 100% sensitivity, may help correct this bias by providing new epidemiological information on parasitic infections and by promoting the determination of higher prevalence rates.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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