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DETECTION OF *MYCOBACTERIUM* IN CLINICAL SAMPLES BY MULTIPRIMER POLYMERASE CHAIN REACTION

Abdulmonam Saied Barouni; Halha Ostrensky Saridakis; Marilda Carlos Vidotto*

Universidade Estadual de Londrina, Departamento de Microbiologia, Londrina, PR, Brasil.

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ABSTRACT

We investigated the use of multiprimer-PCR for detection of mycobacteria species in clinical samples. Three different mycobacterial genomic fragments were investigated: the IS6110 insertion sequence, present in *M. tuberculosis* complex; the genus specific fragment (32kDa); and from *M. tuberculosis* species-specific *mtp*40 gene. The sensitivity and specificity using 135 clinical isolates were 94.5% and 95.9%, respectively, compared with culture in Löwenstein-Jensen medium; the detection limit was 0.05ng of DNA. In conclusion, this assay is reliable and rapid for detection of *Mycobacterium* species in clinical samples, and differentiates *M. tuberculosis* from *M. bovis* strains in a single-step assay.

Key words: *Mycobacterium tuberculosis*, multiprimer-PCR, molecular diagnosis

INTRODUCTION

Tuberculosis remains a worldwide public health problem; its incidence has increased after the rise of human immunodeficiency virus (HIV) infection and bacterial drug resistance (9). In Brazil, the population presents high rates of tuberculosis, 100.000 cases per year. A differential identification of the type of mycobacteria present in patients is important for the control of the disease and effective antituberculosis treatment. Differentiation between *M. tuberculosis* and other members of the *M. tuberculosis* complex, which includes human-pathogenic species such as *M. bovis* e *M. africanum*, would also be desirable epidemiologically. *M. bovis,* the cause of tuberculosis in cattle, is a pathogen for a large number of others animals, and its transmition to humans constitutes a public health problem (6).

The definitive diagnosis depends on culture of the mycobacteria, which is a time-consuming and not always sensitive enough. Polymerase chain reaction (PCR), a highly sensitive DNA amplification technique, has been evaluated for detection of *M. tuberculosis* in either cultured strains or noncultured clinical samples, being of special interest for use with those specimens containing few mycobacteria (5,12,13). Although rapid and more sensitive than culture, the use of PCR has been hindered by problems such as contamination and inhibition of the reaction (2). Use of PCR for rapid diagnosis of tuberculosis in a developing country showed 90% of sensitivity and 81% of specificity, requiring a standardization of the technique (1). The choice of the target sequence, which should be present in all tuberculosis mycobacteria and absent in all other bacteria, is crucial for the successful use of DNA amplification for detection of those bacteria. Multiprimer PCR system, which involves the use of three sets of primers, each annealing to different bacterial genomic fragments, allowed discrimination between *M. tuberculosis*, *M. bovis*, and other nontuberculous mycobacteria (4,7).

In this study, the multiprimer PCR system described by Del Portillo *et al.* (4) was applied to clinical samples in an epidemiological study in Brazil. Sputum specimens (n=135) obtained from patients with respiratory symptoms at the Hospital of Londrina University and at the Central Health Laboratory at Maringá (Paraná State), during 1997 and 1998, were analysed by standard microbiology methods and by PCR. Specimens were digested and decontaminated by the standard protocol with N-acetyl-L-cysteine-NaOH (3) and used for microscopy by Ziehl-Neelsen staining, culture, and PCR. The samples were

*Corresponding author. Mailing address: Universidade Estadual de Londrina, Departamento de Microbiologia - CCB, Campus Universitário, C.P. 6001. 86051-970, Londrina, PR, Brasil. Tel.: (+5543) 3714396. Fax: (+5543) 3714207. E-mail: macarlos@uel.br

inoculated on Löwenstein-Jensen slants and incubated at 37ºC and 25ºC for 8 weeks, and all colonies isolated were identified by biochemical tests (3). The following strains were used as standards to test the specificity and sensitivity of the reaction: *Mycobacterium tuberculosis* H37Rv, *M. bovis* clinical isolate from Brazil-SP, *M. avium-intracellulare* ATCC 13950, *M. chelonae* ATCC 946, *M. fortuitum* ATCC 6841, and *M. kansasii* ATCC 12478, *M. smegmatis* (clinical isolate). *Staphylococcus aureus* ATCC 6538, *Streptococcus hemolyticus* (clinical isolate) and *Acinetobacter baumannii* (clinical isolate).

DNA from clinical specimens and bacterial strains were isolated as previously described (4), with some modifications: bacterial strains or pellet from 400ml of treated sputum were washed in 10x TE (1x TE is 10mM Tris-HCl, pH 8.0; 1mM EDTA) and the pellet was resuspended in 300ul of TE containing 2mg/ml of lysozyme, and incubated for 1.5 h at 37ºC. This was followed by incubation with 4 ul of proteinase K (20mg/ml), and 30 ul of sodium dodecyl sulfate (SDS) (10%) for 1.5 h at 65ºC. DNA was purified from the aqueous phase by phenol-cloroform (1:1) and precipitated with 0.6 V of isopropanol and 100mM NaCl. The pellet was washed with 70% ethanol and resuspended in 30 ml milli Q water, from which 5 ml were used for the multiprimer PCR.

The multiprimer PCR reaction mixture was prepared in a final volume of 25ml: PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5mM MgCl2), 2.5U of Taq polymerase, 0.5mM each deoxynucleoside triphosphate, 10pmol of MT1: TTCCTGACCAGCGAGCTGCCG and MT2: CCCCAGTACTCCCAGCTGTGC; 15pmol of IS5: CGGAGACGGTGCGTAAGTGG and IS6: GATGGACCGC CAGGGCTTGC; 20pmol of PT1: CGGCAACGCGCCGTC GGTGG and PT2: CCCCCCACGGCACCGCCGGG; and 5ml of sample DNA. Cycles were initiated at 95ºC for 5 min, followed by 30 cycles with denaturation at 94ºC for 1 min, annealing at 68ºC for 2 min, and extension at 72ºC for 1 min. The samples were kept for 5 min at 72ºC for final extension, and kept at 4ºC in the thermal cycler until removed for analysis. In each experiment, we included a positive control of *M. tuberculosis* DNA extracted from a culture, as well as a negative control obtained by replacing the sample with milli Q water. The amplification products were visualized by direct analysis of 10ml on 2.0% agarose gels containing 0.5 mg/ml of ethidium bromide, which was electrophoresed in Tris-borate-EDTA (TBE) (89 mM Tris-base, 89 mM boric acid, 2.5mM EDTA). The gel was run at 100V for 1h and the DNA was visualized by UV transilumination and photographed.

Southern blot hybridization was used to confirm the specificity of PCR products. PCR products, from reference *Mycobacterium,* electrophoretically separated on agarose gels were transferred onto nylon membranes (Gene Screen Plus). Nylon membranes were incubated with pre-hybridization solution containing 50% formamide (v/v) deionized, 5x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent (Boehringer Mannheim) at 42ºC for 1 h. The probes were labeled with digoxigenin-dUTP by using the primers IS5 and IS6, MT1 and MT2, and PT1 and PT2, and PCR DIG Probe Kit (Boehringer Mannheim) according to the instructions of the manufacturer.

The multiprimer PCR assay showed three DNA bands, which correspond to a 984-pb amplification fragment from the IS6110 insertion sequence present in *M. tuberculosis* complex, a 506-bp amplification fragment from the gene for the 32-KDa antigen, which is present in most of the species belonging to the genus *Mycobacterium*, and a 396-bp fragment from the species-specific MTP40 gene of *M. tuberculosis* (Fig. 1).

What concerns specificity of this assay, considering standard mycobacterial species and other bacterial species, only *M. tuberculosis* presented all three bands, whereas the 506-bp band was detected in all *Mycobacterium*. No amplification products were observed in *Staphylococcus aureus*, *Streptococcus hemolyticus* and *Acinetobacter baumannii*. Southern blot hybridization confirmed the specificity of the amplified products. *M. tuberculosis* and *M. bovis* hybridized with the DIG labeled 984 bp fragment IS, whereas hybridization was not observed with samples of nontuberculous mycobacteria and other strains. DIG labeled 506 bp MT probe hybridized with all mycobacteria, and DIG labeled 396 bp PT probe hybridized only with *M. tuberculosis* (data not shown). The sensitivity of this assay was evaluated by using serial dilutions of DNA (50ng to 50 fg) from three clinical samples, quantified by Fluorometer DyNA (200 Hoefer Pharmacia); the limit of detection was 0.05 ng (data not shown).

Of the 135 sputum samples examined, 26 presented acid-fast bacilli by Ziehl-Neelsen staining (positive smears). Growth on Löwenstein-Jensen slants (positive culture) was obtained with 37 samples, 35 of which were identified as *M. tuberculosis*, one sample was identified as *M. avium* and another as *M. fortuitum*. Thirty-nine samples were positive by multiprimer PCR. Fig. 1 shows multiprimer PCR amplified bands of DNA from *M. tuberculosis* and *M. bovis* in clinical samples, some of which were negative in Ziehl-Neelsen stain or in culture.

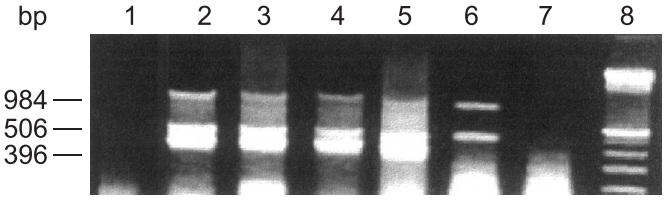

Figure 1. Detection of *M. tuberculosis* and *M. bovis* in clinical samples after amplification with PCR multiprimer. The PCR products were then analyzed by eletrophoresis in 2% agarose gel stained with ethidium bromide. Lane 1, negative control without target DNA; lane 2, 4, 5, 6 and 7, smear +/ culture +; lane 3, smear -/ culture +; lane 8, smear -/ culture - samples identified as *M.bovis*; lane 9, smear - / culture - ; lane 10, MW bp100.

The correlation between multiprimer PCR and the smears and culture is shown in Table 1. With the exception of two samples, all the samples positive by Ziehl-Neelsen staining or in culture were also positive by multiprimer PCR. In contrast, four samples, which had given negative results in convencional tests, were positive by multiprimer PCR. Thirty five samples (25 smear-positive and 10 smear-negative) were multiprimer PCR and culture positive. The sensitivity of this method for sputum, compared to that of cultures, was 94.5% and the specificity was 95.9%. Only two samples with positive cultures were PCR negative, but four samples with negative cultures were PCR positive. The presence of two false-negative samples by this method can be due to insuficient amount of samples or the presence of inhibitors. Other authors found lower sensitivity (88%) for two-band multiprimer-PCR to detect *M. tuberculosis* in clinical specimens (7).

The results obtained in this study with multiprimer PCR are similar to those reported by other authors using single step PCR. However, it is difficult to compare the results obtained by those groups, since not only extraction and purification procedures were different, but the methodology and target sequence were also different. Pao *et al.* (8), using primers from 65kDa protein, found a correlation between PCR and bacteriology data with sensitivity of 100% and specificity of 62.6%. Querol *et al.* (10) studied 314 respiratory samples and found a correlation between culture and PCR in 97%, using primers from IS6110. Thoe *et al.* (13), evaluating Amplicor- and IS6110-PCR for detection of *M. tuberculosis* complex in Singapore, detected 86.5% and 83.6% of sensitivity and specificity, respectively; and suggested that size of inoculum, nonuniform samples due to clumping effect of mycobacteria, and the absence of target gene sequences for IS6110-PCR, were the reasons for false negative results. Rajalahti *et al.* (11) using automated Cobas Amplicor MTB PCR found sensitivity of 83% for this method.

Table 1. Detection of *M. tuberculosis* in sputum by multiprimer PCR.

Microscopy	Multiprimer	Number of samples with the following culture result		
		Positive	Negative	Total
Positive	Positive	25	0	25
	Negative	1	0	1
	Total	26	0	26
Negative	Positive	10	4	14
	Negative	1	94	95
	Total	11	98	109
Total	Positive	35	4	39
	Negative	2	94	96
	Total	37	98	135

Similar to the results of Del Portillo *et al.* (4), we also identified *M. tuberculosis* and *M. bovis* in addition to other nontuberculosis mycobacteria. This multiprimer system has the advantage of discriminating *M. tuberculosis* and *M. bovis* from nontuberculosis mycobacteria in a single step within 48 h, and should be easy to use in most microbiology laboratories since detection can be achieved by simple agarose electrophoresis. This is important due to an increase in tuberculosis, mycobacterial diseases caused by nontuberculous mycobacteria, and infections by more than one mycobacterial species in immunosuppressed patients. Also, *M.* bovis has not been eradicated in Brazil and *M.* bovis BCG strains are widely used as a vaccine to prevent tuberculosis; therefore its differentiation from *M. tuberculosis* has additional public health implication (9).

The results presented in this study indicate that the multiprimer method improves tuberculosis diagnosis, with the advantage that this method is more rapid and more sensitive than classical bacteriology methods. This method could be applied directly to clinical samples in medical and veterinary laboratories, and can be used to differentiate *M. tuberculosis* from *M. bovis* strains.

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RESUMO

Detection of *Mycobacterium* in clinical samples by multiprimer polymerase chain reaction

PCR utilizando vários oligonucleotídeos para detecção de espécies de micobactérias em espécimes clínicas foi padronizado. Três diferentes fragmentos genômicos: da seqüência de inserção IS6110, presente no complexo *M. tuberculosis*, do gene responsável por proteína específica (32kDa) do gênero e do gene *mtp*40 espécie-específico do *M. tuberculosis* foram estudados. A sensibilidade e especificidade do método em 135 amostras clínicas foram de 94.5% e 95.9%, respectivamente, comparados com os resultados da cultura em meio Löwenstein-Jensen, e o limite de detecção foi de 0.05 ng of DNA. O ensaio mostrou-se confiável e rápido para detecção de espécies de *Mycobacterium* em amostras clínicas, diferenciando *M. tuberculosis* de *M. bovis* em uma única reação.

Palavras-chave: *Mycobacterium tuberculosis*, diagnóstico molecular, IS6110, gene *mtp*40, PCR

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