Artigo Original

Using polymerase chain reaction with primers based on the *plcB-plcC* intergenic region to detect *Mycobacterium tuberculosis* in clinical samples*,**

Deteção de *Mycobacterium tuberculosis* em amostras clínicas por reação em cadeia da polimerase utilizando primers baseados na região intergênica *plcB-plcC*

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Abstract

Objective: To develop a system for the molecular diagnosis of tuberculosis by polymerase chain reaction (PCR), constructing primers based on the difference in gene organization of the intergenic region of phospholipase C (*plcB-plcC* region), which differentiates *Mycobacterium tuberculosis* from other mycobacteria. **Methods:** A PCR product of the expected size (432 bp) was obtained from *M. tuberculosis* and *M. africanum* only. A total of 33 mycobacterial isolates and 273 clinical samples from patients suspected of having tuberculosis were examined. These were used in the comparative study of the PCR technique versus culture. **Results:** For PCR versus culture, the data showed 93.8% accuracy ($p < 0.0001$), 93.1% sensitivity (CI: 88.7-96.0), and 96.4% specificity (CI: 96.1-99.4). The Kappa value (0.82) shows that there was a near-perfect concordance between the two tests. **Conclusion:** The use of the *plcB-plcC* region in PCR amplification was found to be an important and reliable tool for the specific diagnosis of tuberculosis in the samples analyzed.

Keywords: Polymerase chain reaction; Diagnosis; Tuberculosis; *Mycobacterium tuberculosis*.

Resumo

Objetivo: Desenvolver um sistema para o diagnóstico molecular da tuberculose por reação em cadeia da polimerase, do inglês *polymerase chain reaction* (PCR), pela construção de *primers* baseados na diferença da organização de uma região intergênica da fosfolipase (*phospholipase*) C (região *plcB-plcC*), que diferencia *Mycobacterium tuberculosis* das outras micobactérias. **Métodos:** Um produto de PCR com o tamanho esperado (432 pb) foi obtido somente de *M. tuberculosis* e *M. africanum*. Um total de 33 isolados micobacterianos e 273 amostras clínicas de pacientes com suspeita de tuberculose foram examinados. Estes foram submetidos ao estudo comparativo da técnica de PCR contra o cultivo. **Resultados:** Os dados mostraram 93,8% de exatidão para PCR contra o cultivo ($p < 0,0001$), 93,1% de sensibilidade (IC: 88,7-96,0) e especificidade de 96,4% (IC: 96,1-99,4). O valor de Kappa foi de 0,82, demonstrando um alinhamento perfeito para a verificação do grau de concordância entre os testes. **Conclusão:** O uso da região *plcB-plcC* para a amplificação por PCR é mostrado como uma ferramenta importante e de confiança para o diagnóstico específico de tuberculose nas amostras clínicas analisadas.

Descritores: Reação em cadeia da polimerase; Diagnóstico; Tuberculose; *Mycobacterium tuberculosis*.

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Introduction

Fast and accurate diagnosis is an important element of global health measures to control tuberculosis (TB). Moreover, since 90% of TB cases occur in developing countries,[1] a cost-effective diagnostic tool for TB that could be routinely used would be of great benefit. A number of methods based on nucleic acid amplification, including polymerase chain reaction-restriction enzyme analysis (PCR-REA),[2] commercial DNA assays,[3] PCR-based sequencing technologies,[4] and, more recently, real-time PCR assays,[5] have been developed for use in the identification of mycobacteria. A shortcoming of the PCR-REA method is its inability to distinguish the small differences in band size between some species of mycobacteria,[2] whereas a major drawback of commercial assays is their high cost. In addition, it has been reported that a commercial direct amplification test designed for the in vitro diagnostic detection of *Mycobacterium tuberculosis* complex rRNA can produce false-positive results for *M. tuberculosis* in AIDS patients infected with *M. kansasii* or *M. avium*, as well as in HIV-negative patients with chronic lung disease caused by *M. avium*.[6] Another commercial test has such a low sensitivity for smear-negative specimens that the United States Food and Drug Administration has approved it only for direct detection of *M. tuberculosis* in acid-fast bacilli smear-positive respiratory specimens.[7,8] In addition, the ligase chain reaction test has not demonstrated a sufficient degree of accuracy with smear-negative specimens to warrant its use on a routine basis.[9] Although the use of nucleic acid sequencing is highly accurate, it is labor-intensive and requires expensive equipment. Therefore, various laboratories use in-house methods for the detection of *M. tuberculosis*. These assays have a wide variety of nucleic acid targets, such as insertion sequence (IS)*6110*, ribosomal ribonucleic acid genes, 65-kDa antigen, and heat shock protein 65 gene.[8] In such in-house assays, IS6110 is probably the target most widely used in the molecular detection of *M. tuberculosis*.[7,10] However, *M. tuberculosis* strains that do not contain IS*6110* have been reported,[5,11,12] and IS*6110* homologs have been found in *M. fortuitum*, *M. avium*-*M. intracellulare* complex, *M. kansasii*, *M. xenopi*, *M. malmoense*, and *M. chelonei* clinical isolates,[13,14] as well as in *M. bovis*.[15] According to data from the Pan American Health Organization and World Health Organization, 7000 new cases of human TB caused by *M. bovis* are reported in South America each year.[16] Differentiation between *M. tuberculosis* and *M. bovis* is particularly important, since the latter is resistant to pyrazinamide, a drug commonly used in the treatment of TB.[17] However, current methodologies to distinguish between *M. tuberculosis* and *M. bovis* have been largely unsuccessful due to their lack of resolution,[18] as well as to the fact that skilled laboratory personnel and relatively expensive consumable reagents are required.[19,20]

Upon performing comparative hybridization experiments, eleven regions present in *M. tuberculosis* H37Rv were found to be absent from one or more virulent strains of *M. bovis*[21]; among them, a cluster of three phospholipase C genes (*plcA*, *plcB*, and *plcC*).[21] Accordingly, we have attempted to develop a rapid, low-cost, direct PCR method based on the amplification of a DNA fragment including the *plcB*-*plcC* intergenic region for detection of *M. tuberculosis*, which will allow *M. tuberculosis* to be the differentiated from *M. bovis* in clinical samples.

Methods

A total of 33 mycobacterial isolates and 273 clinical samples were tested. All mycobacterial strains (*M. tuberculosis* H37Rv, *M. africanum*, *M. bovis*, *M. abscessus*, *M. aichiense*, *M. avium*, *M. asiaticum*, *M. aurum*, *M. chitae*, *M. chubuense*, *M. duvalii*, *M. flavescens*, *M. fortuitum*, *M. gastri*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, *M. lentiflavum*, *M. mageritense*, *M. marinum*, *M. microti*, *M. neoaurum*, *M. nonchromogenicum*, *M. parafortuitum*, *M. peregrinum*, *M. porcinum*, *M. scrofulaceum*, *M. simiae*, *M. smegmatis*, *M. szulgai*, *M. triviale*, *M. vaccae*, and *M. xenopi*) were identified by conventional culturing and biochemical tests in the Molecular Biology Mycobacteria Laboratory of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). The clinical samples were obtained patients treated at the Sanatório Partenon Hospital (Porto Alegre, Brazil) or the Espírito Santo State Center for Infectious Diseases (Vitória, Brazil). Sputum specimens were collected from the respiratory tract of patients. One aliquot was inoculated in Löwenstein-Jensen medium, and 200 µl were used for DNA purification/PCR amplification. All clinical isolates

in this study were identified on the basis of conventional techniques, including the determination of microbiological characteristics and biochemical tests.

The mycobacterial strains and clinical samples were inactivated at 95 °C for 10 min. The DNA was extracted using QIAamp Tissue DNA Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Purified DNA (10 µl) was added to 40 µl of reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 1.0 U Platinum Taq DNA polymerase (Invitrogen Life Technologies, Gaithersburg, MD, USA), and 0.2 mM dNTPs (Invitrogen Life Technologies), together with 10 pmol each of the primers BC3 (5'-TCGACGGCAGAAAGCGTGCC-3') and BC5 (5'-GCGGCTCAATGCGCTTCCG-3'). The primers BC3 (complement of bases 200266 to 200285) and BC5 (bases 200679 to 200697), which are based on the *M. tuberculosis* H37Rv sequence (EMBL accession number BX842579), amplified a 432-bp DNA fragment comprising the 3' end of the *plcB* gene, the intergenic region, and the 5' end of the *plcC* gene. PCR amplification was performed as follows: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, extension at 72 °C for 30 s; and a final extension step at 72 °C for 5 min. The PCR products were analyzed by agarose gel electrophoresis (2% w/v) and stained with ethidium bromide. The clinical samples were also amplified with specific primers for human β-globin gene GH20 and PC04[22] in order to detect inhibitory samples.

Direct sequencing of PCR fragments was performed in a commercial laboratory (CATG, Porto Alegre, Brazil) using the ABI PRISM BigDye Terminator kit (PE Applied Biosystems, Foster City, CA, USA), and the output was analyzed with an ABI 3700 DNA sequencer. The PCR amplification primers were also used as sequencing primers, and at least a single base overlap from two directions for each was usually achieved. The FASTA program[23] was used to compare the amplified sequence with a DNA sequence from the GenBank database.

The collected data were analyzed using SPSS software (version 1.0 for Windows). Pearson's chi-square was used to assess relationships between categorical variables, and the data were interpreted using the Kappa table.

Results

In order to optimize the PCR, we tested amplifications with different concentrations of $MgCl_2$ (1.5-2.5 mM). The best amplification efficiency was achieved with $MgCl_2$ at a concentration of 2 mM. A strong, 432-bp product, without nonspecific fragments and without abundant primer dimers, was observed. A PCR product of the expected size was obtained for *M. tuberculosis* and *M. africanum*. Particularly encouraging was the absence of any PCR product observed for *M. bovis* (Figure 1). In addition, no amplification was observed using DNA from other organisms, such as humans, *Escherichia coli*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*. When we tested amplification with $MgCl_2$

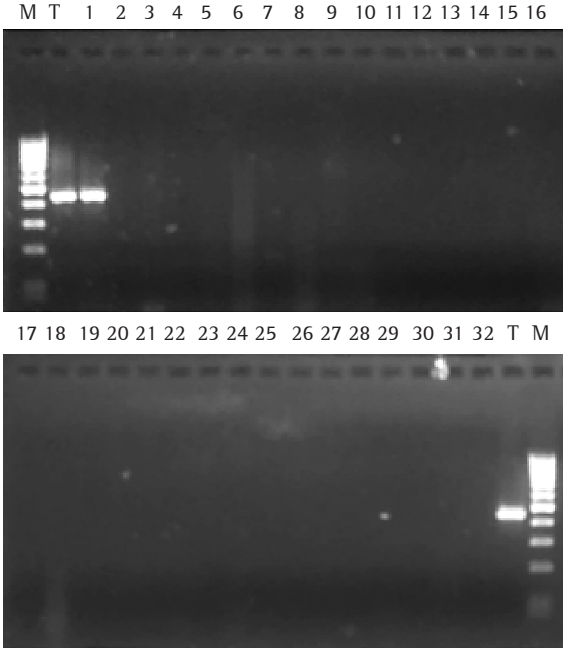

Figure 1 - Analysis of mycobacterial strains by PCR using BC3-BC5 primers - Lanes: M, 100-bp DNA Ladder (Invitrogen); T, *M. tuberculosis* H37Rv; 1, *M. africanum*; 2, *M. bovis*; 3, *M. abscessus*; 4, *M. aichiense*; 5, *M. avium*; 6, *M. asiaticum*; 7, *M. aurum*; 8, *M. chitae*; 9, *M. chubuense*; 10, *M. duvalii*; 11, *M. flavescens*; 12, *M. fortuitum*; 13, *M. gastri*; 14, *M. gordonae*; 15, *M. intracellulare*; 16, *M. kansasii*; 17, *M. lentiflavum*; 18, *M. mageritense*; 19, *M. marinum*; 20, *M. neoaurum*; 21, *M. nonchromogenicum*; 22, *M. parafortuitum*; 23, *M. peregrinum*; 24, *M. porcinum*; 25, *M. scrofulaceum*; 26, *M. simiae*; 27, *M. smegmatis*; 28, *M. szulgai*; 29, *M. triviale*; 30, *M. vaccae*; 31, *M. xenopi*; 32, water.

concentration of 2.5 mM, a faint 445-bp band was amplified with *M. marinum*, whereas all other mycobacterial strains tested resulted either in PCR products with sizes different from those expected or in no observable PCR product (data not shown). All further amplifications were then performed with 2 mM $MgCl_2$. The 432-bp DNA fragment was isolated and sequenced. It was found to be identical to the 200266-200697 nucleotide sequence of *M. tuberculosis* H37Rv deposited in the GenBank (BX842579).

Of the 273 clinical samples analyzed by PCR (Table 1), 93.8% were in agreement with the culture results ($p < 0.001$). Only 2 of the 55 culture-negative samples were identified as positive in the PCR. However, 15 of the 203 culture-positive samples were identified as negative in the PCR. Comparing the PCR results with the culture results, the sensitivity, specificity, positive predictive value, and negative predictive value were, respectively, 93.1% (CI: 88.7-96.0), 96.4% (CI: 96.1-99.4), 99% (CI: 96.1-99.8), and 77.9% (CI: 65.9-86.7). The Kappa value (0.82) showed that there was a near-perfect concordance between the two tests. These results compare favorably with those compiled in a recent review of the literature.[8]

Table 1 - Comparison between PCR results and culture results for clinical samples.

	Culture results		Total
	Positive	Negative	
Positive PCR	203	2	205
Negative PCR	15	53	68
Total	218	55	273

Discussion

The rapid identification of *M. tuberculosis* is crucial to optimizing patient recovery. Species identification can take several weeks to complete, during which time the patient might receive inappropriate antimycobacterial agents. This is particularly worrisome if the patient is infected with *M. bovis* rather than *M. tuberculosis*, since *M. bovis* is resistant to pyrazinamide, a first-line antitubercular drug.[19] In the present study, we designed and tested the primers BC3 and BC5, which differentiate *M. tuberculosis* from *M. bovis*. The genomic fragment designated *mtp40* has been tested as a species-specific DNA target for the identification of *M. tuberculosis*.[24] However, the *mtp40* sequence has been shown to be absent from a number of *M. tuberculosis* strains.[18,25] Interestingly, the *mtp40* sequence has been shown to be part of the *plcA* gene.[25] In the present study, we used primers flanking the junction of the 3' end of the *plcB* gene and the 5' end of the *plcC* gene. In addition, our attempts to amplify a DNA fragment comprising the 3' end of the *plcA* gene, the intergenic region, as well as one comprising the 5' end of the *plcB* gene, failed (data not shown).

The absence of any observed PCR product for *M. bovis* was expected, since the deletion of three *plc*-encoding genes (*plcA*, *plcB*, and *plcC*) in *M. bovis* (virulent strains and BCG strains) had already been reported by some authors.[21]

Our PCR assay cannot differentiate *M. tuberculosis* from *M. africanum*, but both are susceptible to the same chemotherapeutic agents. Some authors[26] developed a PCR-based method to differentiate the subspecies of the *M. tuberculosis* complex on the basis of genomic deletions. Seven primer pairs were used in order to construct the *M. tuberculosis* complex PCR typing. However, the authors found that their method was unable to differentiate *M. tuberculosis* from *M. africanum*. The PCR method described in the present study was also unable to differentiate between *M. tuberculosis* and *M. africanum*. However, we used only one pair of primers, and the interpretation of results was straightforward. Real-time PCR assays for the detection, differentiation, and quantitation of *M. tuberculosis* have recently been developed.[5] However, none of them can differentiate *M. tuberculosis* from *M. africanum* or from *M. bovis*.

The PCR results are in good agreement with the culture data, except for the 15 samples that were identified as negative in the PCR and positive in culture (out of 203 culture-positive samples) and the 2 samples that were identified as positive in the PCR and negative in culture (out of 55 culture-negative samples). In the case of the false-negatives, sensitivity might have been increased by the fact that more than one sample was collected from each patient.[10] In contrast, the false-positives might be attributable to the inclusion of paucibacillary patients presenting symptoms indicating infection with TB.

Within the *M. tuberculosis* complex, bacterial *plc*-encoding genes have been reported to play a role in the pathogenesis of many bacteria. Genetic

variations (IS*6110* insertions and deletions) have been observed in the *plcA-plcB-plcC* and *plcD* regions.[27,28] At *M. tuberculosis plcC* positions 19849 and 19668,[27] as well as 19589, 19645, and 19848,[29] IS*6110* insertions have been observed. These findings indicate that this domain is a preferential integration region, defined as a <500-bp chromosomal domain.[27] One group of authors[30] reported that, in *M. tuberculosis*, there was a much higher frequency of IS*6110* insertion and deletion in the *plcD* gene than in the *plcA*, *plcB*, and *plcC* genes. However, these alterations did not affect the analyses of the clinical samples studied. The BC3/BC5 primer pair amplified a 432-bp fragment from *plcB-plcC* at position 19384-19403 (GenBank accession number Z83860). Therefore, it is out of the chromosomal domain.

Herein, we have describes a PCR method based on amplification of the *plcB-plcC* intergenic sequence. This sequence appears to be a promising target for use in differentiating *M. tuberculosis* from *M. bovis* in clinical samples.

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