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Rapid molecular diagnosis of tuberculosis and other mycobacteriosis in smear-negative clinical specimens

Leite, S.R.A.[1*]; Malaspina, A.C.[2]; Hirata, M.H.[3]; Dubuc, M.A.[2]; Leite, C.Q.F.[2]

[1]*Departamento de Química Geral e Inorgânica, Instituto de Química, Universidade Estadual Paulista, UNESP, Araraquara, SP, Brasil*
[2]*Departamento de Ciências Biológicas, Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, UNESP, Araraquara, SP, Brasil.*
[3]*Departamento de Análises Clínicas e Toxicológicas , Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, USP, São Paulo, SP, Brasil.*

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ABSTRACT

Delay in diagnosis of pulmonary and other forms of tuberculosis (TB) can be fatal, particularly in HIV-infected patients. Hence, techniques based on nucleic acid amplification, which are both rapid and of high specificity and sensitivity, are now widely used and recommended for laboratories that diagnose TB. In the present study, diagnostic methods based on mycobacterial DNA amplification were evaluated in comparative trials alongside tradicional bacterial methods, using negative smear samples from patients with clinically-suspected TB (sputum samples from 25 patients with suspected pulmonary TB, urine samples from two patients with suspected renal TB and cerebrospinal fluid samples from one patient with suspected meningeal TB). A specificity of 100% was achieved with DNA amplification methods and tradicional culture/identification methods, in relation to clinical findings and treatment results. For the smear-negative sputa, conventional PCR for *M. tuberculosis* was positive in 62% of suspected lung TB case, showing the same sensitivity as bacterial identification. Both techniques failed in the detection of extra-pulmonary samples. Nested PCR showed, after species-specific amplification, a sensitivity of 100% for *M. avium* and 85% for *M. tuberculosis*. For extra-pulmonary smear-negative samples, only Nested PCR detected M. tuberculosis and all cases were confirmed clinically. Nested PCR, in which two-step amplification reactions are performed, can identify the two most important mycobacteria in human pathology quickly and directly from clinical spicimens.

Keywords: *tuberculosis*; *M. avium*; Nested PCR; Smear-negative specimens

INTRODUCTION

Tuberculosis (TB) remains an important public health problem worldwide, accounting for eight million new cases per year (Dye et al., 1999). Ninety-five percent of TB cases occur in developing countries, where human immunodeficiency virus (HIV) infection is common. Recent studies showed that 24.5% of TB patients in Rio de Janeiro city, Brazil, are coinfected with HIV (Ferreira et al., 2002). HIV infection not only increases the overall incidence of TB, but also exacerbates the clinical course of the illness, leading to the sprouting of extra-pulmonary varieties and severe miliary forms. Infection by other species of mycobacteria is also common in immunodeficient patients. This situation, allied to the rising incidence of *M. tuberculosis* strains resistant to multiple drugs, demands more sensitive and specific laboratory methods of diagnosis. Techniques based on nucleic acid amplification have been adopted widely and are recommended for this purpose.

Mycobacteria can be identified by PCR within a few hours and this amplification method may enhance the detection of smear-negative disease cases of TB. The amplified target DNA is easily detected by agarose gel electrophoresis, using DNA size markers to identify the amplified expected fragments obtained by PCR. However, qualitative reviews (Woods, 2001) have pointed out the low sensitivity of PCR for the diagnosis of clinical specimens. For this reason, the amplified target DNA can be subjected to repeated PCR, using a second set of primers that flank regions inside the fragment amplified in the first step, in a strategy called "nesting". Nested PCR increases sensitivity because two different reactions are performed, each one involving 20-30 cycles, giving an increased amplification overall (Pierre et al., 1991).

The goal of this study was to evaluate the usefulness of PCR and Nested PCR for the detection of *M. tuberculosis*

**Autor Correspondente*: Sergio Roberto de Andrade Leite - Departamento de Química Geral e Inorgânica - Instituto de Química - Universidade Estadual Paulista, UNESP - Rua Professor Francisco Degni s/nº - CEP: 14800-060 - Araraquara - SP, Brasil - Telefone: (16) 3301-6633 - E-mail: leite@iq.unesp.br

and *M. avium* directly from sputum, urine and cerebrospinal fluid specimens with negative smear results.

MATERIAL AND METHODS

Materials

Presence of mycobacteria was evaluated by traditional culture/identification methods and by two DNA amplification techiniques (PCR and Nested-PCR) in specimens considered negative for acid fast bacilli. The specimens were obtained from 28 patients with a strong clinical indication of TB (25 of pulmonary TB, two of renal TB and one of meningeal TB), but whose diagnosis was unconfirmed. The patients were selected from 270 attended during a year at a Public Health Service (SESA, Araraquara, SP, Brazil). The assays were tested on negative controls using specimens obtained from healthy people (50 sputum and five urine samples), and on positive controls, using cultures of reference strains M. tuberculosis H37Rv ATCC 27294, *M. avium* ATCC 25291 and *M. chelonae* ATCC 19977. In all 75 sputum, seven urine, and one cerebrospinal fluid (CSF) samples were analyzed.

Culture and identification

Specimens were decontaminated by the Petroff method (Brasil, 2005), concentrated by centrifugation (15 min, 3000 x g, 4ºC) and cultured on Löwestein-Jensen medium. Cultures were incubated at 37ºC for 60 days and were inspected weekly for bacterial growth. Eugonic mycobacterial isolates were identified by conventional methods, namely, rate of growth, colonial morphology, pigmentation, and biochemical properties (CDC, 1985).

PCR and Nested-PCR analysis

Specimens decontaminated by the Petroff method were also used for the nucleic acid amplification methods. The concentrated samples were resuspended in sterile distilled water and DNA was obtained by boiling the suspension for 10 min.

PCR was performed by the Van Embden et al., (1993) method, using internal primer sequences (specific for *M. tuberculosis*) of the insertion sequence IS6110 (*primer sense* 5' - CGT GAG GGC ATC GAG GTG GC - 3' and antisense 5' - GCG TAG GCG TCG GTG ACA AA - 3') with a product length of 245bp. Reagents were added to a 0.2 mL centrifuge tube: 5.0 µL 10 x PCR buffer, 4.0 µL pooled dNTPs (1.25 mM each), 0.6 µL of 5.0 µM stocks of each primer, 0.125 µL Taq polymerase (2.5 U/µL), 5.0 µL of DNA template and water to a final volume of 50 µL. All reagents used were from BioAgency Biotecnologia Ltda (São Paulo, Brazil). Cycles were performed in a PTC-1196 thermocycler (MJ Research Inc., Massachussets, USA) as follows: 95º C for 10 min, 35 cycles of amplification (94° C for 1 min, followed by 58º C for 1 min and 72º C for 1 min) and finally 72º C for 10 min. Amplified product (5 µL) was separated by 2% (w/v) agarose gel electrophoresis, developed by staining with 0.03% ethidium bromide and vizualied by UV light on a transilluminator (Pharmacia Biotech).

Nested PCR was performed as described by Abanto et al. (2000). In the first DNA amplification step, primers encoding sequences of the 16S rRNA of the Mycobacterium genus were used, sense 5' - GAG AGT TTG ATC CTG GCT CAG - 3' and antisense 5' - TGC ACA CAG GCC ACA AGG GA - 3' (Persing et al., 1993), which amplified fragments of 1030 bp. Reagents were added to 0.2 mL centrifuge tubes: 5.0 µL 10 x PCR buffer, 4.0 µL pooled dNTPs (1.25 mM each), 0.6 µL of 5.0 µM stocks of each primer, 0.125 µL Taq polymerase (2.5 U/µL), 5.0 µL DNA template and water to a final volume of 50 µL. Cycles were as follows: 95º C for 10 min, 25 cycles of amplification (94º C for 1 min, followed by 58º C for 1min and 70º C for 1 min) and lastly 72º C for 10 min. The amplified product (5 µL) was analysed by 2% (w/v) agarose gel electrophoresis and the bands of DNA stained with 0.03% ethidium bromide and visualized under UV light.

In the second amplification, the sense primer for the genus and species-specific *antisense primers* for *M. tuberculosis* Complex (5' - ACC ACA AGA CAT GCA TCC CG - 3') and *M. avium Complex* (5' - ACC AGA AGA CAT GCG TCT TG - 3') were used. This resulted in fragments of 180 and 375bp respectively. One microliter of PCR product from the first amplification was used as template. The same amplification cycles and conditions as in the first step were used in the second. Five microliters of the amplified product were identified, as in the first step, by electrophoresis, ethidium bromide staining and UV visualization.

RESULTS

By culture, PCR and Nested PCR techniques, 19 cases of mycobacteriosis (14 *M. tuberculosis*, four *M. avium* and one *M. chelonae*) were stablished from smear-negative specimens of 28 patients. The clinical findings and the response to antiTB treatment confirmed two more cases of pulmonary TB and also confirmed all cases of extra-pulmonary TB.

The results of culture/identification and PCR methodologies are compared in Table 1. Nested PCR bands for pulmonary samples are displayed in Figure 1. PCR and Nested PCR bands of extra-pulmonary samples are showed in Figure 2. All *M. tuberculosis* detected directly in specimens by PCR presented a 245bp amplification product (data not shown). For Nested PCR, the genus-specific primers generated positive signals for the samples containing *M. tuberculosis*, *M. avium* and *M. chelonae* (amplified product of 1030bp), and the species-specific primers showed positive results only for *M. tuberculosis* (180 bp) and *M. avium* (375bp) (Figure 1), but not for *M. chelonae*. All 50 negative sputum controls and five negative urine controls showed no amplification for mycobacteria. In this sense, the both

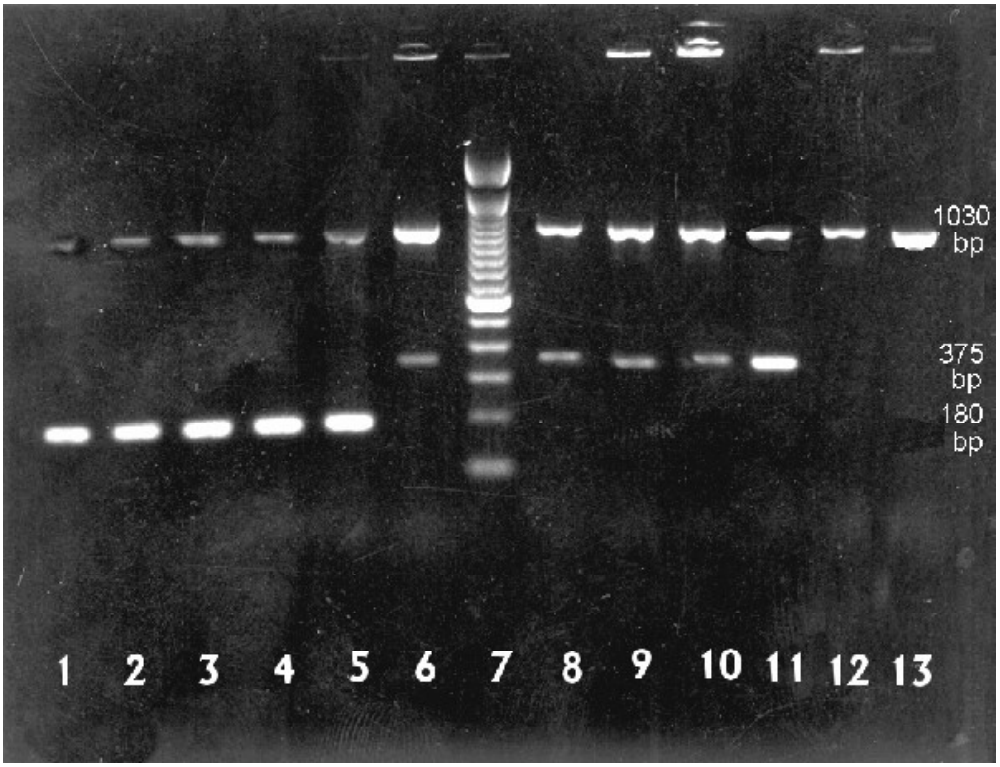

Figure 1. Nested PCR for *M. tuberculosis* and *M. avium* detection in smear-negative sputum samples. Lanes 1-4: sputa with *M. tuberculosis*; 5: reference strain *M. tuberculosis* $H_{37}R_v$; 6, 8-10: sputa with *M. avium*; 7: 100 bp ladder; 11: reference strain *M. avium*; 12: reference strain *M. chelonae*; 13: sputum with *M. chelonae*.

amplification methods and the culture/identification assay employed here showed specificity of 100%. The analytical specificity of primers was also confirmed with DNA samples from cultured reference strains. From 25 suspected pulmonary TB patients, Nested PCR confirmed *M. tuberculosis* in 11. PCR and culture/identification were positive for *M. tuberculosis* in samples from eight patients. In four cases, culture/identification and Nested PCR identified the *M. avium* complex. PCR could not identify *M.avium* and *M.chelonae*. This last mycobacterium could be detected only by culture/identification. In specimens from three patients with suspected extra-pulmonary TB (two renal and one meningial), *M. tuberculosis* was detected only by Nested PCR (Figure 2).

DISCUSSION

TB is one of the most prevalent infectious diseases worldwide and, with an incidence of 129,000 new cases per year, *M. tuberculosis* is the commonest agent of human TB in Brazil (Conselho Nacional de Desenvolvimento Científico e Tecnológico, 2005). Smear-negative pulmonary TB cases represent a serious public health hazard and a heavy burden, accounting for as much as 17% of *M. tuberculosis* transmission (Behr et al., 1999). Approximately 20 to 50% of patients with pulmonary tuberculosis are smear-negative and 10% are culture-negative (Dye et al., 1999).

In this study, we detected 19 mycobacteriosis cases (14 *M. tuberculosis*, four *M. avium* and one *M. chelonae* strains) by analyzing smear-negative samples taken from 28 patients with clinically suspected TB. Our results showed that DNA amplification methods such as PCR and Nested PCR reduce the time required for the identification of *M. tuberculosis* and may enhance the detection of smear-negative pulmonary cases. Detection of TB cases at an early stage of the disease is very important for the care of patients, enabling an optimal course of treatment and avoiding unnecessary and expensive isolation of patients. PCR gave, within six hours, results similar to those found in culture (Table 1), with detection of *M. tuberculosis* in eight out of 25 smear-negative sputum samples. Nested PCR, with two PCR steps, showed a significantly higher detection-rate than either culture or simple PCR, detecting 11 strains of *M. tuberculosis* and four strains of *M. avium*. Abanto et al. (2000) obtained reproducible Nested PCR results from specimens containing three bacterial cells per milliliter. For extra-pulmonary samples, only Nested PCR was able to identify the TB cases. Positive results obtained from urine and cerebrospinal fluid samples demonstrate that Nested PCR can be used in the diagnosis of both pulmonary and extra-pulmonary tuberculosis.

False negative Nested PCR results were observed in sputa from two pulmonary cases in wich TB was confirmed

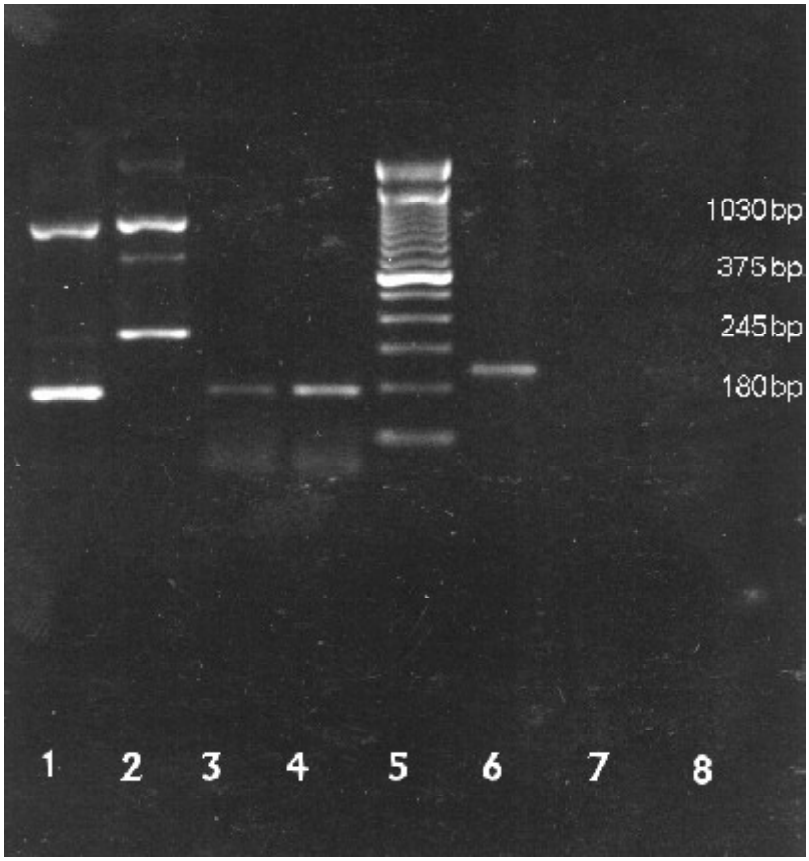

Figure 2. Nested PCR and PCR for *M. tuberculosis* and *M. avium* detection in extra-pulmonary smear-negative samples. Lanes 1-4: amplification by Nested PCR for 1) reference strain *M. tuberculosis* $H_{37}R_v$; 2) reference strain *M. avium*; 3) urine sample; 4) CSF sample. Lane 5: 100 bp ladder. Lanes 6-8: amplification by PCR for 6) reference strain *M. tuberculosis* $H_{37}R_v$; 7) urine sample; 8) CSF sample.

by clinical findings and therapeutic proof. Simple PCR and culture failed to identify five cases of pulmonary TB. In specimens that contain few organisms and are smear-negative, PCR is positive in 48-53% of patients with culture-positive tuberculosis (American Thoracic Society, 2000; Sarmiento et al., 2003). The two false negative results might be explained by the presence of some inhibitory factors in the samples (Woods, 2001). By carring out DNA purification with phenol-chloroform-isoamyl alcohol, Abanto et al. (2000), using the same Nested PCR protocol as here, found positivity in 92.9% of sputa analyzed, compared to our 85%. On the other hand, no false amplification by PCR methods and absence of growth in culture, for all the negative sputa and urine controls, is a indication that the methods used here showed a specificity of 100%.

In this study we identified other species of mycobacteria such as *M. avium* and *M. chelonae*. The former species is the commonest isolated non-tuberculous mycobacterium (NTM) in Brazil, and its presence may be associated with AIDS (Ferreira et al., 2002). In a national survey from 1994 to 1999, *M. avium* and *M. chelonae* were detected respectively in 44.4% and 11.0% of isolates (Barretos & Campos, 2000).

M. avium was identified equally well by the conventional method and Nested PCR, but much more rapidly by Nested PCR, which detected this mycobacterium directly from sputum. The introduction of species-specific primers in the second step allows simultaneous identification of *M. tuberculosis* and *M. avium* in a single PCR run. Tanaka et al. (2003) found that there was no amplification inhibition in the multiplex PCR technique when a mixture of two different primer pairs was used. They verified that the DNA was selectively amplified.

The Nested PCR technique used in the present study increased the versatility of the method, without loss of sensitivity, by amplifying two different reaction targets for the two most important mycobacteria in human pathology, within the 1030bp genus fragment. Since the method is a fast, cost-effective, and reliable way of identifying *M. tuberculosis* and *M. avium*, the Nested PCR assay may be used as a confirmatory diagnosis, improving the clinical diagnosis of pulmonary tuberculosis. The results also suggest

Table 1 - Mycobacteria identification by culture/identification, PCR and Nested PCR from smear-negative patients' specimens.

Specimens/Mycobacteria	**Culture/identification**		**PCR**		**Nested PCR**	
(total)	Number	%	Number	%	Number	%
Sputum (25)						
Absence (07)						
M. tuberculosis (13)	8	61.5	8	61.5	11	84.6
M. avium (04)	4	100.0	0	…	4	100.0
M. chelonae (01)	1	…	0	…	*	*
Urine (02)						
M. tuberculosis (02)	0	…	0	…	2	100.0
Cerebrospinal fluid (01)						
M. tuberculosis (01)	0	…	0	…	1	…

* *Detected in genus amplification* (Figure 1)

the usefulness of Nested PCR for detection of extra-pulmonary *M. tuberculosis* and *M. avium*, making its future use for this purpose very promising.

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RESUMO

Técnica molecular no diagnóstico rápido da tuberculose e de outras micobacterioses em amostras clínicas com baciloscopia negativa

Tradicionalmente, o diagnóstico laboratorial da tuberculose (TB), baseado na cultura da micobactéria, é um procedimento demorado, o que é um problema sério no caso de pacientes graves, mormente aqueles portadores de HIV. Por este motivo, técnicas de execução rápida, baseadas na amplificação de ácidos nucléicos, sensíveis e específicas, foram muito bem-vindas para o diagnóstico das micobacterioses e têm sido rapidamente assimiladas e utilizadas pelos laboratórios desta área. No presente estudo, métodos baseados na amplificação do ADN micobacteriano foram comparados entre si e com a metodologia tradicional. Foram estudados 28 pacientes com diagnóstico clínico de TB, porém com baciloscopia negativa (25 amostras de escarro, duas de urina e uma de liquor) e 55 controles normais (50 amostras de escarro e 5 de urina). Foi obtida uma especificidade de 100%, tanto para o método clássico quanto para os de amplificação de ADN. Para o caso dos pacientes portadores de TB pulmonar e com baciloscopia negativa no escarro, a cultura e a PCR convencional apresentaram, ambas, uma sensibilidade de 62% na detecção do *Mycobacterium tuberculosis*. Ambas falharam na demonstração deste agente nos casos de TB extra-pulmonar. Por outro lado, a técnica de *Nested PCR*, mediante uma amplificação específica para espécie, apresentou uma sensibilidade de 85% para o *M. tuberculosis* e de 100% para o *M. avium*. No caso das amostras de origem extra-pulmonar, somente este último método foi capaz de detectar o *M. tuberculosis* em todos os casos confirmados clinicamente. Concluíndo, a técnica de *Nested PCR*, na qual são realizadas reações de amplificação em duas etapas, é capaz de identificar, de forma rápida e diretamente do espécime clínico, as duas mais importantes micobacterioses humanas.

Palavras-chave: *M. tuberculosis*; *M. avium*; Nested PCR; Baciloscopia negativa em escarro.

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