Detection of *Mycobacterium tuberculosis* complex by nested polymerase chain reaction in pulmonary and extrapulmonary specimens*,**

Detecção do complexo *Mycobacterium tuberculosis* por *nested polymerase chain reaction* em espécimes pulmonares e extrapulmonares

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Abstract

Objective: To compare the performance of nested polymerase chain reaction (NPCR) with that of cultures in the detection of the *Mycobacterium tuberculosis* complex in pulmonary and extrapulmonary specimens. **Methods:** We analyzed 20 and 78 pulmonary and extrapulmonary specimens, respectively, of 67 hospitalized patients suspected of having tuberculosis. An automated microbial system was used for the identification of *Mycobacterium* spp. cultures, and *M. tuberculosis* IS*6110* was used as the target sequence in the NPCR. The kappa statistic was used in order to assess the level of agreement among the results. **Results:** Among the 67 patients, 6 and 5, respectively, were diagnosed with pulmonary and extrapulmonary tuberculosis, and the NPCR was positive in all of the cases. Among the 98 clinical specimens, smear microscopy, culture, and NPCR were positive in 6.00%, 8.16%, and 13.26%, respectively. Comparing the results of NPCR with those of cultures (the gold standard), we found that NPCR had a sensitivity and specificity of 100% and 83%, respectively, in pulmonary specimens, compared with 83% and 96%, respectively, in extrapulmonary specimens, with good concordance between the tests (kappa, 0.50 and 0.6867, respectively). **Conclusions:** Although NPCR proved to be a very useful tool for the detection of *M. tuberculosis* complex, clinical, epidemiological, and other laboratory data should also be considered in the diagnosis and treatment of pulmonary and extrapulmonary tuberculosis.

Keywords: Tuberculosis/diagnosis; Tuberculosis/microbiology; Mycobacterium tuberculosis; Polymerase chain reaction.

Resumo

Objetivo: Comparar o desempenho da técnica *nested polymerase chain reaction* (NPCR) com aquele de culturas na detecção do complexo *Mycobacterium tuberculosis* em espécimes pulmonares e extrapulmonares. **Métodos:** Analisamos 20 e 78 espécimes pulmonares e extrapulmonares, respectivamente, de 67 pacientes hospitalizados com suspeita de tuberculose. Um sistema automatizado foi utilizado na identificação de culturas de *Mycobacterium* spp., e *M. tuberculosis* IS*6110* foi utilizada como sequência alvo na NPCR. A estatística kappa foi utilizada para verificar a concordância entre os resultados.**Resultados:** Entre os 67 pacientes, 6 e 5, respectivamente foram diagnosticados com tuberculose pulmonar e extrapulmonar, e a NPCR foi positiva em todos os casos. Entre os 98 espécimes clínicos, a baciloscopia, cultura e NPCR foram positivas em 6,00%, 8,16% e 13,26%, respectivamente. Comparando-se os resultados da NPCR com aqueles da cultura (padrão ouro) nos espécimes pulmonares, a sensibilidade e a especificidade foram 100% e 83%, respectivamente, enquanto essas nos espécimes extrapulmonares foram 83% e 96% respectivamente, com boa concordância entre os testes (kappa, 0,50 e 0,6867, respectivamente). **Conclusões:** Embora a NPCR tenha se mostrado uma ferramenta muito útil na detecção do complexo *M. tuberculosis*, No entanto, os resultados positivos da NPCR devem ser associados à clínica, dados clínicos, epidemiológicos e outros dados laboratoriais devem também ser considerados no diagnóstico e tratamento da tuberculose pulmonar e extrapulmonar.

Descritores: Tuberculose/diagnóstico; Tuberculose/microbiologia; Mycobacterium tuberculosis; Reação em cadeia da polimerase.

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Financial support: This study received financial support from the Brazilian *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq, National Council for Scientific and Technological Development; Process no. 576297/2008-9)
Submitted: 1 February 2013. Accepted, after review: 24 September 2013.
**A versão completa em português deste artigo está disponível em www.jornaldepneumologia.com.br

Introduction

The World Health Organization estimates that there were between 8.8 and 9.2 million new tuberculosis cases in 2010.[1] In addition, it is estimated that 1.2 million of those cases are infected by HIV. Furthermore, it is estimated that 1.1 million deaths occurred in HIV-negative tuberculosis patients, which equates to 15 deaths per 100,000 population. Mortality is the most sensitive indicator of tuberculosis control measures[2]; tuberculosis remains among the top ten causes of death worldwide. The goal of curing 85% of tuberculosis cases, set by the World Health Assembly in 1991, was not achieved in 2009 in 7 of the 22 countries with the highest burden of the disease, including Brazil (at 72%).[3]

Diagnosis in an early stage of the disease is of paramount importance for treatment initiation, with direct consequences for individual disease control and, therefore, for public health initiatives aimed at the prevention of tuberculosis transmission. Therefore, it is necessary that clinical microbiology laboratories be able to quickly identify mycobacteria by means of microscopy and culture. However, sputum smear tests, although rapid and cost-effective, have low sensitivity and specificity, particularly in paucibacillary samples, and culture, even though it is considered the gold standard due to its high sensitivity, requires several weeks to produce a result.[4-8]

Currently, Brazil is one of the countries that, together, accounts for 80% of tuberculosis cases, with an incidence of 70,977 cases in 2010.[4,6] For the majority of those cases, the diagnosis was based only on sputum smear test results; chest X-rays, cultures, and biochemical tests for *Mycobacterium* spp. were only carried out in patients with negative sputum smear results but with respiratory symptoms.[8,9] These diagnostic limitations have encouraged the use of molecular tools with improved sensitivity, specificity, and speed, in order to detect mycobacteria in all clinical specimens.[5-8,10] The new technologies that are being developed have recently redefined the diagnosis of tuberculosis, providing a basis for diagnostic laboratory techniques.[5,8] The molecular diagnosis of tuberculosis by polymerase chain reaction (PCR) and primers with high specificity (98%), with high variations in sensitivity (20-100%), has been used in order to identify genetic targets in the bacillus.[7,11,12]

Despite the widespread use of conventional PCR, modifications in the technique, such as the addition of one extra reaction (nested PCR), have increased its sensitivity and specificity.[6] This might be due to the fact that it dilutes potential PCR inhibitors, which are commonly present in biological samples.[11] Therefore, the possibility of having access to a molecular tool that leads to a more rapid diagnosis and that is effective for the detection of cases that are difficult to elucidate by conventional tests certainly helps decrease morbidity and improve tuberculosis control. The aim of the present study was to evaluate the technique of nested PCR targeting the insertion sequence IS*6110* in *Mycobacterium tuberculosis* and to compare the results with those obtained in cultures of samples from patients suspected of having pulmonary or extrapulmonary tuberculosis.

Methods

This study was carried out between February and December of 2009. The patients included in the study were submitted to physical evaluation and sample collection at the *Hospital de Base*, a referral center for the diagnosis and treatment of tuberculosis located in the city São José do Rio Preto, Brazil. Epidemiological and clinical data were obtained from medical records in accordance with a protocol approved by the Research Ethics Committee of *Faculdade de Medicina de São José do Rio Preto* (São José do Rio Preto School of Medicine; Protocol no. 064/2009). The presence of HIV antibodies, identified by ELISA and confirmed by Western blot, indicated HIV seropositivity.

All of the patients included in the study were over 18 years of age, were immunosuppressed (due to immunosuppression therapy, autoimmune disease, organ transplantation, or HIV-positivity), and presented with clinical symptoms and signs suggestive of pulmonary or extrapulmonary tuberculosis. Our sample comprised 67 hospitalized patients, and 98 clinical specimens were collected, of which 20 were pulmonary specimens (sputum, BAL fluid, or gastric lavage fluid), and 78 were extrapulmonary specimens (blood, cerebrospinal fluid, lymph node aspirate, urine, pleural fluid, secretion from ganglia, pleura fragment, liver fragment, ascitic fluid, bone marrow aspirate, or biopsy specimens). The number of specimens collected from the patients ranged from one

to three, according to physician requests. The diagnostic confirmation of tuberculosis was based on the following criteria: clinical and radiological evidence of tuberculosis confirmed by laboratory tests, isolation of *M. tuberculosis* in clinical specimens by direct smear microscopy or culture (gold standard), and evident clinical improvement after antimycobacterial treatment.

In brief, direct smear microscopy was performed using Ziehl-Neelsen staining, and an automated microbial system (BacT/ALERT MP; Organon Teknika Corp., Durham, NC, USA) was used for the identification of *Mycobacterium* spp. in cultures. The strains were identified by phenotypic methods.[13] Genotyping was carried out by PCR-restriction enzyme analysis in accordance with Chimara et al.,[14] although with modifications.

Blood samples were collected in 5-mL tubes containing EDTA, and peripheral blood mononuclear cells were isolated by density gradient centrifugation (Ficoll-Histopaque) for future extraction of the DNA.[15-17] For solid organs, 2.0-mm punch biopsy samples were collected. All clinical samples were kept at −20°C until DNA extraction, which was performed in accordance with the method described by Rossetti et al.[18] with modifications by Lima et al.[11,18,19] In brief, a 500-µL aliquot of the sample was centrifuged at 13,000 rpm for 10 min and washed three times in Tris-EDTA (TE). The pellet was resuspended in 100 µL of TE and heated at 100°C for 10 min. The supernatant was transferred to a different tube, and 5 µL of resin were added (Sephaglas BandPrep Kit; Amersham-Pharmacia Biotech, Uppsala, Sweden); an aliquot of sodium iodide solution (0.9 g/mL) was added to the final volume. The tube was shaken for 5 min and incubated at room temperature for 5 min. After centrifuging the tube for 1 min and discarding the supernatant, we added 200 µL of iced 70% ethanol; the tube was then shaken, after which it was centrifuged for 1 min. The resulting pellet was kept at room temperature for 60 min, in order to complete the drying process, and resuspended in 40 µL of 1×TE. The tube was incubated in a water bath at 50°C for 10 min. Subsequently, the tube was centrifuged for 1 min, and the supernatant was transferred to another tube and stored at −20°C until processing.[11,18]

For the nested PCRs, IS*6110* of *M. tuberculosis* was used as the target sequence (GenBank accession no. 215310.1). The reactions were performed in accordance with Ritis et al.[17] The following primers were used: sense (TJ3 5'-ATC CCC TAT CCG TAT GGT G-3'); antisense (TJ5 5'-CCG CAA AGT GTG GCT AAC-3'); sense (*STAN3* 5'-GTC GAG TAC GCC TTC TTG TT-3'); and antisense (*OLI 5* 5'-AAC GGC TGA TGA CCA AAC-3'). The PCR used a final volume of 50 µL (1× buffer, 50 mM $MgCl_2$, 10 pmol/µL of each oligonucleotide, 0.2 µM dNTP, and 2 U Taq DNA polymerase [Invitrogen Life Technologies, Carlsbad, CA, USA]). The amplification process consisted of an initial denaturation step of 94°C for 3 min, 30 denaturing cycles (at 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min), followed by a final extension at 72°C for 5 min. The second PCR was performed using 3 µL of the product of the first PCR under similar conditions to those described above, but with an annealing temperature of 60°C. DNA of the H37Rv strain of *M. tuberculosis* and PCR mix alone were used as positive and negative controls, respectively. The result was analyzed by electrophoresis on 2% agarose gel, stained with ethidium bromide, and visualized in an ultraviolet transilluminator (Fisher-Biotech, Fairlawn, NJ, USA), resulting in a 316-bp fragment.

Statistical analyses were performed with the Epi Info statistical package, version 6.0. The kappa statistic was used in order to assess the level of agreement among the results.[20] The level of significance was set at 5%.

Results

Our sample comprised 67 individuals, 63.7% being male. The mean age of the patients was 40.10 ± 3.66 years (range, 18-87 years). The most common comorbidity was HIV/AIDS, in 41 patients.

Tuberculosis was diagnosed in 11 individuals (16.41%), 5 being diagnosed with pulmonary tuberculosis and the remaining with extrapulmonary tuberculosis (pleural tuberculosis in 3, meningeal tuberculosis in 2, and miliary tuberculosis in 1). All culture isolates were confirmed by *M. tuberculosis* genotyping. Nested PCR was positive in all of the cases of confirmed tuberculosis, as shown in Table 1. In 4 of the tuberculosis patients, positive laboratory results were obtained only by the molecular technique (p = 0.110; Fisher's exact test).

Of the 98 clinical specimens analyzed, 6.00%, 8.16%, and 13.26%, respectively, showed positive

results in smear microscopy, culture, and nested PCR, as summarized in Table 1. Culture was negative in 2 of the samples with positive smear microscopy results, whereas smear microscopy was negative in 4 of the samples with positive culture results.

By comparing the results of nested PCR with those of cultures in pulmonary samples (Table 2), we found that the sensitivity and specificity of nested PCR were 100% and 83%, respectively. The positive and negative predictive values were 40% and 100%, respectively, with good concordance between the tests (kappa = 0.50; p = 0.25; McNemar's test). Regarding extrapulmonary samples (Table 2), sensitivity, specificity, positive predictive value, and negative predictive value were, respectively, 83.0%, 96.0%, 62.5%, and 98.5%, with good concordance between the tests (kappa = 0.6867; p = 0.625; McNemar's test).

Discussion

The early diagnosis of tuberculosis is essential for prompt treatment and effective control of the disease.[5] This is particularly true in cases of extrapulmonary tuberculosis, because various factors can complicate the diagnosis of the disease. Due to the paucibacillary nature of extrapulmonary tuberculosis, studies have shown a variability in positive culture results (from 12% to 80%) and a variety of clinical samples/biological tissues, which implies a non-uniform distribution of the bacillus, as well as the presence of nonspecific signs and symptoms.[10,12,19-21] In our study, pleural tuberculosis was diagnosed in 2 HIV-negative

Table 1 - Clinical, epidemiological, and laboratory data of the eleven patients with confirmed pulmonary or extrapulmonary tuberculosis.

Patient[a]	Form of tuberculosis	Type of sample	Results			Outcome
			Smear microscopy	Culture	Nested PCR	
1	Pulmonary	Blood	–	–	+	Death/NTB
2	Pulmonary	Blood	–	–	+	Cure
3	Pleural	Blood	–	–	+	Death/NTB
4	Pleural	Pleural fluid	+	–	+	Cure
5	Pulmonary	Blood	+	+	+	Death/TB
6	Pulmonary	Sputum	+	–	+	Death/NTB
7	Pulmonary	Sputum	+	+	+	Cure
		CSF	+	+	+	
		Blood	–	–	–	
8	Meningeal	CSF	–	+	+	Cure
9	Meningeal	CSF	–	+	+	Cure
10	Miliary	Sputum	+	+	+	Death/NTB
		CSF	–	+	–	
		Blood	–	+	+	
11	Pleural	Pleural fluid	–	–	+	Abandonment

PCR: polymerase chain reaction; Death/NTB: death due to causes other than tuberculosis; Death/TB: death due to tuberculosis; and CSF: cerebrospinal fluid. [a]Patients 1 to 4 were HIV-negative, and patients 5 to 11 were HIV-positive. Culture vs. nested PCR (negative/positive): 7 × 8/2 × 13 (p = 0.110; Fisher's exact test).

Table 2 - Culture and nested polymerase chain reaction for the detection of *Mycobacterium tuberculosis* in pulmonary and extrapulmonary samples.

Nested PCR	Culture				Total
	Extrapulmonary samples (n = 78)		Pulmonary samples (n = 20)		
	(+)	(-)	(+)	(-)	
(+)	5	3	2	3	13
(-)	1	69	0	15	85
Total	6	72	2	18	98

PCR: polymerase chain reaction.

patients, corroborating the findings of a study reporting that pleural tuberculosis is prevalent in extrapulmonary cases in HIV-negative patients.[22] Approximately 50% of HIV-positive individuals with tuberculosis develop extrapulmonary forms.[12,20,21,23] Despite the small number of HIV-positive patients included in the present study, our results show a predominance of extrapulmonary tuberculosis (57%).

Various tests based on PCR techniques using commercial kits and in-house tests are being evaluated. The nested PCR technique, by targeting IS*6110* in order to identify the *M. tuberculosis* complex,[4] provides variable sensitivity and specificity, depending on the laboratory, clinical specimen, bacillary load, cell lysis, and technical parameters.[10] Molecular techniques have greatly improved the detection of mycobacteria in lymph nodes and in various body fluids (aspirates, cerebrospinal fluid, ascitic fluid, and pleural fluid). However, due to the variable sensitivity and specificity in different studies, positive results should be interpreted in conjunction with clinical findings.[21] In the present study, the proportion of positive results in the individuals diagnosed with pulmonary or extrapulmonary tuberculosis using nested PCR (100%) was higher than in other studies using the same target gene and carried out in Brazil,[6] India,[21] and Greece.[17]

The discordance between the molecular method and smear microscopy in pulmonary and extrapulmonary samples in our study (in 1 and in 6 samples, respectively) might be directly related to the low sensitivity of the phenotyping technique, the paucibacillary nature of samples, or even the absence of infection by AFB, as observed by other authors.[12,15,20] Negi et al.[19] reported that a molecular technique targeting IS*6110* showed high positivity in pulmonary and extrapulmonary samples (90% and 77%, respectively), whereas smear microscopy showed low positivity (49% and 24%, respectively). Similarly, Barani et al.,[10] when studying 19 pulmonary and 104 extrapulmonary samples, obtained higher positivity with a molecular technique targeting IS*6110* and TCR4 than with the smear method (17 similar and 12 divergent results). It is of note that a reported 50% of tuberculosis cases are classified as negative on the basis of smear microcopy results.[24] In addition, smear microcopy was not performed in 32.43% of the reported cases of tuberculosis in Brazil in 2010.[1]

When we compared nested PCR and culture results in pulmonary samples, we found that 3 of the samples showed positive nested PCR results but negative culture results. The negative results in the cultures might be due to co-infection with HIV in 2 of the patients and kidney transplantation in 1 (Table 1) or to the characteristics of paucibacillary infections.[6,12] Various factors can influence the result of cultures, such as the number of organisms present in the specimen, the methods of sample collection, previous treatments, and the processing method. In addition, the solutions used for digestion/decontamination of the samples can damage the mycobacteria.[4,25]

In the present study, the molecular results supported the clinical and diagnostic criteria widely accepted for the diagnosis of tuberculosis. The sensitivity and specificity found for nested PCR in our study (100% and 83%, respectively) are higher than those reported in previous studies using molecular techniques targeting the IS*6110* gene in sputum samples (88-98% and 15-100%, respectively).[5,12,23] This difference might be attributable to the volume and type of samples, as well as to the different molecular typing protocols employed in different laboratories.[11,26,27] Hence, the correlation of the results with the clinical profile of the patient is essential for the diagnosis of tuberculosis, and the definition of the disease can therefore be established from negative cultures after the therapeutic test.[26] This was found in 3 of our patients who had positive nested PCR results and negative culture results in their pulmonary samples, 2 of whom were cured after treatment and 1 of whom was noncompliant with the treatment. In addition, 2 patients died before the beginning of the recommended tuberculosis treatment. As to the concordance between nested PCR and culture, although the kappa coefficient (0.50) was lower than that reported in other studies, carried out in India (kappa = 0.6-0.8) and in Brazil (kappa = 0.78), the concordance was good.

Regarding extrapulmonary samples, nested PCR was positive in 3 of the blood samples with negative culture results, and negative in 1 of the cerebrospinal fluid samples with a positive culture result. This false-negative result in a cerebrospinal fluid sample (Table 1) might be

related to the absence of copies of the IS*6110* gene, as previously described in studies conducted in southeast Asia, Denmark, Tunisia, India, and Vietnam, indicating the need to incorporate additional targets, such as TRC4, in order to improve molecular detection.[7,10] However, to our knowledge, no study to date has reported the absence of this element in *M. tuberculosis* strains in Brazil, and various studies have reported that this sequence is the most sensitive in order to detect the *M. tuberculosis* complex.[9,17] Another factor could be the presence of molecular reaction inhibitors in up to 18.6% of extrapulmonary specimens.[5]

In Brazil, the prevalence of *M. tuberculosis* isolated in cultures ranges from 15.0% to 25.6%,[28,29] the highest values being obtained prior to the highly active antiretroviral therapy era. Positive nested PCR results in blood samples led to the diagnosis of pulmonary tuberculosis in 2 HIV-negative patients and in 1 HIV-positive patient, as well as to the diagnosis of extrapulmonary tuberculosis in 1 HIV-negative patient and in 1 HIV-positive patient. In those blood samples, smear microscopy and culture were positive in only 1 and 2 samples, respectively; this might be due to the small number of bacilli in the circulation.[6,7] In fact, the sensitivity of PCR in peripheral blood mononuclear cells, as used here, has been reported to be better than is that of culture, both having similar specificity.[11,16,29] In addition, clinical specimens, such as cerebrospinal fluid, blood, and sputum, have been described as good substrates for PCR[26] with good concordance (kappa = 0.6867; p = 0.625; McNemar's test), as in our results, in which the level of positivity in extrapulmonary samples was higher when nested PCR was used (p = 0.0042), thus corroborating the findings of Noussair et al.[21] Different rates of sensitivity were described using the molecular methodology for this type of sample in studies conducted in France[21] and India[12] (86.6% and 90%, respectively). If standardization studies using the same molecular target, DNA extraction method, and PCR optimization were validated in different laboratories, the sensitivity of the test could be improved and, consequently, so could its concordance.

In the present study, a positive nested PCR result, associated with the clinical features, was used as the single laboratory criterion for the diagnosis of 4 patients (2 with pulmonary tuberculosis and 2 with extrapulmonary tuberculosis), because it was the only test producing positive results (Table 1); the lack of isolates in cultures might be mainly due to the paucibacillary character of the samples. Although the difference is not significant in this situation, diagnosis is often established only by clinical and radiological data, even if the culture and sputum smear testing are negative. In such cases, molecular analysis can help establish specific antimycobacterial therapy and, consequently, contribute to reduce empirical treatment, which has been currently used in almost 27% of suspected pulmonary tuberculosis cases. In addition, it can help control the spread of the bacillus[11] and prevent more severe clinical evolution, mainly in HIV-positive patients. However, the current Brazilian guidelines on tuberculosis do not include positive molecular results in the definition of tuberculosis cases; as a rule, the use of this method has been restricted to certain referral and research centers in Brazil.[9]

It is worth mentioning that clinical screening that is carefully designed to study the disease caused by mycobacteria, together with similar attention to the collection and transport of biological samples, are factors that contributed to the isolation of a higher rate of *M. tuberculosis* strains than was expected at our hospital. The present study corroborates the results obtained at a referral center for the diagnosis of tuberculosis in the city of São José do Rio Preto (XV Subdivision of the São Paulo State Health Department), in which a 50% increase in the number of isolates was observed during the study period.[25]

The results of the nested PCR assay revealed good agreement with those of the culture. The specificity and sensitivity achieved with this relatively simple molecular approach can be seen as an important contribution to the future establishment of a protocol for the molecular detection of *M. tuberculosis* complex in pulmonary and extrapulmonary samples. In addition, this methodology could reduce the time required for the appropriate diagnosis of tuberculosis.

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