Conventional and molecular techniques in the diagnosis of pulmonary tuberculosis: a comparative study*

Métodos convencionais e moleculares para o diagnóstico da tuberculose pulmonar: um estudo comparativo

Stella Sala Soares Lima, Wanessa Trindade Clemente, Moisés Palaci, Reinaldo Vieira Rosa, Carlos Mauricio de Figueiredo Antunes, José Carlos Serufo

Abstract

Objective: To compare four laboratory methods in the diagnosis of pulmonary tuberculosis. Methods: Respiratory secretion specimens were collected from 160 patients suspected of having pulmonary tuberculosis. Direct testing for Mycobacterium tuberculosis was carried out using Ziehl-Neelsen staining, auramine staining, culture on Löwenstein-Jensen (LJ) medium and polymerase chain reaction (PCR). The strains isolated were identified by means of a radiometric method using p-nitro-alpha-acetylaminobeta-hydroxypropiofenone (NAP) and classical methods. The sensitivity of the methods was compared to the gold standard for the diagnosis of pulmonary tuberculosis, based on clinical, radiological and microbiological criteria. Results: Of the 160 patients, 142 were diagnosed with pulmonary tuberculosis according to the gold standard. The sensitivity of Ziehl-Neelsen staining, auramine staining, culture on LJ medium and PCR was 54.2%, 58.4%, 67.6% and 77.5%, respectively, when compared with the diagnostic criterion adopted. All four methods presented 100% specificity. In the identification of mycobacteria, there was high (96.8%) concordance between PCR and the radiometric method using NAP. The sensitivity of PCR was 50.8% in samples with negative sputum smear microscopy results and 98.8% in those with positive results. The sensitivity of PCR was lower in specimens with negative results in sputum smear microscopy and culture than in those with positive results (25.6% and 99.0%, respectively). Conclusions: We found PCR to be a promising method for the diagnosis of pulmonary tuberculosis, even in paucibacillary specimens. Simultaneous identification and faster results are additional advantages of this method.

Keywords: Tuberculosis, pulmonary/diagnosis; Culture media; Polymerase chain reaction; Sputum/microbiology.

Resumo

Objetivo: Comparar quatro métodos laboratoriais no diagnóstico de tuberculose pulmonar. Métodos: Foram realizadas pesquisa direta pelas colorações de Ziehl-Neelsen e auramina, cultura para micobactérias em meio Löwenstein-Jensen (LJ) e polymerase chain reaction (PCR, reação em cadeia da polimerase) para Mycobacterium tuberculosis em 160 amostras de secreção respiratória de pacientes com suspeita de tuberculose pulmonar. As cepas isoladas foram identificadas por método radiométrico utilizando-se p-nitro-alfa-acetilaminobeta-hidroxipropiofenona (NAP) e métodos clássicos. A sensibilidade dos métodos foi comparada com o padrão ouro para o diagnóstico da tuberculose pulmonar, definido por critérios clínicos, radiológicos e microbiológicos. Resultados: Dos 160 pacientes, 142 foram diagnosticados com tuberculose pulmonar de acordo com o padrão ouro. As técnicas de Ziehl-Neelsen e auramina, cultura em meio LJ e PCR apresentaram sensibilidade de 54,2%, 58,4%, 67,6% e 77,5%, respectivamente, quando comparados ao critério diagnóstico adotado. A especificidade dos quatro métodos foi de 100%. A concordância na identificação da micobactéria entre PCR e o método radiométrico utilizando NAP foi alta (96,8%). A sensibilidade da PCR foi de 50,8% nas amostras com baciloscopia negativa e de 98,8% naquelas com baciloscopia positiva. Nas amostras com resultados negativos na baciloscopia e cultura, a sensibilidade da PCR foi menor que nas com resultados positivos (25,6% e 99,0%, respectivamente). Conclusões: A PCR é método promissor no diagnóstico da tuberculose pulmonar, mesmo em amostras paucibacilares. Além disso, apresenta a vantagem da identificação simultânea e rápida do resultado.

Descritores: Tuberculose pulmonar/diagnóstico; Meios de cultura; Reação em cadeia da polimerase; Escarro/microbiologia.

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Methods

Sputum samples collected from patients with presumed pulmonary TB who were treated at the Júlia Kubitschek Hospital, Belo Horizonte, Brazil, between January of 2001 and January of 2002, were analyzed. The patients underwent clinical evaluation, chest X-rays (anteroposterior and lateral) and HIV serology, as well as completing a form regarding epidemiological data. After a minimum of 18 months, the medical charts were reevaluated in order to confirm the diagnosis and determine the clinical evolution of the patients. Sample collection and all laboratory procedures were performed following safety regulations, and standardized handling criteria were met.

The samples were decontaminated using monosodium phosphate and trisodium phosphate (modified Corper & Stoner method) and were concentrated by centrifugation. Subsequently, smears prepared from an aliquot of the sediment were submitted to staining (Ziehl-Neelsen and auramine), after which they were examined under microscopy. A quantity (0.1 mL) of the sediment was added to at least two tubes containing Löwenstein-Jensen (LJ) medium, which were incubated at 37°C for up to eight weeks. The results were described using a semiquantitative scale. The strains isolated were identified by means of the growth inhibition test using p-nitro-alpha-acetylamino-beta-hydroxypropiophenone (NAP) in the BACTEC 460 system (Becton Dickinson Microbiology Systems, Sparks, MD, USA) and by means of other phenotyping tests (morphological analysis of colonies and classical biochemical tests). The Amplicor® PCR kit for M. tuberculosis (Roche Molecular Systems, Branchburg, NJ, USA), with an internal control, was used according to the manufacturer specifications.

The final criterion for the diagnosis of TB was defined using a combination of clinical, radiological and microbiological data, which were obtained by specialists at the Júlia Kubitschek Hospital, together with the response to the use of antituberculosis drugs.

All patients included in the investigation gave written informed consent. The present study was approved by the Ethics in Research Committee of the Hospital Foundation of the State of Minas Gerais.
The data initially collected on the questionnaires were entered into a database and analyzed using the Epi Info 2002 program, version 3.2.2. The kappa coefficient was used to compare the methods. Categorical variables were analyzed using the chi-square test with Yates’ correction. When there were fewer than five events or a null value, Fisher’s exact test was used. The level of statistical significance required to reject the null hypothesis was set at 5% (p ≤ 0.05) for all tests.

Results

Between January of 2001 and January of 2002, 161 patients suspected of having pulmonary TB were evaluated, and one sputum sample was collected per patient. There was one sample loss due to insufficient volume. The mean age of the 160 patients included was 40.0 ± 12.8 years (range, 19-78 years). The general characteristics of the patients are described in Table 1.

Of the 160 patients included, 142 (88.8%) presented pulmonary TB according to the criteria adopted at the Júlia Kubitschek Hospital. Of those 142, 3 had silicotuberculosis and 12 had pulmonary multidrug-resistant TB (MDR-TB), according to the World Health Organization criteria. After the medical charts had been reevaluated, the clinical evolution of pulmonary TB was confirmed in 106 patients (74.6%). Of those, 35 (24.6%) improved after treatment with regimen I (rifampin + isoniazid + pyrazinamide), 19 (13.4%) improved after treatment with regimen IR (regimen I + ethambutol), 10 (7.0%) improved after treatment with regimen III (streptomycin + ethionamide + ethambutol + pyrazinamide), and 10 (7.0%) improved after treatment with the MDR-TB treatment regimen (ethambutol + ofloxacin + clofazimine + terizidone + amikacin). Of the 106 patients, 18 (12.7%) abandoned treatment, 13 (9.1%) died, and 1 (0.7%) presented no improvement after treatment with the MDR-TB treatment regimen. Of the 142 patients with pulmonary TB, 36 (25.4%) were referred for treatment at referral centers close to their home, and it was not possible to ascertain their clinical evolution.

The results obtained using the laboratory methods evaluated are shown in Figure 1. The sensitivity of direct testing using Ziehl-Neelsen staining and auramine staining, as well as of culture on LJ medium and PCR, was 54.2%, 58.4%, 67.6% and 77.5%, respectively. All four methods presented 100% specificity. The kappa coefficients of the comparisons of the methods used are presented in Table 2.

The identification of M. tuberculosis, by means of a radiometric method using NAP, was performed in 95 of the 96 positive LJ medium cultures (one culture was contaminated, and it was not possible...
In samples with negative results in direct testing and culture, the sensitivity of PCR was 15.6%, whereas, in those with positive results in direct testing and culture, the sensitivity of PCR was 99.0% (p < 0.0001).

In total, 77 patients reported a history of pulmonary TB (median time since previous diagnosis, 36 months), and, of those, 44 (57.1%) abandoned treatment. The sensitivity of PCR was 78.6% in patients with a history of pulmonary TB and 77.6% in those without such a history (p = 0.94). No false-negative results were obtained in patients with a history of TB but without active pulmonary TB at the time of the study.

**Discussion**

The present study was developed at a referral center for the treatment of pulmonary TB where there are high proportions of symptomatic patients, patients with comorbidities and patients with a history of TB who report treatment abandonment, as well as a considerable frequency of MDR-TB and death. In our
In the present study, culture on LJ medium, which allows the definitive confirmation of the diagnosis of pulmonary TB, had a sensitivity of 67.6%, which is lower than the 80%-100% rate typically described, and the influence of the collection of a single sample should be considered. The main advantage of other culture media, as well as of automated and semi-automated detection methods, is the shorter time required to detect mycobacteria (approximately 15 days rather than 3-8 weeks). However, LJ medium, which is approved by the World Health Organization, remains the most widely used in Brazil. Additional benefits include the fact that the strains can be stored for future studies, and that some strains grow only in this medium. For these reasons, the use of new methods does not dispense with the use of conventional culture.

In relation to the other methods, PCR had higher sensitivity (77.5%), which is within the range (42%-90.9%) established in the literature, the variation depending principally on the characteristics of the patient sample. Results similar to those obtained in other studies, in which the sensitivity of sputum smear microscopy ranges from 50% to 80%,. The higher sensitivity of direct testing using auramine staining in relation to that of direct testing using Ziehl-Neelsen staining was not statistically significant. These two methods presented excellent concordance of results, which was expressed by a kappa coefficient of 0.93, as well as equivalence in clinical practice, although some authors suggest that the sensitivity of direct testing is higher when auramine staining is used. Therefore, the choice of the method is based on the characteristics and resources of the laboratory, since the time required to analyze the smears using auramine staining is shorter than that required to perform sputum smear microscopy using Ziehl-Neelsen staining.

Table 3 - Sensitivity and specificity of nucleic acid amplification tests in the diagnosis of pulmonary tuberculosis.

<table>
<thead>
<tr>
<th>Test</th>
<th>Respiratory secretion</th>
<th>Positive sputum smear microscopy results</th>
<th>Negative sputum smear microscopy results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity, %</td>
<td>Specificity, %</td>
<td>Sensitivity, %</td>
</tr>
<tr>
<td>Amplicor</td>
<td>97</td>
<td>&gt; 95</td>
<td>65.5-85.3</td>
</tr>
<tr>
<td>Cobas Amplicor</td>
<td>73.6-93.3</td>
<td>80-100</td>
<td>56.5-75</td>
</tr>
<tr>
<td>AMTD</td>
<td>92-100</td>
<td>&gt; 95</td>
<td>40-93</td>
</tr>
<tr>
<td>EMTD</td>
<td>83.8-99.9</td>
<td>99</td>
<td>50.6-87.9</td>
</tr>
<tr>
<td>Real-time PCR system</td>
<td>78</td>
<td>100</td>
<td>78</td>
</tr>
</tbody>
</table>

*Source: Reproduced with permission from Roche Molecular Systems, Branchburg, NJ, USA; Amplified Mycobacterium tuberculosis direct test (AMTD) and Enhanced Amplified Mycobacterium tuberculosis direct test (EMTD): produced by Gen-Probe Inc, San Diego, CA, USA; and Real-time PCR system: produced by Shanghai Hongshi Medical Tech. Co., Shanghai, China.*

In the present study, culture on LJ medium, which allows the definitive confirmation of the diagnosis of pulmonary TB, had a sensitivity of 67.6%, which is lower than the 80%-100% rate typically described, and the influence of the collection of a single sample should be considered. The main advantage of other culture media, as well as of automated and semi-automated detection methods, is the shorter time required to detect mycobacteria (approximately 15 days rather than 3-8 weeks). However, LJ medium, which is approved by the World Health Organization, remains the most widely used in Brazil. Additional benefits include the fact that the strains can be stored for future studies, and that some strains grow only in this medium. For these reasons, the use of new methods does not dispense with the use of conventional culture.

In relation to the other methods, PCR had higher sensitivity (77.5%), which is within the range (42%-90.9%) established in the literature, the variation depending principally on the characteristics of the patient sample. The sensitivity of PCR was found to be similar to that of culture (p = 0.09), as described in most of the studies comparing these two methods, and there was good concordance of results, which was expressed by a kappa coefficient of 0.78. Culture and PCR were superior to the direct testing methods (p = 0.048 and p = 0.00007, respectively), probably due to the greater capacity of culture and PCR for bacillus detection in paucibacillary samples. Direct testing detects bacilli in samples containing at least 5,000-10,000 bacilli, whereas culture requires only 10-100 bacilli, and PCR requires only 1-20 bacilli. Ziehl-Neelsen staining and auramine staining demonstrated good concordance of results with culture and PCR (kappa coefficient, 0.54-0.66; Table 2).
The collection of more than one sample per patient would probably increase the sensitivity of all of the methods evaluated in the present study. However, the comparison of the methods using a single sample portrays common situations in clinical practice, as occurs in emergency care clinics.

The sensitivity of PCR was statistically lower in samples with negative sputum smear microscopy results than in those with positive results (50.8% and 98.8%, respectively), which is in accordance with the results of other studies (Table 3). The sensitivity of PCR in samples with negative results in direct testing and culture was even lower (25.6%), being statistically lower than that found in samples with positive conventional test results (99.0%). The decreased sensitivity in samples with negative sputum smear microscopy results is due to the presence of a reduced number of bacilli, the lack of homogeneity of the patient sample and the use, in PCR, of a volume lower than that used in culture. We highlight the fact that, to date, the PCR technique used (Amplicor) has not been approved by the Food and Drug Administration for use in samples with negative sputum smear microscopy results, since the sensitivity in this type of sample varies. Although the decreased sensitivity of PCR in samples with negative direct testing results is one of the greatest limitations of the method, it is of note that, in the present study, PCR detected bacilli in approximately half of the patients with negative sputum smear microscopy results and in approximately one fourth of the patients whose diagnosis would not have been clarified by direct testing or culture. In samples with negative direct testing results, the sensitivity of PCR was statistically higher than was that of culture (50.8% and 34.0%, respectively), considering that the sensitivity of culture in the present study was lower than that usually reported. Despite its higher cost, PCR yields faster results and has advantages such as simultaneous detection and identification of mycobacteria, which is desirable in some situations, even in patients with positive direct testing results.

In the identification of M. tuberculosis, the concordance between PCR and the radiometric method using NAP was 96.8%. The identification of mycobacteria using conventional culture requires 6 to 8 weeks, whereas the identification using culture by the radiometric method reduces the time required to approximately 15 days. The molecular techniques require only approximately 2 h. Reducing the time required to identify TB patients is important for controlling the dissemination of the disease, allowing the early institution of treatment, and this can have a positive impact on public health.

However, PCR results can remain positive for more than 12 months after diagnosis and initiation of treatment, even 6 months after conversion (direct testing and culture). This precludes the use of PCR in follow-up treatment. In the present study, there was no statistically significant difference between patients with a history of pulmonary TB and those without in terms of the sensitivity of PCR (78.6% and 77.6%, respectively). Among the patients with a history of TB, the rate of abandonment of previous treatment was high (44 of 77 patients; 57.1%), as was the incidence of active disease at the time of the study, which might have contributed to the absence of a difference between patients with a history of pulmonary TB and those without in terms of the sensitivity of PCR. In patients with a history of TB but without active disease, the median time since previous diagnosis was 36 months, longer than the 6- and 12-month intervals described in the literature as necessary for PCR detection of bacilli to persist.

Due to the small number of patients without pulmonary TB and the absence of infection by other pathogens, the present study is not adequate to evaluate specificity, although no false-positive results were observed, and other studies confirm the high specificity of nucleic acid amplification tests (Table 3).

In the present study, direct testing was found to remain the method of choice for the initial evaluation of patients suspected of having TB, since it has high sensitivity (even in a single sample), is inexpensive and is easily performed. The most sensitive method for the diagnosis of TB proved to be PCR, being equivalent to culture, with the advantages of faster results and simultaneous identification of M. tuberculosis, although having the disadvantage of being more costly. It has been said that, although the sensitivity of PCR in samples with negative sputum smear microscopy results is lower than desirable, this method can still present an advantage, when compared with conventional methods, for the rapid diagnosis of paucibacillary pulmonary TB. Currently, there is no other, more effective method when the combined analysis of conventional clinical, radiological and microbiological findings does not establish the diagnosis.
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