Identification of nontuberculous mycobacteria isolated from clinical sterile sites in patients at a university hospital in the city of Rio de Janeiro, Brazil*.

Identificação de micobactérias não tuberculosas isoladas de sítios estéreis em pacientes em um hospital universitário na cidade do Rio de Janeiro

Simone Gonçalves Senna, Ana Grazia Marsico, Gisele Betzler de Oliveira Vieira, Luciana Fonseca Sobral, Philip Noel Suffys, Leila de Souza Fonseca

Abstract

Objective: To identify nontuberculous mycobacteria (NTM) isolated from sterile sites in patients hospitalized between 2001 and 2006 at the Clementino Fraga Filho University Hospital, located in the city of Rio de Janeiro, Brazil. Methods: During the study period, 34 NTM isolates from sterile sites of 14 patients, most of whom were HIV-positive, were submitted to phenotypic identification and hsp65 PCR-restriction enzyme analysis (PRA). Results: Most isolates were identified as Mycobacterium avium, followed by M. monacense, M. kansasii, and M. abscessus. Conclusions: The combination of PRA, a relatively simple and inexpensive method, with the evaluation of a few phenotypic characteristics can allow NTM to be accurately identified in the routine of clinical laboratories.

Keywords: Mycobacteria, atypical; Molecular biology; Polymerase chain reaction.

Resumo

Objetivo: Identificar micobactérias não tuberculosas (MNT) isoladas de sítios estéreis em pacientes internados no Hospital Universitário Clementino Fraga Filho, Rio de Janeiro (RJ) entre 2001 e 2006. Métodos: Durante o período do estudo, 34 isolados de MNT de sítios estéreis de 14 pacientes, a maioria HIV positivos, foram submetidos a identificação fenotípica e hsp65 PCR-restriction enzyme analysis (PRA, análise por enzimas de restrição por PCR do gene hsp65). Resultados: A maioria dos isolados foi identificada como Mycobacterium avium, seguida por M. monacense, M. kansasii e M. abscessus em menores proporções. Conclusões: A combinação de PRA, um método relativamente simples e de baixo custo, com algumas características fenotípicas pode fornecer a identificação correta de MNT na rotina de laboratórios clínicos.

Descritores: Micobactérias atípicas; Biologia molecular; Reação em cadeia da polimerase.

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Introduction

In recent years, the number of nontuberculous mycobacteria (NTM) isolated from clinical specimens has increased, partly due to opportunistic infections accompanying immunosuppression. Traditionally, the definitive diagnosis of mycobacterial infections has been dependent on the isolation and identification of causative agents and has required a series of specialized physiological and biochemical tests. Because mycobacterium species have different drug susceptibilities, precise identification is crucial for the adoption of appropriate drug therapy and can ultimately influence patient outcome. Plikaytis et al. and Telenti et al. using separate gene regions, described the successful identification of mycobacteria by using restriction digest analysis of amplified \textit{hsp65} fragments—\textit{hsp65} PCR-restriction enzyme analysis (PRA). Most of the DNA patterns generated by restriction enzyme analysis of \textit{Mycobacterium}-specific PCR products are species-specific, and PRA of part of the \textit{hsp65} gene is just one approach that has been used as a means of identifying mycobacterium species. The objective of the present study was to identify, using the PRA method, isolates collected between 2001 and 2006 from sterile sites of clinical inpatients at a university hospital in the city of Rio de Janeiro, Brazil.

Methods

Between 2001 and 2006, 34 atypical mycobacterial isolates from various biological specimens (at least one from a sterile site in each patient) were identified. These samples were collected from 14 patients hospitalized at the Clementino Fraga Filho University Hospital, located in the city of Rio de Janeiro, Brazil. The medical records of the 14 patients were reviewed retrospectively. Of the 14 patients, 12 were HIV-positive.

The isolates were cultured on Löwenstein-Jensen medium. The phenotypic identification was carried out by means of standard biochemical tests (arylsulfatase, catalase, Tween hydrolysis, tellurite reduction, niacin production, urease, and nitrate reduction), as well as by evaluation of pigment production, growth rate, and colony characteristics.

Nucleic acid from clinical isolates was extracted by submitting a loopful of a colony growth from the solid culture in 50 µL of ultrapure water. The mixture was boiled once for 10 min and frozen at −20°C overnight. After a brief centrifugation, 10 µL of the supernatant was used for amplification.

The preparation of PCR was carried out with 10 µL of the genomic DNA extract, 20 pmol of \textit{hsp65}-specific primers—\textit{tb11} (ACCAACGATGGTGTGTCCAT) and \textit{tb12} (CTTGTCGAACCGCATACCCT)—50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl$_2$, 50% glycerol, 200 µM deoxynucleotide triphosphate, and 1.5 U of Taq polymerase (Invitrogen, Karlsruhe, Germany) to yield a final volume of 50 µL. The reactions were performed using a DNA analyzer system (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed as follows: initial denaturation at 94°C for 1 min; 45 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min; and a final extension step at 72°C for 7 min, followed by a cooling step at 4°C. In order to verify the success of the amplification, the PCR product was run on a 1% agarose gel containing ethidium bromide. Subsequently, 15 µL of PCR products were separately digested with restriction enzymes \textit{Bst}EII (incubated at 60°C for 2 h) and \textit{Hae}III (at 37°C for 2 h) in accordance with the manufacturer instructions. The digestion products were visualized on 3% agarose ultrapure gel + (89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8.0) + ethidium bromide. The DNA size markers—50-bp and 25-bp DNA ladders—were applied in three lines (one at each extreme of the gel and one in the center). After electrophoresis at 5 V/cm, the gels were visualized on a UV transilluminator and photographed.

Results

Most of the 14 patients selected were HIV-positive. The 34 isolates were biochemically tested (Table 1), and PRA, using \textit{Bst}EII and \textit{Hae}III, generated the \textit{hsp65} PRA patterns shown in Figure 1. The isolates were submitted to complementary speciation by conventional biochemical tests, and the majority showed the biochemical profile typical of \textit{Mycobacterium avium} complex (MAC). The results of the PRA and of the biochemical identification were mostly concordant; however, for one isolate, there

Discussion

In immunocompromised individuals, infections due to NTM have been shown to be a major cause of morbidity and mortality.\textsuperscript{a} We employed phenotypic and molecular techniques to study NTM isolated from sterile sources in.

Table 1 - Patient characteristics and identification of nontuberculous mycobacteria isolated from sterile sites in patients hospitalized at the Clementino Fraga Filho University Hospital, Rio de Janeiro, Brazil, 2001-2006.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, years</th>
<th>HIV status</th>
<th>Strains, n</th>
<th>Clinical specimens</th>
<th>Biochemical tests</th>
<th>Molecular method (PRA-\textit{hsp65})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26</td>
<td>+</td>
<td>2</td>
<td>cerebrospinal fluid</td>
<td>Mycobacterium flavescens</td>
<td>M. monacense</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>+</td>
<td>3</td>
<td>blood</td>
<td>MAC</td>
<td>M. avium type I</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
<td>+</td>
<td>2</td>
<td>pleural fluid</td>
<td>MAC</td>
<td>M. avium type I</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
<td>+</td>
<td>1</td>
<td>lymph node</td>
<td>MAC</td>
<td>M. avium type I</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>+</td>
<td>1</td>
<td>pleural biopsy</td>
<td>MAC</td>
<td>M. avium type I</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>+</td>
<td>2</td>
<td>blood</td>
<td>MAC</td>
<td>M. avium type I</td>
</tr>
<tr>
<td>7</td>
<td>41</td>
<td>+</td>
<td>1</td>
<td>blood</td>
<td>MAC</td>
<td>M. fortuitum</td>
</tr>
<tr>
<td>8</td>
<td>39</td>
<td>Unknown</td>
<td>4</td>
<td>lymph node</td>
<td>MAC</td>
<td>M. avium type I</td>
</tr>
<tr>
<td>9</td>
<td>33</td>
<td>+</td>
<td>1</td>
<td>blood</td>
<td>MAC</td>
<td>M. avium type I</td>
</tr>
<tr>
<td>10</td>
<td>26</td>
<td>+</td>
<td>5</td>
<td>blood</td>
<td>MAC</td>
<td>M. avium type I</td>
</tr>
<tr>
<td>11</td>
<td>50</td>
<td>−</td>
<td>2</td>
<td>cardiac valves</td>
<td>M. chelonae complex</td>
<td>M. abscessus type I</td>
</tr>
<tr>
<td>12</td>
<td>52</td>
<td>+</td>
<td>1</td>
<td>bone marrow</td>
<td>MAC</td>
<td>M. avium type I</td>
</tr>
<tr>
<td>13</td>
<td>33</td>
<td>+</td>
<td>2</td>
<td>blood</td>
<td>MAC</td>
<td>M. avium type I</td>
</tr>
<tr>
<td>14</td>
<td>35</td>
<td>+</td>
<td>2</td>
<td>blood</td>
<td>M. kansasii</td>
<td>M. kansasii type I</td>
</tr>
</tbody>
</table>

MAC: \textit{M. avium} complex; BALF: bronchoalveolar lavage fluid.

was a discrepancy between the PRA-generated pattern and the results of the phenotypic identification. The isolate from patient 1 (in a cerebrospinal fluid sample) was biochemically identified as \textit{M. flavescens}, whereas the PRA pattern was indicative of \textit{M. monacense}. The latter strain is a novel species within the genus \textit{Mycobacterium}: it is pigmented and grows in less than one week on solid medium; therefore, its differentiation from \textit{M. flavescens} by the phenotypic method is very difficult. However, the PRA method easily differentiates the two species, because the PRA band patterns of \textit{M. flavescens} and of \textit{M. monacense} are, respectively, \textit{BstEII} 440/0/0-\textit{HaeIII} 150/85/55/0 and \textit{BstEII} 235/130/85-\textit{HaeIII} 140/55/50/0. The overall concordance between the two methods was 95.9% (for 47 of 49 isolates). \textit{M. avium} was mostly isolated from blood, liquor, lymph nodes, biopsy specimens, and disseminated infection. All of the patients were being treated for HIV, except for patients 11 (an HIV-negative patient) and 8 (whose HIV status was unknown).

![Figure 1 - The \textit{hsp65} PRA patterns obtained using \textit{BstEII} and \textit{HaeIII}. Lanes 1 to 3 and 5 to 8: \textit{Mycobacterium avium} type I; lane 4: \textit{Mycobacterium intracellulare}; M: contains 25-bp and 50-bp molecular weight markers.](image-url)
patients hospitalized between 2001 and 2006. The patients were HIV-positive under treatment, except for patients 11 (an HIV-negative patient) and 8 (whose HIV status was unknown). In the present study, we opted to use the PRA method because it is rapid and easy to perform, as well as because it can identify multiple mycobacterium species within a single experiment. The PRA method has a broader spectrum than do other molecular methods because it amplifies a conserved hsp65 gene that is present in all mycobacteria and in some other bacteria, such as Nocardia spp. Mycobacteria are ubiquitous in nature and can be found in soil, dust, rocks, bioaerosols, and water. These organisms have been increasingly identified in environments in which the conditions are harsh (low nutrients, low pH, and extreme temperatures).

Biofilm formation is a successful survival strategy for these very hydrophobic organisms. In the present study, two isolates from cardiac valves in patient 11 (a cardiac surgery patient) were identified as M. abscessus, which was probably introduced via intraoperative contamination. Rapidly growing mycobacteria have been implicated in outbreaks of surgical wound infections and post-injection abscesses. Catheter-related infections have been also associated with such mycobacteria. Pseudo-outbreaks of diseases, defined as clusters of false infections or artifactual clustering of real infections, have been associated with contaminated bronchoscopes and contaminated tap water used in automated endoscopic cleaning machines. Patient 14, who was HIV-positive, developed disseminated M. kansasii infection. This pathogen has frequently been isolated in water distribution networks that feed domestic sinks and showers, and it can be acquired from the environment rather than from human-to-human transmission. M. kansasii commonly causes pulmonary infection, similarly to pulmonary tuberculosis, the highest incidence of such infection being in patients with COPD. However, M. kansasii can infect the blood, which can lead to death. The identification of the two strains from the cerebrospinal fluid of patient 1 showed discordant results. The strains were identified as M. flavescens by biochemical tests and as M. monacense by the PRA method. M. monacense is a novel, fast-growing, yellow-pigmented, non-photochromogenic species. Based on the phenotypic investigation alone, the distinction of this species from other known rapidly growing scotochromogenic strains is problematic. However, it differs from any other mycobacterial species due to its PRA band patterns. One M. monacense strain was reported to be responsible for a severe, post-traumatic wound infection in a healthy boy. We obtained 29 M. avium isolates that infected the majority of the HIV-positive patients. Prior to the AIDS pandemic, MAC was mostly responsible for pneumopathy in patients with basic chronic pulmonary diseases, such as emphysema and chronic bronchitis. In 1981, with the advent of AIDS, MAC came to represent one of the most common bacterial diseases among AIDS patients, the disseminated form of the disease being the major clinical manifestation of the infection. In 1997, Gadelha et al. evaluated the incidence of mycobacterial disease and the MAC colonization of the respiratory and gastrointestinal tracts in AIDS patients in the city of Rio de Janeiro, Brazil. Although the blood cultures of the patients were negative, they were all under treatment with highly active antiretroviral therapy (HAART). In São Paulo, da Silva et al. isolated 103 strains from blood, lymph node, and biopsy samples and, using the PRA method, identified 24 isolates as M. avium. Ferreira et al. determined the prevalence of NTM isolates at a referral hospital for AIDS in Rio de Janeiro during a one-year period, prior to the HAART era. The authors isolated 83 specimens from 35 patients, using biochemical tests to determine the species and IS1245 amplification to identify M. avium. In that study, M. avium seemed to predominate in sterile blood isolates from hospitalized patients.

The present study described the NTM isolated from sterile sites over a six-year period in the HAART era, at the same university hospital where the Ferreira et al. study was conducted. We identified 34 isolates from 14 patients, a mean of 2.3 patients/year, which points out the impact of HAART on NTM infections. The Mycobacterium genus includes a great number of species, and the actual number of taxonomically valid species is fluctuating because of the use of synonyms and the frequent discovery of new mycobacterium species. The conventional differentiation of NTM by culture and biochemical characteristics is virtually impossible
today. In the present study, all of the strains identified as MAC by phenotypic characteristics showed a PRA band pattern of *M. avium* type I. Silva et al.(1) compared the PRA method and the phenotypic identification technique in a routine setting. Among the 24 *M. avium* isolates, only 6 belonged to PRA site type I. In our study, PRA was compared with biochemical identification, and the results showed that the PRA method is also a valid alternative to conventional (nonmolecular) methods of identification. The time needed to complete PRA was less than one week, shortening the time to the final diagnosis. The main drawback in the routine use of PRA is the analysis of patterns. Differences between calculated sizes of restriction fragments and published patterns have been reported, and this could be due to differences in running conditions, type of agarose gel used, or type of computer program used. In our experience, using PRA in combination with the evaluation of phenotypic characteristics resolves the problem of PRA identification; for example, *M. gordonae* type V and *M. avium* type I have very similar PRA patterns (*Bst*II 235/210/0-*Hae*III 130/115/0/0 and *Bst*II 235/210/0-*Hae*III 130/105/0/0, respectively). However, *M. gordonae* is yellow-pigmented and *M. avium* is nonpigmented.

In a routine setting, the rapid identification of *M. tuberculosis* complex, *M. avium, M. kansasii, M. fortuitum,* and *M. abscessus* is important because the type of treatment is different for each of these species. In our study, the PRA-based identification of these species was performed by simple visualization on agarose gels. This confirms the usefulness of this technique, even at facilities where computerized systems are unavailable.

In conclusion, the present study demonstrated that PRA is a rapid, inexpensive, and accurate method for the identification of pathogenic and potentially pathogenic mycobacteria. For final identification and the best speciation of NTM, the PRA band patterns should be considered together with a few phenotypic characteristics, such as pigment production and growth rate.

References


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