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.

* Study carried out at the *Universidade Federal do Rio de Janeiro* – UFRJ, Federal University of Rio de Janeiro – Rio de Janeiro, Brazil. Correspondence to: Simone Gonçalves Senna. Departamento de Microbiologia Médica, Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho, 373, CCS, Bloco I, Lab. 011, Cidade Universitária, Ilha do Fundão, CEP 21941-902, Rio de Janeiro, RJ, Brasil
Tel. 55 21 2560-8344, extension 134. E-mail: simonegsenna@yahoo.com.br
Financial support: This study received financial support from the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq, Brazilian National Council for Scientific and Technological Development) and *Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro* (FAPERJ, Rio de Janeiro Research Foundation).
Submitted: 28 January 2011. Accepted, after review: 2 May 2011.
** A versão completa em português deste artigo está disponível em www.jornaldepneumologia.com.br

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.[1] Plikaytis et al.[2] and Telenti et al.,[3] using separate gene regions, described the successful identification of mycobacteria by using restriction digest analysis of amplified *hsp65* fragments—*hsp65* PCR-restriction enzyme analysis (PRA). Most of the DNA patterns generated by restriction enzyme analysis of *Mycobacterium*-specific PCR products are species-specific, and PRA of part of the *hsp65* gene is just one approach that has been used as a means of identifying mycobacterium species.[4] 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),[5] 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 *hsp65*-specific primers[3]—tb11 (ACCAACGATGGTGTGTCCAT) and 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 *Bst*EII (incubated at 60°C for 2 h) and *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 *Bst*EII and *Hae*III, generated the *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 *Mycobacterium avium* complex (MAC). The results of the PRA and of the biochemical identification were mostly concordant; however, for one isolate, there

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.

Patient	Age, years	HIV status	Strains, n	Clinical specimens	Biochemical tests	Molecular method (PRA-*hsp*65)
1	26	+	2	cerebrospinal fluid	*Mycobacterium flavescens*	*M. monacense*
2	30	+	3	blood	MAC	*M. avium* type 1
3	32	+	2	pleural fluid	MAC	*M. avium* type 1
4	33	+	1	lymph node	MAC	*M. avium* type 1
			1	BALF		
5	25	+	1	pleural biopsy	MAC	*M. avium* type 1
6	15	+	2	blood	MAC	*M. avium* type 1
			1	lymph node		
			1	ascitic fluid	*M. fortuitum*	*M. fortuitum*
7	41	+	1	blood	MAC	*M. avium* type 1
			1	lymph node		
8	39	Unknown	4	lymph node	MAC	*M. avium* type 1
9	33	+	1	blood	MAC	*M. avium* type 1
10	26	+	5	blood	MAC	*M. avium* type 1
			1	lymph node		
11	50	–	2	cardiac valves	*M. chelonae* complex	*M. abscessus* type 1
12	52	+	1	bone marrow	MAC	*M. avium* type 1
13	33	+	2	blood	MAC	*M. avium* type 1
14	35	+	2	blood	*M. kansasii*	*M. kansasii* type 1

MAC: *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 *M. flavescens*, whereas the PRA pattern was indicative of *M. monacense*. The latter strain is a novel species within the genus *Mycobacterium*: it is pigmented and grows in less than one week on solid medium; therefore, its differentiation from *M. flavescens* by the phenotypic method is very difficult. However, the PRA method easily differentiates the two species, because the PRA band patterns of *M. flavescens* and of *M. monacense* are, respectively, *Bst*EII 440/0/0-*Hae*III 150/85/55/0 and *Bst*EII 235/130/85-*Hae*III 140/55/50/0. The overall concordance between the two methods was 95.9% (for 47 of 49 isolates). *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).

Discussion

In immunocompromised individuals, infections due to NTM have been shown to be a major cause of morbidity and mortality.[6] We employed phenotypic and molecular techniques to study NTM isolated from sterile sources in

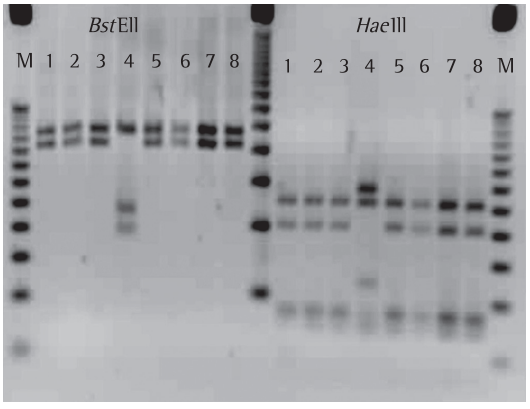

Figure 1 - The *hsp65* PRA patterns obtained using *Bst*EII and *Hae*III. Lanes 1 to 3 and 5 to 8: *Mycobacterium avium* type 1; lane 4: *Mycobacterium intracellulare*; M: contains 25-bp and 50-bp molecular weight markers.

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.[7] 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[8-11] and post-injection abscesses.[12,13] Catheter-related infections have been also associated with such mycobacteria.[14] 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.[15,16] 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.[6,17] *M. kansasii* commonly causes pulmonary infection, similarly to pulmonary tuberculosis, the highest incidence of such infection being in patients with COPD.[18,19] 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.[20] 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.[21] 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*.[1] 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.[22] The authors isolated 83 specimens from 35 patients, using biochemical tests to determine the species and *IS*1245 amplification to identify *M. avium*. In that study, *M. avium* seemed to predominate in sterile blood isolates from hospitalized patients.[22]

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.[22] 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,[4,23] 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*EII 235/210/0-HaeIII 130/115/0/0 and *Bst*EII 235/210/0-*Hae*III 130/105/0/00, 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

1. da Silva CF, Ueki SY, Geiger Dde C, Leão SC. hsp65 PCR-restriction enzyme analysis (PRA) for identification of mycobacteria in the clinical laboratory. Rev Inst Med Trop Sao Paulo. 2001;43(1):25-8.
2. Plikaytis BB, Plikaytis BD, Yakrus MA, Butler WR, Woodley CL, Silcox VA, et al. Differentiation of slowly growing Mycobacterium species, including Mycobacterium tuberculosis, by gene amplification and restriction fragment length polymorphism analysis. J Clin Microbiol. 1992;30(7):1815-22.
3. Telenti A, Marchesi F, Balz M, Bally F, Böttger EC, Bodmer T. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol. 1993;31(2):175-8.
4. Devallois A, Goh KS, Rastogi N. Rapid identification of mycobacteria to species level by PCR-restriction fragment length polymorphism analysis of the hsp65 gene and proposition of an algorithm to differentiate 34 mycobacterial species. J Clin Microbiol. 1997;35(11):2969-73.
5. Kent PT, Kubica GP. Public Health Mycobacteriology: A Guide for the Level III Laboratory. Atlanta: U.S. Dept. of Health and Human Services, Public Health Service, Centers for Disease Control; 1985.
6. Falkinham JO 3rd. Epidemiology of infection by nontuberculous mycobacteria. Clin Microbiol Rev. 1996;9(2):177-215.
7. De Groote MA, Huitt G. Infections due to rapidly growing mycobacteria. Clin Infect Dis. 2006;42(12):1756-63.
8. Wallace RJ Jr, Brown BA, Griffith DE. Nosocomial outbreaks/pseudo-outbreaks caused by nontuberculous mycobacteria. Annu Rev Microbiol. 1998;52:453-90.
9. Wallace RJ Jr, Musser JM, Hull SI, Silcox VA, Steele LC, Forrester GD, et al. Diversity and sources of rapidly growing mycobacteria associated with infections following cardiac surgery. J Infect Dis. 1989;159(4):708-16.
10. Freitas D, Alvarenga L, Sampaio J, Mannis M, Sato E, Sousa L, et al. An outbreak of Mycobacterium chelonae infection after LASIK. Ophthalmology. 2003;110(2):276-85.
11. Vijayaraghavan R, Chandrashekhar R, Sujatha Y, Belagavi CS. Hospital outbreak of atypical mycobacterial infection of port sites after laparoscopic surgery. J Hosp Infect. 2006;64(4):344-7.
12. Galil K, Miller LA, Yakrus MA, Wallace RJ Jr, Mosley DG, England B, et al. Abscesses due to mycobacterium abscessus linked to injection of unapproved alternative medication. Emerg Infect Dis. 1999;5(5):681-7.
13. Villanueva A, Calderon RV, Vargas BA, Ruiz F, Aguero S, Zhang Y, et al. Report on an outbreak of postinjection abscesses due to Mycobacterium abscessus, including management with surgery and clarithromycin therapy and comparison of strains by random amplified polymorphic DNA polymerase chain reaction. Clin Infect Dis. 1997;24(6):1147-53.
14. Wallace RJ Jr, Swenson JM, Silcox VA, Good RC, Tschen JA, Stone MS. Spectrum of disease due to rapidly growing mycobacteria. Rev Infect Dis. 1983;5(4):657-79.
15. Fraser VJ, Jones M, Murray PR, Medoff G, Zhang Y, Wallace RJ Jr. Contamination of flexible fiberoptic bronchoscopes with Mycobacterium chelonae linked to an automated bronchoscope disinfection machine. Am Rev Respir Dis. 1992;145(4 Pt 1):853-5.
16. Fraser V, Wallace RJ Jr. Nontuberculous mycobacteria. In: Mayhall CG, editor. Hospital Epidemiology and Infection Control. Philadelphia: Lippincott Williams & Wilkins; 1996.

17. Collins FM. Mycobacterial disease, immunosuppression, and acquired immunodeficiency syndrome. Clin Microbiol Rev. 1989;2(4):360-77.
18. Martínez-Moragón E, Menéndez R, Palasí P, Santos M, López Aldeguer J. Environmental mycobacterial diseases in patients with and without HIV infection: epidemiology and clinical course [Article in Spanish]. Arch Bronconeumol. 2001;37(6):281-6.
19. Koh WJ, Kwon OJ, Lee KS. Nontuberculous mycobacterial pulmonary diseases in immunocompetent patients. Korean J Radiol. 2002;3(3):145-57.
20. Reischl U, Melzl H, Kroppenstedt RM, Miethke T, Naumann L, Mariottini A, et al. *Mycobacterium monacense* sp. nov. Int J Syst Evol Microbiol. 2006;56(Pt 11):2575-8.
21. Gadelha A, Accácio N, Grinzstejn B, Veloso V, da Silveira LB, Fandinho F, et al. Low incidence of colonization and no cases of disseminated Mycobacterium avium complex infection (DMAC) in Brazilian AIDS patients in the HAART era. Braz J Infect Dis. 2002;6(5):252-7.
22. Ferreira RM, Saad MH, Silva MG, Fonseca Lde S. Non-tuberculous mycobacteria I: one year clinical isolates identification in Tertiary Hospital Aids Reference Center, Rio de Janeiro, Brazil, in pre highly active antiretroviral therapy era. Mem Inst Oswaldo Cruz. 2002;97(5):725-9.
23. da Silva Rocha A, da Costa Leite C, Torres HM, de Miranda AB, Pires Lopes MQ, Degrave WM, et al. Use of PCR-restriction fragment length polymorphism analysis of the hsp65 gene for rapid identification of mycobacteria in Brazil. J Microbiol Methods. 1999;37(3):223-9.

About the authors

Simone Gonçalves Senna
Researcher. Department of Medical Microbiology, Professor Paulo de Góes Microbiology Institute, *Universidade Federal do Rio de Janeiro* – UFRJ, Federal University of Rio de Janeiro – Rio de Janeiro, Brazil.

Ana Grazia Marsico
Head. Laboratory of Mycobacteriology, *Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro* – HUCFF/UFRJ, Clementino Fraga Filho University Hospital, Federal University of Rio de Janeiro – Rio de Janeiro, Brazil.

Gisele Betzler de Oliveira Vieira
Head. Laboratory of Mycobacteriology, *Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro* – HUCFF/UFRJ, Clementino Fraga Filho University Hospital, Federal University of Rio de Janeiro – Rio de Janeiro, Brazil.

Luciana Fonseca Sobral
Researcher. Laboratory of Mycobacteriology, *Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro* – HUCFF/UFRJ, Clementino Fraga Filho University Hospital, Federal University of Rio de Janeiro – Rio de Janeiro, Brazil.

Philip Noel Suffys
Head. Laboratory of Molecular Biology applied to Mycobacteria, *Fundação Oswaldo Cruz* – FIOCRUZ, Oswaldo Cruz Foundation – Rio de Janeiro, Brazil.

Leila de Souza Fonseca
Full Professor. *Universidade Federal do Rio de Janeiro* – UFRJ, Federal University of Rio de Janeiro – Rio de Janeiro, Brazil.