Nontuberculous Mycobacteria in Clinical Samples with Negative Acid-Fast Bacilli

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Abstract

Background: There is a progressive increase in nontuberculous mycobacteria (NTM) in pulmonary and extrapulmonary infections that might cause confusion with the Mycobacterium tuberculosis complex. To determine the frequency of finding NTM in clinical samples from patients diagnosed with active tuberculosis, with negative acid-alcohol-resistant bacilli (AFB) in a third-level specialty hospital’s mycobacterial laboratory between January 2013 and December 2014. Methods: This is a prospective, descriptive study where isolated strains of biological material were studied in Löwenstein–Jensen and BACTEC MGIT 960 cultures. Results: Clinical samples of 120 patients were studied, with pulmonary samples of 99/120 (82%) and extrapulmonary samples of 21/120 (18%). We identified NTM in 37/120 samples (30.8%), of which 16 in pulmonary, 13 in genitourinary, 3 in bone marrow, and 5 in various specimens. Mycobacterium avium was isolated in 20 samples, Mycobacterium intracellulare in seven samples, and various other species of NTM in the other 10 samples. Conclusion: To establish adequate treatment, we point out the importance of identifying the presence of NTM in the clinical samples of active tuberculosis patients with negative AFB, as possibly becoming confused with M. tuberculosis and which is essential in deciding which treatment is the most adequate.

Keywords: Mycobacterium tuberculosis, nontuberculosis mycobacteria, PCR sequencing

INTRODUCTION

Tuberculosis is a global public health problem that coexists with a progressive increase in nontuberculous mycobacteria (NTM). However, there are no hard data on the prevalence of these in the general population because NTM cases are not on the list of obligatory reporting to health authorities. There has been an increase in frequency of patients infected by NTM as of 2004, worrisome to health officials due to the high cost of treatment, especially in emerging countries. The incidence of NTM is higher in countries with greater tuberculosis prevalence. In Mexico, there are only limited reports of immunodeficient patients, in whom Mycobacterium avium, Mycobacterium kansasii, Mycobacterium gordonae, Mycobacterium fortuitum, and Mycobacterium simiae have been found.

The NTM phylogenetically identified form a group of >148 species, among which there is a group of 25 different species isolated from various biological products of individuals presenting with pulmonary and extrapulmonary pathology. NTM are saprophyte environmental microorganisms indistinguishable from the Mycobacterium tuberculosis complex, few of which have been classified as producing illness. However, when these are found in pathologic processes, the source of infection is rarely found. The American Thoracic Society mentions 20 strains of NTM that have been isolated as pathogenic in active illness. Pulmonary infection is more frequent in older adults and in childhood lymphadenopathies. Immunodepressed patients, such as with HIV/AIDS, are
the most susceptible and frequently present disseminated types.\textsuperscript{[15-17]}

These mycobacteria also affect the immunocompetent, usually localizing in the lung and in other organs, such as the lymph nodes, kidneys, pleura, meninges, and others.\textsuperscript{[18,19]} NTM do not respond to primary treatment with antituberculosis drugs and must be treated with other medication. Their presence is suspected when patients do not improve and requiring their identification to establish their specific therapy.\textsuperscript{[20]} NTM cases are no longer considered clinical “curiosities”\textsuperscript{[21]} and have been classified as causing emerging infections in countries where tuberculosis has decreased.\textsuperscript{[22,23]} The pathogenesis of the infections produced by NTM has not been demonstrated clearly, and they are not transmitted from person-to-person and occur more frequently in women older than 40 of age with tall, thin phenotype and \textit{pectus excavatum} or bronchiectasis.\textsuperscript{[24]}

The diagnosis and precise identification of the species of NTM require special molecular techniques, which are necessary for establishing treatment.

The objective of this study is to investigate the frequency in one third-level specialty hospital of NTM in samples taken from patients with clinical diagnosis of active tuberculosis, negative acid-fast bacillus (AFB), carried out in the hospital’s mycobacteria laboratory between January 2013 and December 2014.

**Methods**

The prospective study of clinical samples of consecutive cases was carried out to study the frequency of NTM, as causal agent of disease in patients diagnosed clinically with tuberculosis in a third-level hospital in Mexico City, Mexico.

Specimens were taken from patients over the age of 18, with HIV negative, with diagnosis of active tuberculosis and negative AFB, without previous treatment with antituberculosis drugs, and without evidence of pulmonary or extrapulmonary lesions.

Bronchial lavage and extrapulmonary biological material were obtained from each patient and cultivated in Lowenstein–Jensen (L–J) and BACTEC MGIT 960 medium. Each sample was stained with Ziehl–Neelsen (Z–N) for AFB, and polymerase chain reaction (PCR) sequencing was conducted and directed to gene \textit{hsp65} that codes for the thermic shock protein, in patients negative for the \textit{M. tuberculosis} complex.

Bacteriologic studies were carried out in the samples previously homogenized in isotonic saline solution, phosphate buffer, and decontaminated with a solution of sodium hydroxide at 4%. After neutralization and centrifugal spinning, each of the homogenized specimens was suspended again in distilled water. For the cultures, 0.2 mL of this was inoculated in bottles containing BACTEC MGIT 960 liquid fluorescent medium (\textit{Mycobacterium} Growth Indicator Tube, Becton Dickinson, San José, CA, USA) and in solid L–J medium. The cultures were incubated at 37°C for 8 weeks and inspected weekly to evaluate mycobacterial growth.

DNA was extracted by cell disruption with heat shock. PCR was carried out with Tb11 y Tb12 primers of \textit{hsp65} fragment gene, as described by Telenti,\textsuperscript{[25]} and PCR products of 440 pb were obtained. Subsequent sequencing was achieved using the ABI PRISM_3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Phylogenetic analysis was performed with MEGAv. 6.0 software (distance Neighbor Joining method) using the data base for NTM species reported by Escamilla-Escobar,\textsuperscript{[10]} including the GenBank sequences with ID as follows: HM056121, KY337278, KF432467, HM454220 and sequences from Table 1.

### Table 1: Phylogenetic identification of isolates

| Isolates | Year | Isolation source | Phylogenetic identification | GenBank accession number |
|----------|------|------------------|-----------------------------|-------------------------|
| HGM 139/13 | 2013 | Urine | \textit{M. avium} | KX824022 |
| HGM 400/13 | 2013 | Urine | \textit{M. avium} | KX824023 |
| HGM 411/13 | 2013 | Urine | \textit{M. xenopi} | KX824024 |
| HGM 420/13 | 2013 | Bronchial lavage | \textit{M. kubai} | KX824025 |
| HGM 506/13 | 2013 | Gastric lavage | \textit{M. parascrofulaceum} | KX824026 |
| HGM 522/13 | 2013 | Urine | \textit{M. intracellulare} | KX824027 |
| HGM 1533/13 | 2013 | Bronchial lavage | \textit{M. avium} | KX824028 |
| HGM 1730/13 | 2013 | Bronchial lavage | \textit{M. triplex} | KX824029 |
| HGM 1796/13 | 2013 | Urine | \textit{M. avium} | KX824030 |
| HGM 1815/13 | 2013 | Urine | \textit{M. avium} | KX824031 |
| HGM 1849/13 | 2013 | Bone marrow | \textit{M. simiae} | KX824032 |
| HGM 1921/13 | 2013 | Bronchial lavage | \textit{M. intracellulare} | KX824033 |
| HGM 2057/13 | 2013 | Bone marrow | \textit{M. avium} | KX824034 |
| HGM 2319/13 | 2013 | Bronchial lavage | \textit{M. intracellulare} | KX824035 |
| HGM 1486/13 | 2013 | Ascites fluid | \textit{M. avium} | KX824036 |
| HGM 2404/13 | 2013 | Bronchial lavage | \textit{M. avium} | KX824037 |
| HGM 2424/13 | 2013 | Bronchial lavage | \textit{M. avium} | KX824038 |
| HGM 2464/13 | 2013 | Bronchial lavage | \textit{M. avium} | KX824039 |
| HGM 2557/13 | 2013 | Urine | \textit{M. avium} | KX824040 |
| HGM 2588/13 | 2013 | Urine | \textit{M. avium} | KX824041 |
| HGM 2614/13 | 2013 | Bronchial lavage | \textit{M. senueanse} | KX824042 |

Contd...
The frequency of NTM in this study was 30.8% in our samples. Of this group, four patients presented with >1 infected site by NTM as follows: (1) female patient, age 28, with peritoneal and pulmonary localization, and *M. avium* was isolated; (2) male patient, age 26, and had miliary (*M. simie*) and urogenital (*M. avium*) TB; (3) male patient, age 32, and with pulmonary and meningeal with *M. avium*; and (4) male patient, age 31, with pulmonary, and urogenital with *M. avium*.

**DISCUSSION**

The *M. tuberculosis* complex causes the greatest number of tuberculous infections. However, NTM are increasingly being considered more frequent in both immunocompetent and immunocompromised subjects. The infection by NTM is suspected when there are risk factors such as advanced age, bronchiectasis, and poor response to specific and supervised antituberculosis treatment (directly observed treatment). In such cases, the possibility of pathogenic or pharmacologically resistant NTM infection should be considered [7,15,26,27].

Patients in our clinical samples might have NTM infection in spite of negative Z–N for AFB, which means that cultures and PCR are essential for a definitive diagnosis of the species of pathogenic mycobacteria involved and necessary for ruling out possible colonization [29]. Negative AFB does not imply absence of tuberculosis because its sensitivity is 50%–60%. Bronchial lavage specimens provide an alternative for obtaining useful specimens for bacteriologic diagnoses in cases of pulmonary affectionations [29,30].

In Mexico, the increase in the frequency and characterization of nontuberculous disease-causing mycobacterial species is unknown. Studies in >30 countries worldwide found that *M. avium* complex is predominant, which is consistent with the findings in our research. Pulmonary infections and the increase in their extrapulmonary presentations are reported to be a growing problem in many countries, especially in the developing world where uncontrolled tuberculosis is a major issue. Although difficult, it is important to determine the sources and modes of transmission of NTM strains to implement effective prophylactic measures [30].

A delay in accurate diagnosis of NTM leads to an increased mortality, especially in immunocompromised patients. The medication employed may have significant side effects that can lead to treatment withdrawal and an increased resistance to the first-line drugs, and less than 50% of these drugs are successful, and relapses are estimated at >50% within 3 years [31].

The frequency of NTM in this study was 30.8% in our samples. Males were more affected, different from that informed by other authors [32,33] that report predominance in females. Systematic detection of NTM in samples from cases suspected of active tuberculosis with negative AFB is important to implement...
treatment according to sensitivity to antituberculosis drugs, with long-term follow-up and for the evaluation of results.\textsuperscript{[34,35]}

Increasing hospital costs for inpatients and outpatients are currently a priority for the most countries. The high costs affect the public hospitals. Diseases caused by NTM require a prolonged treatment for success, and the total cost involves many other expenses that are both financial and human, such as lost wages. Knowledge of the presence of NTM as a cause of illness in our region will enable the improvement of current prevention guidelines as well as treatment strategies.\textsuperscript{[36]}

**Conclusion**

The possibility of NTM existence in clinical samples in pulmonary and extrapulmonary cases suspected of active tuberculosis should be investigated by culture and PCR, because the NTM can become confused with the *M. tuberculosis* complex and each requires special treatment/species. The frequency of NTM in samples in our study was 30.8%. We identified 13 different species of NTM with *M. avium* and *M. intracellulare* predominating.

**Study limitations**

This study confirms the existence of NTM in Mexico with patients diagnosed with active tuberculosis. However, since our sample is relatively small, our findings establish the need for further controlled studies to determine the actual correct frequency of these infections in the country.

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Nil.
Conflicts of interest
There are no conflicts of interest.

References
1. Glassroth J. Pulmonary disease due to nontuberculous mycobacteria. Chest 2008;133:243-51.
2. Martín-Casabona N, Bahrmand AR, Bennedsen J, Thomsen VO, Cucurio M, Fauville-Dufaux M, et al. Non-tuberculous mycobacteria: Patterns of isolation. A multi-country retrospective survey. Int J Tuberc Lung Dis 2004;8:1186-93.
3. van der Werf MJ, Kődmön C, Katalinić-Janković V, Kummik T, Soini H, Richter E, et al. Inventory study of non-tuberculous mycobacteria in the European Union. BMC Infect Dis 2014;14:62.
4. Adjemian J, Olivier KN, Seitz AE, Holland SM, Prevots DR. Prevalence of nontuberculous mycobacterial lung disease in U.S. Medicare beneficiaries. Am J Resp Crit Care Med 2012,185:881-6.
5. Thomson R, Donnan E, Unwin S. Nontuberculous mycobacterial lung disease. Time to get a grip? Ann Am Thorac Soc 2015;12:1425-7.
6. Strollo SE, Adjemian J, Adjemian MK, Prevots DR. The burden of pulmonary nontuberculous mycobacterial disease in the United States. Ann Am Thorac Soc 2015;12:1456-64.
7. Jankovic M, Samaranjia M, Saboll J, Jakopovic M, Katalinić-Janković V, Zmak L, et al. Geographical distribution and clinical relevance of non-tuberculous mycobacteria in Croatia. Int J Tuberc Lung Dis 2013;17:836-41.
8. Cicero R, Olivera H, Hernández-Solis A, Ramírez-Casanova E, Escobar-Gutiérrez A. Frequency of Mycobacterium bovis as an etiologic agent in extrapulmonary tuberculosis in HIV-positive and -negative Mexican patients. Eur J Clin Microbiol Infect Dis 2009;28:455-60.
9. Cortés-Torres N, González-Y-Merchand JA, González-Bonilla C, García-Elorriaga G. Molecular analysis of mycobacteria isolated in Mexican patients with different immunodeficiencies in a tertiary care hospital. Arch Med Res 2013;44:562-9.
10. Escobar-Escamilla N, Ramírez-González JE, González-Villa M, Torres-Mazadiego P, Mandujano-Martínez A, Barrón-Rivera C, et al. Hsp65 phylogenetic assay for molecular diagnosis of nontuberculous mycobacteria isolated in Mexico. Arch Med Res 2014;45:90-7.
11. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, et al. An official ATS/IDSA statement: Diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Resp Crit Care Med 2007;175:367-416.
12. Cassidy PM, Hedberg K, Saulson A, McNelly E, Winthrop KL. Nontuberculous mycobacterial disease prevalence and risk factors: A changing epidemiology. Clin Infect Dis 2009;49:e124-9.
13. White MP, Bangash H, Goel KM, Jenkins PA. Non-tuberculous mycobacterial lymphadenitis. Arch Dis Child 1986;61:368-71.
14. Cruz AT, Edwards MS, Torchia MM. Overview of Nontuberculous Mycobacterial Lymphadenitis in Children. UpTo Date; June 2015. Available from: https://www.uptodate.com/contents/nontuberculous-mycobacterial-lymphadenitis-in-children. [Last accessed on 2017 Oct].
15. Horsburgh CR Jr, Selik RM. The epidemiology of disseminated nontuberculous mycobacterial infection in the acquired immunodeficiency syndrome (AIDS). Am Rev Respir Dis 1989;139:4-7.
16. Piersimoni C, Scarparo C. Pulmonary infections associated with non-tuberculous mycobacteria in immunocompetent patients. Lancet Infect Dis 2008;8:323-34.
17. Martinez S, McAdams HP, Batchu CS. The many faces of pulmonary nontuberculous mycobacterial infection. AJR Am J Roentgenol 2007;189:177-86.