Disease caused by non-tuberculous mycobacteria: diagnostic procedures and treatment evaluation in the North of Buenos Aires Province

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ABSTRACT

Non-tuberculous mycobacteria (NTM) have emerged as pathogens frequently associated to HIV co-infection. The aims of this study were to describe the clinical importance of NTM in patients from the North of Buenos Aires Province and the drug-susceptibility patterns in relation with the therapy used. A total of 23,624 clinical specimens were investigated during the period 2004-2010. Ziehl-Neelsen stain and cultures were used for diagnosis. Molecular and biochemical tests were performed to identify the mycobacteria. TB and mycobacterioses cases were 2118 and 108 respectively. Sixteen NTM species were found: Mycobacterium avium and Mycobacterium intracellulare as the main causative agents. Infections produced by more than one species at the same time were confirmed (4 cases). Macrolides and fluoroquinolones were the most active in vitro drugs. Treatment evaluation showed that 68.0% of the cases completed the therapy, 20% died; and 12% were relapses. The cases in which the treatment outcome was evaluated received an individual tailor-made therapeutic scheme including those drugs showing in vitro activity and presumed in vivo usefulness. More than a quarter of the patients had HIV co-infection and the majority of the deaths were associated with this co-infection.

Key words: Non-tuberculous mycobacteria, diagnosis and treatment

INTRODUCTION

In opposition to Mycobacterium tuberculosis complex members, non-tuberculous mycobacteria (NTM) are free-living and ubiquitously distributed microorganisms with diverse ability to cause disease in humans. NTM have emerged in the last decades as pathogens frequently associated to the HIV co-infection (11). Nevertheless they can also be found as colonizers of the respiratory tract usually in persons affected by tuberculosis (TB) in the past and having sequel lesions, or they can cause real disease with different localization as lymph adenitis mainly occurring in young children. Patients´ pre-existing conditions such as severe lung infection or past TB, bronchiectasis, emphysema, cystic fibrosis, rheumatoid arthritis and other chronic diseases with pulmonary manifestations can frequently predispose to acquiring a NTM and developing a mycobacterioses (MB). In addition, NTM can cause disseminated disease in severely immunocompromised patients and affect organs such as skin and soft tissues (7, 11).

A large variety of species have been identified as belonging to the mycobacterium genus. Classically, they have been classified into four groups according to the...
Runyon system. Groups I to III contain slowly growing mycobacteria. Groups I, II and III represent the photochromogens, the scotochromogens and nonchromogenic mycobacteria, respectively. Group IV group includes the rapid growers (colonies are visibly in agar in 7 days or less), some of which can produce pigment (23, 24).

Another classification is according to the risk of producing infection and disease in humans. Four risk groups have been defined: group I comprising mycobacteria classified as rare pathogens, with low risk to produce infection for the individual and the community; group II includes the potential pathogens or opportunistic organisms which have moderate risk of causing disease; group III comprises strict pathogens with high risk of causing infection in individuals but moderate risk for the community, and group IV composed of more recently characterized species, isolated from human and environmental sources (5).

An increasing number of infections due to NTM had been previously reported. Although MB cases are generally associated to immunocompromised patients or to patients with pre-existing lung disease, the infection may also occur in an immunocompetent person (10, 12, 16).

In case a NTM is recovered from clinical specimens, it is very important to establish whether this mycobacterium is actually the causative agent of the disease. If the NTM is isolated from a sputum sample belonging to a non-immunocompromised host, the mycobacterium must be isolated at least three times for establishing it as the etiological agent of the disease, but if the NTM is isolated once from an immunocompromised patient or from a sample collected from sterile body sites, it is assumed that the NTM is the real causative agent of the MB (2, 11).

Nevertheless, and in order to avoid treatment mismanagement, the diagnosis of TB caused by members of the *M. tuberculosis* complex must be properly studied and dismissed before assuming the MB diagnosis (2, 11).

Measurement of the NTM burden as etiological agents of a disease different from TB has great relevance mainly in health-care centers where the prevalence of the MB is unknown.

The aims of this study were to describe the load and the clinical importance of NTM in patients from the North of Buenos Aires Province and the drug-susceptibility patterns in relation with the therapeutic schemes used, and to evaluate the successful rate of pharmacological treatments.

**MATERIALS AND METHODS**

**Mycobacteria clinical isolates**

During the period 2004 to July 2010 a total of 23,624 respiratory and non-respiratory clinical specimens were investigated at the Hospital Zonal Especializado de Agudos y Crónicos Dr. A. Cetrángolo for lung diseases. This hospital is located in a Northern Buenos Aires City area with almost four million inhabitants and approximately 1400 new TB cases per year. The Hospital Interzonal General de Agudos Petrona V. de Cordero, San Fernando, Buenos Aires, is a general hospital in the same region. This centre works in cooperation with Hospital Dr. A. Cetrángolo for lung diseases and mycobacteriosis cases and contributes with almost 100 new TB cases per year to the total amount of the region. The disease diagnosis was performed by direct smear examination using the Ziehl-Neelsen stain and cultures. To obtain the isolates, specimens from both non-sterile and sterile body sites were processed as previously described (11, 21). Cultures were recovered from solid media in Löwenstein Jensen, Stonebrink and/or Middlebrook 7H11 (M7H11) and liquid media using the M7H9 and the fully automated BACTEC MGIT960 system (BD Argentina) (13, 17, 21).

**Patient data recovery**

Epidemiological data such as gender, age, site of the lesion, HIV co-infection status and other co-morbidities, previous history of TB or MB treatment, and chemotherapeutic scheme designed for each MB episode were collected. Medical records stored at both Dr. Cetrángolo and P. V. de Cordero Hospitals were used to collect all the epidemiological and clinical information while laboratory records were the source document for microbiological data.

**Mycobacteria identification**

NTM were identified by biochemical and molecular tests using an algorithm previously reported (18). Biochemical tests included the temperature and time of growth, the cultural characteristics of the colonies, the production of pigment and several enzymatic reactions like niacin production, nitrate reduction, catalase production at room temperature and at 68 °C, telurite reduction and Tween 80 hydrolysis (6).

Molecular identification was performed by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene (PRA) (4, 9). In this work, the gold standard for mycobacteria identification was the combination of biochemical tests and PRA. When the correct identification by PRA was not possible or discordant results were obtained between PRA and phenotypic methods, the 16S-rRNA gene sequencing was performed to properly identify the mycobacterial species (6, 26).

**Drug-susceptibility testing (DST)**

The indirect proportion method on M7H11 was used to determine the drug-resistance (DR) profile of the isolates to first and second line anti-TB drugs (8, 14, 15, 22).

The microdilution colorimetric method (CMM) using resazurin as vital dye (REMA) was also used in order to determine the minimal inhibitory concentration (MIC) values for the same drugs (22, 25). *M. tuberculosis* H37Rv ATCC 27294 and *Mycobacterium avium* D4 reference strains were used as DST controls.

Table 1 shows the drug concentrations used in both methods as well as the cutoff values proposed to consider the strain susceptible or resistant when tested by CMM and after a comparison with the proportion method. This comparison and the cutoff values obtained by CMM were performed by the ROC curve method (20, 29).

A strain was considered susceptible or resistant to a particular agent when the results obtained by both methods were in full agreement and following the criteria given by the literature (14, 15, 22).

Drugs such as cefoxitin, imipenem, doxycycline and tobramycin frequently used for the treatment of rapid growers, were not properly standardized under our working conditions and therefore were not tested for *in vitro* DST.
RESULTS

From a total of 23,624 clinical specimens analyzed, 2,914 (12.3 %) yielded mycobacterial isolates. *M. tuberculosis* and NTM represented 93.9 % (n = 2,736) and 6.1 % (n = 178) of the isolates respectively. The TB cases during the study period were 2,118 while 108 (4.9 %, 108/2226) were identified by PRA and/or sequencing and 16 different NTM species were found. *M. avium* (n = 28, 32.2 %) was the main causative agent found in this study, and *M. intracellulare* I ranked second (n = 23, 26.4 %). In addition, 50.0 % (n = 14) of the cases produced by *M. avium* occurred in HIV+ patients while only 17.4 % (n = 4) of the cases due to *M. intracellulare* belonged to HIV co-infected patients. These findings are in concordance with the literature (1, 5).

Table 2 shows the frequency of different mycobacteria species and their sub-types in relationship with the physical localization of the disease found in this work. *Mycobacterium kumamotonense* and *Mycobacterium sherrisii* were found among all of them.

Infections produced by more than one species at the same time were confirmed in 4 cases. In one case of an HIV+patient, the infection was produced simultaneously by *M. tuberculosis* and *M. avium*; in 2, one HIV+ and the other a non co-infected HIV patient, the infection was produced by *M. tuberculosis* plus *M. intracellulare* I; and the last case was caused by *M. fortuitum* and *M. intracellulare* I in a patient suffering from both diabetes and systemic erythematous lupus.

In 5 (16.7 %) different HIV+ patients, a first TB episode was confirmed and opportunely treated. However, in a second episode occurring far apart in time, a MB caused by *M. avium* was also diagnosed.

After being properly treated during a year and after at least two consecutive negative cultures, 6 out of 87 (6.9 %) MB cases were reported as relapses, being three of them HIV+ individuals. The etiological agents found were: *M. avium* (n = 3), *M. kansasii* I (n = 1) and *M. intracellulare* (n = 2).

Localization of mycobacteriosis cases

Most of the cases in which the etiological agent was properly identified presented pulmonary localization (68/87, 78.2 %) (See Table 2). The analyzed specimens were sputa, broncho-alveolar and bronchial lavages. Only 26.5 % (18/68) of pulmonary cases presented AFB smear positive by Ziehl-Neelsen stain. Of the 19 (21.8 %) extra pulmonary cases, 9 corresponded to blood and bone marrow, 6 biopsies, 2 cerebrospinal fluid, 1 ascitic liquid, and 1 feces. Only 1 case (1.1 %) had both pulmonary and extra pulmonary localization of the disease. Only 1 extra pulmonary case (5.2 %) showed positive results in the AFB smear examination. Twelve out of 19 (63.0 %) of the patients with extrapulmonary disease had HIV co-infection.

Drug susceptibility testing

Figure 1 shows the percentage of resistant isolates found for each one of the drugs. DST on M7H11 and REMA results showed an important resistance level of the NTM to several first and second line anti-TB drugs. The macrolides ([azithromycin, (AZ), and clarithromycin, (CLA)], the fluoroquinolones, (FQ), [levofloxacin, (LX),

### Table 1. First and second line drug-concentrations tested in proportion and colorimetric methods

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug concentration (µg/ml)</th>
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<tr>
<td></td>
<td>PM7H11</td>
</tr>
<tr>
<td>Amikacin</td>
<td>4.0</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>10.0</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>10.0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>30.0</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>7.5</td>
</tr>
<tr>
<td>Ethionamide</td>
<td>10.0</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>0.2-1.0</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>6.0</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>2.0</td>
</tr>
<tr>
<td>Linezolide</td>
<td>1.0</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>10.0</td>
</tr>
<tr>
<td>P Amino-salicylic acid</td>
<td>8.0</td>
</tr>
<tr>
<td>Rifabutin</td>
<td>0.5</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>1.0</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>2.0</td>
</tr>
</tbody>
</table>

PAS: para amino-salicylic acid; INH: isoniazid; CS: cycloserine; TTH: ethionamide; Rif: rifampicin; SM: streptomycin; M7H11: proportion method on Middlebrok 7H11; CMM: colorimetric microplate method
Figure 1. Proportion of drug-resistant non-tuberculous mycobacteria to first and second-line drugs. 
PAS: paraamino-salicylic acid; INH: isoniazid; CS: cycloserine; ETH: ethionamide; RIF: rifampicin; SM: streptomycin; 
EMB: ethambutol; LZ: linezolide; RBT: rifabutine; AMK/KM: amikacin/kanamycin; OFX/LX: ofloxacin/levofloxacin; AZ/ 
CLA: azithromycin/ clarithromycin.
ofloxacin, (OFX) and the aminoglycosides [amikacin, (AMK), and kanamycin, (KAN)] were the most active drugs tested in vitro, exerting their inhibitory action in around 70.0 % of the isolates. On the other hand, isoniazid (INH), para amino-salicylic acid (PAS), cycloserine (CS), ethionamide (ETH) and rifampicin (RIF) showed less than 40.0 % inhibition while streptomycin (SM), linezolid (LZ), and ethambutol (EMB) showed their inhibitory activity in around 50.0 % of the isolates.

Table 3 shows the drug resistance profile for each one of the tested drugs and for the main mycobacterial species found in the study.

### Treatment evaluation

A cohort comprising data at the beginning of the therapy, the drug regimens and a final evaluation was obtained from 62 out of 87 (71.3 %) fully identified MB cases. No available data regarding MB treatment were obtained from the remaining 25 MB cases, so they were excluded from the final cohort. A total of 12 (19.4 %) patients were removed from the cohort because 10 of them still remained under treatment at the evaluation time, and the other 2 were lost from the register; therefore the final analysis was performed in 50 cases with complete therapy. All the complete treatments lasted a median of 20 (range: 18-30) months.

A cohort of 50 cases with treatment results was obtained. This evaluation showed that 34 (68 %) cases completed the prescribed therapy and they were considered cured; 10 (20 %) patients (7 HIV+) died; 6 (12 %) were classified as relapses.

Table 4 shows the proportion of drug utilization and its relationship with the treatment success.

| Drug | Cured (n = 34) | Death (n = 10) | Relapse (n = 6) | Total (%)
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<tbody>
<tr>
<td>OFX</td>
<td>20</td>
<td>3</td>
<td>-</td>
<td>23 (46.0)</td>
</tr>
<tr>
<td>LX</td>
<td>26</td>
<td>1</td>
<td>-</td>
<td>27 (54.0)</td>
</tr>
<tr>
<td>AMK</td>
<td>23</td>
<td>4</td>
<td>1</td>
<td>27 (54.0)</td>
</tr>
<tr>
<td>KM</td>
<td>10</td>
<td>1</td>
<td>-</td>
<td>11 (22.0)</td>
</tr>
<tr>
<td>CLA</td>
<td>19</td>
<td>1</td>
<td>-</td>
<td>20 (40.0)</td>
</tr>
<tr>
<td>AZ</td>
<td>26</td>
<td>4</td>
<td>4</td>
<td>30 (60.0)</td>
</tr>
<tr>
<td>EMB</td>
<td>28</td>
<td>6</td>
<td>2</td>
<td>34 (68.0)</td>
</tr>
<tr>
<td>CS</td>
<td>10</td>
<td>3</td>
<td>-</td>
<td>13 (26.0)</td>
</tr>
<tr>
<td>RIF</td>
<td>12</td>
<td>3</td>
<td>-</td>
<td>15 (30.0)</td>
</tr>
</tbody>
</table>

The remaining eight and seven cases caused by M. avium and M. intracellulare respectively were still under treatment during the study period. One patient with a mixed infection by M. tuberculosis and M. intracellulare, 2 with M. gordonae, and one with M. simiae and another one with M. lentiflavum also died.
DISCUSSION

On several occasions, physicians have to assume a case as TB with the sole evidence of the positive AFB smear results until therapy failure and/or when the worsening of the patient’s clinical condition pose a doubt about diagnostic accuracy. The evidence of a TB diagnosis based on M. tuberculosis identification from clinical samples allows the setting up of a proper treatment and furthermore expect it to be successful. Nevertheless, when a NTM is the causative agent of disease, the currently anti-TB therapy is often worthless with the subsequent patient’s health impairment and life-threatening risk. Thus, the detection of NTM as etiological agent is very important still in settings with high incidence rate of TB.

Disease caused by NTM is quite common in the HIV co-infected population, mainly in developed countries. In addition, immunosuppressive therapies are more frequently used not only for cancer therapy but for autoimmune and inflammatory diseases which very often present lung manifestations and can be associated to NTM infection. Therefore, a wider epidemiology of NTM as agents producing MB is increasing in interest and importance (30). In this study period, NTM were reported in 4.9 % of the cases caused by mycobacteria (TB plus NTM) affected patients with and without HIV co-infection; more than a quarter of the cases (26.8 %) were associated to HIV co-infection. Although the North of Buenos Aires is not a region with tropical characteristics, a broad spectrum of species (16) was found. Nevertheless, the main etiological agent was M. avium, followed by M. intracellulare, M. kansasii and M. gordonae. Recently described species such as M. kumamotonense, M. sherrissii and Mycobacterium nonchromogenicum I were also found among these patients. M. sherrissii had previously been reported as etiological agent of MB cases in Argentina (3).

Interestingly, mixed infections caused by two different mycobacteria species at the same time were confirmed in 4 (4.6 %) cases.

NTM DST results showed a high resistance level to the main anti-TB drugs, especially INH and Rif. All the isolates were also completely resistant to PAS and almost to CS and ETH. The susceptibility to the studied FQ, macrolides, aminoglycosides (different to SM), and RBT was more than 50.0 %. Nevertheless, between 40.0 to 49.0 % of the isolates showed EMB and LZ MIC values compatible with those of drug-susceptible strains.

The cohort for the evaluation of treatment success comprised almost 50.0 % (patients n = 108) of the original number initially included in this study. The facts that caused this decreased cohort to be effectively evaluated were multiple and mostly related to the underestimation of the proper disease at the diagnostic time. On many occasions, the patients were diagnosed after having received an inadequate standardized treatment for TB for several months. The delay in proper detection of the causative agent as well as no proper treatments in most of those cases, led to the losses in the final cohort. In contrast, HIV+ cases were followed up more closely and deaths occurred in these cases under proper treatment.

The first worldwide publication of M. kumamotonense, from clinical isolates was in 2006, in Kumamoto Prefecture, Japan, by Masaki T et al. (19). To our knowledge, it is the first time that M. kumamotonense is reported in Argentina as an etiological agent.

M. tuberculosis and M. avium simultaneous infection was reported in one HIV+ patient who died after starting appropriate therapy. The M. tuberculosis isolate was recovered from a solid medium that allowed the discrimination between two different colony morphology which, in the end, belonged to two different species. A mixture culture of both species was obtained in the MGIT 960 liquid medium. This mixed infection findings had previously been reported (27, 28) but it underscores the importance of using more than one culture system when investigating clinical specimens from severe immunocompromised patients. In other 2 cases (1 HIV+ and 1 non co-infected with HIV) the infection was produced by M. tuberculosis plus M. intracellulare I. The latter case was caused by M. fortuitum and M. intracellulare I in a patient suffering from both diabetes and systemic erythematous lupus.

All the cases in which the treatment outcome was evaluated received an individual tailor-made therapeutic scheme including those drugs showing in vitro activity and presumed in vivo usefulness. Nevertheless, the majority of the deaths caused by NTM were associated to HIV co-infection (83.0 %).

The disease caused by NTM is not currently considered a public health problem in countries where its prevalence is unknown or presumably low. In spite of that, the NTM are causing serious clinical problems and they must be taken into consideration at the time of making a proper diagnosis to provide the best treatment, offering the cure opportunity. In conclusion, a timely detection of the mycobacteria species as the etiological agent is of paramount clinical importance because delays in proper treatment might cause impaired health in people usually affected by other severe illnesses such as HIV co-infection.

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