Pulmonary Nontuberculous Mycobacterial Infections in the State of Para, an Endemic Region for Tuberculosis in North of Brazil

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1. Introduction

Traditionally, *Mycobacterium* species are divided in those belonging to the *Mycobacterium tuberculosis* complex (MTBC), *M. leprae* and the nontuberculous or atypical mycobacteria (NTM), the latter consisting of either rapid (RGM) or slow growing (SGM) species, forming colonies, respectively, within seven days of culture or requiring longer incubation time (Runyon, 1959). Among the RGM are the *M. chelonae* complex, including *M. chelonae*, *M. abscessus*, *M. mucogenicum*, *M. salmoniphilum*, *M. bolletii* and *M. massiliense* (Brown-Elliot & Wallace, 2002; Whipps et al., 2007) and the *M. fortuitum* complex, including *M. fortuitum*, *M. peregrinum*, *M. septicum*, *M. mageritense*, *M. houstonense* and *M. boenikei* (Adékambi & Drancourt, 2004), containing the NTM commonly encountered in human specimens. Clinically important SGM are the *M. avium* complex (MAC), which include *M. avium*, *M. intracellulare*, *M. colombiense* and *M. chimaera* (Tortoli, 2003; Tortoli et al., 2004).

Until now, 130 *Mycobacterium* species, with a considerable variability in pathogenicity have been described, being isolated from natural water reservoirs and drinking water distribution systems in buildings, hospitals, household plumbing, hot tubs, spas, building aerosols, boreal forest soils and peats, acidic, brown-waters swamps, potting soils and metal removal fluid systems (Tortoli, 2009; Euzéby, 2011, Falkinham, 2009). The lack of evidence for person-to-person transmission suggests that the environment is the most likely source of NTM infection (Marra & Daley, 2002).

Unlike the bacterial species that belong to the MTBC, NTM are commonly present in the environment and when isolated from human specimens, may either be (i) contaminants during preparation of sputum cultures in the laboratory, (ii) colonizing organisms of the airways without causing disease or (iii) infectious organisms and causing disease and it is
not always easy to distinguish between these situations (Griffith et al., 2007). In the case of real infection with NTM, clinical syndromes are either lymphadenitis, pulmonary or cutaneous infection or disseminated disease, chronic pulmonary infection being the most common (Katoch, 2004; Piersimoni & Scarparo, 2009; Tortoli, 2009). Such NMT infections are frequently observed in immune-compromised patients in developed nations but also in immune-competent individuals with pre-existing structural pulmonary diseases (Griffith et al., 2007; Jeong et al., 2004; Jarzembowski & Young, 2008; Bodle et al., 2008; Glassroth, 2008; Sexton & Harrison, 2008; Griffith, 2010).

The diagnosis of NTM pulmonary disease is often difficult due to the overwhelming presence of environmental organisms, to the indolent nature of disease and the diversity of, mostly, nonspecific clinical symptoms. Therefore, guidelines and criteria for diagnosis of NTM pulmonary disease have been published (American Thoracic Society, 1997; British Thoracic Society, 2000) followed by the publication of a recent update of more lenient criteria (Griffith et al., 2007). Even so, these recommendations do not seem to be satisfactory as most patients with pulmonary disease due to NTM do not match these criteria (Marras et al., 2007; van Ingen et al., 2009). Also, in endemic countries for tuberculosis (TB), the pulmonary disease form is caused also by infection with organisms of the MTBC, presenting similar clinical symptoms. In addition, diagnostic procedures for pulmonary TB are sputum smear microscopy for acid-fast bacilli (AFB) and X-ray, not differentiating between Mycobacterium to the species level. Nonetheless, several case reports and studies on the prevalence of pulmonary disease caused by NTM in North America, Europe and Japan have been published during the last years (Good, 1980; Tsukamura et al., 1988; von Reyn et al., 1993; Falkinham, 2002; Kobashi & Matsushima, 2007; Iseman & Marras, 2008; Billinger et al., 2009; Thomson, 2010; Kendall et al., 2011).

The impact, magnitude and regional dimension of NTM infections in countries where TB is endemic is hardly known (Gopinath & Singh, 2010), such as the case in Brazil, where most cases of infectious NTM have been reported in the southeastern region and, more specifically, in São Paulo (Barreto & Campos, 2000; Ueki et al., 2005; Zamarioli et al., 2008; Pedro et al., 2008). In the Amazon region, North of Brazil, little epidemiological information on this matter is available (da Costa et al., 2009).

We therefore studied the frequency and diversity of NTM isolates, obtained from pulmonary specimens from residents of the Pará State, during a twelve year period.

2. Material and methods

2.1 Study setting and patients

All Mycobacterium isolates evaluated were obtained from sputum samples (n = 119) and bronchial washings (n = 9) from individuals with clinical symptoms of pulmonary TB and residents of the State of Pará, North Brazil (Fig. 1). The study included samples of patients from whom NTM was isolated from at least once, and this between January 1999 and December 2010, at the Evandro Chagas Institute, a reference center for the diagnosis of infections with Mycobacterium. Patient records were reviewed to assess the frequency of isolation and clinical relevance of the presence of NTM and the diagnosis for NTM lung infection was based on the diagnostic criteria published by the American Thoracic Society (Griffith et al., 2007) (Table 1).
Clinical and radiographic analysis
- Pulmonary symptoms that include nodular or cavitary opacities on chest radiograph; multifocal bronchiectasis with multiple small nodules on a high resolution computed tomography (HRCT) scan; lack of abnormalities suggestive for other disease.

Microbiologic analysis
- Positive culture from at least two separate expectorated sputum samples, when initial sputum samples are AFB negative, consider repeated sputum AFB smears and cultures or positive culture results from at least one bronchial wash or lavage

Histopathologic analysis
- Transbronchial or other lung biopsy with mycobacterial histopathologic features (granulomatous inflammation or AFB) and positive culture for NTM.

Table 1. American Thoracic Society diagnostic criteria on NTM pulmonary disease (Griffith et al., 2007).

2.2 Mycobacterium cultures and isolates

Pulmonary specimens were decontaminated using the N-acetyl-L-cysteine-sodium hydroxide procedure (Webb, 1962; Brasil, 2008), inoculated into Lowenstein-Jensen (LJ) medium (Difco, France) and incubated at 35 to 37°C in the absence of light for at least six weeks or until colonies appeared. Conventional procedures for distinguishing between organisms of the MTBC and of the NTM group included macroscopic analysis of aspect of colonies, which MTBC have a rough aspect resemble breadcrumbs or cauliflowers, detection of cord factor from MTBC by Ziehl-Neelsen stain, and the growth inhibition test in medium containing 0.5 mg/mL para-nitrobenzoic acid, a specific inhibitor of MTBC, all according to Kubica (1973).
2.3 Sequence analysis and phylogenetic analysis

Sequencing of part of the 16S ribosomal RNA (16S rRNA) and 65-kilodalton heat shock protein (hsp65) genes was performed as described by previous publications (Kim et al., 2005; Shin et al., 2006). After verification of PCR products on agarose gel Seakem LE 1% (Cambrex, United Kingdom), these were purified using the SNAP TM gel purification kit (Invitrogen). The amplified products were direct sequenced by using both forward and reverse primers of each system and the BigDye Terminator v3.1 cycle sequencing kits (Applied Biosystems, Foster City, CA) and analyzed on an ABI3130 sequencer (Applied Biosystems, Tokyo, Japan).

The 16S rRNA and hsp65 sequences were aligned using the multiple-alignment algorithm of the Bioedit software (version 7.0.9; Tom Hall [http://www.mbio.ncsu.edu/BioEdit/bioedit.html]) with the closest relatives retrieved from the GenBank database across of the Basic Local Alignment Search Tool (BLAST, URL: http://www.ncbi.nlm.nih.gov/BLAST/) and the Ribosomal Differentiation of Medical Microorganisms RIDOM database (RIDOM, URL: http://rdna.ridom.de/). Phylogenetic trees were constructed from the presently-defined 16S rRNA or hsp65 sequences separately using the neighbor-joining algorithm, including sequences of a selection of NTM-type strains, retrieved from GenBank (accession numbers in parenthesis next to the species names in Figs.3 and 4). For this, we used the Kimura’s 2-parameter distance correction model and MEGA software (Version 4.0; Tamura et al. [http://www.megasoftware.net/]). Bootstrap analysis (1,000 repeats) was applied using the Tsukamurella paurometabola (KCTC 9821) sequences as an out-group. The GenBank accession numbers for the Mycobacterium sequences determined in this study included the following: FJ590454-FJ590472, HM056080-HM056113 for the 16S rRNA, and FJ536235-FJ536253, HM056114-HM056147 for the hsp65 gene.

2.4 Statistical analysis

Statistical data were derived by using the nonparametric chi-square test and the Fisher exact test, where appropriate. P values less than 0.05 was considered significant. Statistical analysis was performed with the BioEstat software (version 5.0; Ayres et al. [http: www.mamiraua.org.br]).

3. Results

3.1 Patients and NTM isolates

Between 1999 and 2010, Mycobacterium isolates were recovered from respiratory specimens of 1,580 patients, that were suspected of having pulmonary TB. Among these, 92% (1,453 cases) were infected with MTBC; from the rest (8%, 128 patients) we obtained 249 NTM isolates. Among the NTM-positive patients studied, 57.5% (n=73) presented at least two positive sputum cultures for the same species, or presented at least one bronchial wash positive culture and suffered therefore from infections as defined by the criteria of ATS (Griffith et al., 2007). The clinical significance of NTM pulmonary isolation among 1999-2010 is shown in Fig.2.

The remaining 55 patients could not be confirmed to suffer from NMT infection because (i) only a single sputum sample was collected and delivered to the laboratory (47 patients); (ii)
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Fig. 2. Frequency of NTM isolation from clinical pulmonary specimens of patients from Pará State, Brazil, 1999-2010.

one sample was culture-positive and the others were culture-negative (five cases) or (iii) NTM were found in some patients who were also TB-positive (three patients). Distribution of species according clinical relevance and years of isolation is show in Tab.2 and Fig.3.

<table>
<thead>
<tr>
<th>Species</th>
<th>Clinical relevant Patients</th>
<th>Clinical relevant Isolates</th>
<th>Not relevant Patients</th>
<th>Not relevant Isolates</th>
<th>Total Patients</th>
<th>Total Isolates</th>
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| Total                | 73                        | 194                       | 55                    | 55                    | 128            | 249            |

Table 2. Clinical significance of NTM isolated in Pará State, Brazil, 1999-2010.
Among the 73 patients with bacteriological ATS criteria for NMT infection, 64.4% (n=47; \( p = 0.03 \)) were females and more detailed analysis of their treatment history revealed that 72 had previously been unsuccessfully treated for TB, using the first-line multidrug therapy scheme; one patient had been diagnosed as suffering from allergic bronchitis and therefore submitted to corticosteroid therapy. After confirmation of NTM infection, 70 patients were submitted to a daily regimen of clarithromycin (500-1,000 mg) and ethambutol (25 mg/kg) for 12 months. No therapy information was available for the patient infected with \textit{M. fortuitum} and for the two cases with \textit{M. kansasii}. Treatment outcome was not available for all cases but patients infected with members of the \textit{M. simiae} complex did not present clinical improvement and at the end of our study period, one had died due to progression of disease.

All of the patients described above presented respiratory complaints consistent with TB while additional symptoms were observed (Table 3). Bronchiectasis sequelae occasionally associated with hemoptysis in patients infected with \textit{M. abscessus} (n=1), \textit{M. bolletii} (n=2), \textit{M.}
<table>
<thead>
<tr>
<th>Species</th>
<th>Gender</th>
<th>Clinical characteristics</th>
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</thead>
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<tr>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>chronic cough (14); sputum (14); chest pain (14) weight loss (14); fever (3); hemoptysis (10); malaise (14); dyspnea (3); down syndrome (1); fatigue (2)</td>
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</tr>
<tr>
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<td>chronic cough (11); sputum (11); chest pain (3)</td>
<td>11</td>
</tr>
<tr>
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<tr>
<td></td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. bolletii</td>
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<td>chronic cough (4); hemoptysis (2); decreased lung volume (1); fever (1); loss weight (1)</td>
<td>4</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<tr>
<td>M. colombiense</td>
<td>2</td>
<td>chronic cough (3); chest pain (3)</td>
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<tr>
<td>M. kansasi</td>
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<td>chronic cough (2); chest pain (2)</td>
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<tr>
<td>M. fortuitum</td>
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</tr>
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<td>M. scrofulaceum</td>
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<td>M. szulguai</td>
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</tr>
<tr>
<td>M. terrae</td>
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<td>chronic cough (1); chest pain (1)</td>
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</table>

Table 3. Clinical characteristics of patients with NTM pulmonary infection from Pará State, Brazil, 1999-2010.

massiliense (n=5) and M. simiae complex (n=10) while chronic cough was observed among patients, independent of the infecting Mycobacterium species. The interval between the onset of signs and symptoms and a definitive diagnosis of NTM infection was greater than 12 months, being more pronounced in cases with M. simiae complex isolates, reporting the presence of symptoms for at least 24 months.

3.2 NTM Identification on the genetic level

Based on 16s rRNA gene analysis, the majority of the NTM species isolated from patients could be grouped into three clades, containing sequences from either M. avium, M. chelonae or M. simiae complexes (Fig. 4).
Fig. 4. Relationships between sequences from the type strains and the NTM isolated presently, inferred from partial 16S rRNA gene. Phylogenetic tree was constructed by neighbor-joining method and Kimura's 2-parameter distance correction model. The support of each branch, as determined from 1000 bootstrap samples, is indicated by the value at each node (as a percentage). *T. paurometabola* KCTC 9821 was used as outgroup.

Upon sequence analysis of part of the *hsp65* gene, we observed a higher genetic diversity than that of the 16S rRNA gene; nonetheless, the phylogenetic tree based on *hsp65* gene sequence analysis had the same global topology as that based on 16S rRNA gene (Fig. 5).
Fig. 5. Relationships between sequences from the type strains and the NMT isolated presently, as inferred from partial hsp65 sequences. Phylogenetic tree was constructed as described above.
Among the 249 infectious isolates, 200 NMT sequences, derived from 108 patients, had already been described elsewhere and characteristic for 14 NMT species, the other 49 sequences derived from 19 cases were unpublished in public databases and all phylogenetically classified into the \textit{M. simiae} complex.

4. Discussion

This study demonstrates that among 1,453 cases that were diagnosed between 1999 and 2010 as suffering from pulmonary TB, presence of NTM was observed in 128 (8\%) of these and infection with such species proven to cause disease in 73 cases (5\%). It was observed a steady increase in the number of NTM isolates during the study period, which was more pronounced from 2004 on, when an increase in the demand of culture for AFB at the Evandro Chagas Institute was the case. This latter could be due either to the increase of the physicians’ sensitivity to occurrence of NTM infections in this region and/or to an increase of infection with NMT of the population.

In this study, significantly more females were infected with NTM, and this is contrary to most published data, presenting males as the major risk group for pulmonary NMT disease (Marras & Daley, 2002). However, some recent reports also demonstrated a female predominance (Freeman et al., 2007; Cassid et al., 2009; Prevot et al., 2010; Wintrop et al., 2010), in concordance with the recent data of Chan & Iseman (2010), describe a higher immune susceptibility of women to NTM pulmonary disease. In addition, when stratifying to the NMT species level, it was observed that gender associated infection was even more pronounced in the case of \textit{M. massiliense} (70\% females), \textit{M. simiae} complex (71\%) and \textit{M. avium} (66\%). Griffith et al. (2003) found a predominance of females (65\%) among 154 cases of pulmonary disease by RGM, while descriptions of particular forms of pulmonary disease caused by MAC in women have been reported (Wallace, 1994; Reich & Johnson, 1995). Further studies are needed to elucidate the reasons for female susceptibility. Roughly 40\% (n=55) of the patients with NTM-positive cultures did not meet the diagnostic criteria for NTM pulmonary infection but this does not necessarily mean that the presence of NMT is not the cause of disease. Unfortunately, due to lack of follow-up of patients, it cannot confirm this presently. There is little known about the pathophysiology of NTM-related lung disease what makes it difficult to be certain that colonization is not an indolent or even a slowly-progressive infection. Therefore, such cases need to remain under observation and seek expert consultation (Griffith et al., 2007).

Among the cases with confirmation of NMT infection as a cause of disease, mostly, previous diagnosis and treatment of TB was observed, none demonstrating improvement following treatment. Misdiagnosis of NTM infections as caused by members of the MTBC leads to unsuccessful treatment with anti-TB drugs and because clinicians experiment with various TB therapies without considering a culture-based test, there is a considerable delay in detection of NTM. This is even more a matter of concern in high prevalence countries of TB such as Brazil, where mostly, symptomatic patients with sputum smear positive for acid-fast bacilli are treated with anti-TB drugs without being testing for NTM-related disease, except when co-infected with HIV (Brasil, 2005).

In Brazil, it was common until 2009, to start second line treatment without performing culture test for NTM, when no improvement during the first round of TB treatment was
observed. The high level of pulmonary NTM that was misdiagnosed as TB strongly suggests the need for a different strategy of TB control in the state of Para.

In most countries, NTM-related disease, unlike TB, do not need to be reported unless they are healthcare-associated infections. Therefore, information on the frequency and diversity of NTM infections are usually obtained from laboratory records and surveillance studies (Marras et al., 2007; Parrish et al., 2008). To determine the true epidemiological status of NTM pulmonary disease, well designed population-based studies are needed. However, the financial burden on public health care system in developing countries makes it difficult to perform such surveillance studies. Therefore, laboratory procedures such as the introduction of both liquid and solid culture systems and use of molecular methods such as PCR restriction analysis (PRA) in reference laboratories could be an alternative for more knowledge and improvement of diagnose accuracy in those regions.

Sequence analysis has contributed to the recent description of several new Mycobacterium species and more precise identification and taxonomy of members of this genus. Genotypic taxonomy is typically based on the detection of highly conserved regions within the genome that harbor hypervariable sequences in which species-specific deletions, insertions, or replacements of single nucleotides are present in 16S rRNA, hsp65 gene and more recently on a fragment of the gene coding for the beta sub-unit of RNA polymerase (rpoB) are also contributing to this field, mostly for RGM (da Costa et al., 2009; da Costa et al, 2010). Several amplification molecular methods, have been proposed to correct NTM identification, including specific DNA probes (AccuProbe: GenProbe, Inc., San Diego, CA, U.S.A) and PRA method based on 16S rRNA (Domenech et al., 1994), 16S-23S rRNA internal transcribed spacer (ITS) (Roth et al., 1998), hsp65 (Telenti et al., 1993), rpoB (Lee et al., 2000), cold-shock protein gene (dnaJ) (Takewaki et al., 1994), DNA repair protein gene (recA) (Blackwood et al., 2000) and elongation factor Tu gene (tuf) (Shin et al., 2009), but all have limitations as the variety of mycobacteria to be identified (da Costa et al., 2010a, b).

Based on the 16S rRNA and hsp65 nucleotide sequences, we observed that the most frequent NTM isolates from our pulmonary samples were those of the M. avium, M. chelonae and M. simiae complexes. The most common NTM were M. massiliense, M. intracellulare, followed by Mycobacterium sp. from M. simiae complex. When compared with reports on NMT infections observed in other studies reported on Brazilian NTM cases, the species diversity and frequency is quite particular to the Para State, suggesting that environmental characteristics as temperature, pH and substrate composition may influence the geographical distribution of species. Our findings are in concordance with the fact that isolates of the M. simiae complex are rarely observed in other regions of Brazil.

There are few publications describing NTM in the Amazon region or Brazil. Barreto and Campos (2000) found 35 patients with NTM and showed that isolates of the M. avium complex, M. terrae and M. fortuitum were most common in samples collected between 1994 and 1999 in North of Brazil. A study that evaluated respiratory samples of non-indigenous and indigenous patients from Amazonas State with suspected pulmonary TB identified 19 patients with NTM infection, but the study did not report the identity of the isolates at the species level (Santos et al., 2006). A recent study by da Costa et al. (2009), showed that the M. chelonae complex, which includes the M. massiliense species, is the most frequent cause of pulmonary infections by RGM in Pará State, Amazon region of Brazil, similar to our
observations. Unlikely, in Brazilian southeast, *M. kansasii* and *M. avium* represented the most frequent type of NTM associated with pulmonary infections between 1991 and 1997 in the state of São Paulo (Ueki et al., 2005; Zamarioli et al. 2008; Pedro et al., 2008).

In contrast to other parts of the world, the species variability found in the present study is different. In countries from Latin America like Colombia, MAC, *M. chelonae* and *M. fortuitum* were the NTM isolated with more frequency (León, 1998), while MAC was most frequently isolated from argentinian HIV patients (Di Lonardo, 1995). MAC and *M. kansasii* were predominant in North America, some countries of Europe and South Africa (Griffith et al., 2007). In Asia, MAC, *M. abscessus* and *M. chelonae* were frequently isolated from pulmonary samples (Simons et al., 2011). The knowledge on diversity and epidemiology of species NTM associated to pulmonary in specific region is important because either: (i) it allows the adequate choice of laboratory methods for diagnosis (ii) it allows to recognize the species associated to disease; and (iii) it supplies information that will serve to improve the organization of health service net to attend these patients.

Perhaps the most important finding of this study was the identification of *M. simiae* complex members as the predominant cause of pulmonary infections. In fact, roughly 20% (*n=16*) of the pulmonary infections were caused by members of the *M. simiae* complex and among these, 14 belonged to an unidentified taxon (*n=14*). Currently, this taxonomic group is made up of 17 species including *M. simiae*, *M. genavense*, *M. intermedium*, *M. interjectum*, *M. lentiflavum*, *M. triplex*, *M. heidelbergense*, *M. kubicae*, *M. palustre*, *M. montefioreense*, *M. florentinum*, *M. sherrisi*, *M. parmense*, *M. parascrofulaceum*, *M. saskatchewanense*, *M. stomatepiae* and *M. europaeum* (Tortoli, 2003, 2006; Tortoli et al., 2010). However, among these species, only *M. simiae* is recognized as a real cause of pulmonary infections, as reported in areas such as the Southwest of the United States, Israel and Cuba (Griffith et al., 2007). It is estimated that 9 to 21% of the *M. simiae* isolates from pulmonary specimens have clinical relevance (Rynkiewicz et al. 1998). The findings of this study suggest that members of this group may have pathogenic potential, but further studies are required to assess the characteristics of these isolates, including details on predisposing conditions from patients, as well as the drug susceptibility these NTM.

### 5. Conclusion

In conclusion, although our study is not necessarily representative for the whole Amazon region, it clearly demonstrates the importance of NTM pulmonary infections in this region. Our data also show that a variety of NTM species are involved, and that there is need for bacteriologic diagnosis in patients with TB, especially in patients who have failed TB treatment. We have shown that the lack of species identification in a significant subset (8.0%) of patients with a presumptive diagnosis of TB in a regional reference center can lead to misdiagnosis and may be followed by inadequate treatment.

### 6. Acknowledgment

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7. References


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Pulmonary infections are notorious in causing considerable morbidity and mortality. Caused by bacteria, viruses or fungi, respiratory infections require distinct knowledge of recent advances in pathogenesis. Progress in the understanding of immunopathogenesis of Acinetobacter baumannii infection will explain how an atypical organism establishes infection. The chapter regarding pulmonary nontuberculous mycobacterial infections in the State of Para depicts a unique study in an endemic region for tuberculosis in North of Brazil. The diagnosis and treatment of latent tuberculosis is a formidable challenge. Thus, new developments in diagnosis and treatment of latent tuberculosis are included in this book. Challenging in their diagnosis, nontuberculous mycobacterial pulmonary diseases require special education for management. The problems of respiratory infections in the immunocompromised host are increasing in numbers and in resilience to treatment. Therefore, the chapter describing the host immune responses against pulmonary fungal pathogens comes as a necessary section in this book. The insight brought forth from this book can be valuable for both clinicians and scientists.

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