



Identification and Susceptibility Testing on Samples of *Mycobacterium* spp

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Authors' contributions

This work was carried out in collaboration between all authors. Author RAOC designed the study, performed statistical analysis, and wrote the protocol and the manuscript draft. Author MLS performed bacteria species identification and drug susceptibility testing. Author MAMBR performed laboratorial analysis. Author MPO reviewed the manuscript. Author ALM designed the study, wrote the protocol, and reviewed the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: Tuberculosis is still a major global health problem. Human tuberculosis is caused by species of bacteria belonging to the *Mycobacterium* genus. In this study we determined mycobacterial species affecting patients from Botucatu, Brazil, and tested *M. tuberculosis* sensitivity to different drugs.

Methods: Data were obtained from Clinical Laboratory Analysis records at Botucatu Medical School University Hospital, UNESP. All samples were processed according to standard isolation procedures from the 2008 Brazil Ministry of Health *Mycobacteria* Manual, which consist of staining smears by the Ziehl-Neelsen technique and seeding cultures in the Löwenstein-Jensen medium.

Results: Samples were isolated from sputum (80.5%), bronchoalveolar lavage (13.8%), pleural fluid (4.6%), and cerebrospinal liquor (1.1%). Smears were evaluated in 87 cases and a total of 59

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patients showed positive smears; 55 from 70 sputum samples and 4 from 12 bronchoalveolar lavage samples. No pleural fluid (4) or cerebrospinal liquor (1) samples showed positive smears. The most commonly identified strain was *M. tuberculosis* (61 cases); followed by *M. avium* and *M. gordonae* 2 cases each, and *M. peregrinum* and *M. abscessus* 1 case each. *Mycobacteria* were not identified in 20 patients. Only two strains of *M. tuberculosis* were multidrug resistant; one was resistant to isoniazid, rifampicin, and pyrazinamide. These two patients evolved to cure.

Conclusion: This study highlights a small but troubling percentage of multidrug resistant samples and reveals the occurrence of nontuberculous mycobacteria, emphasizing the importance of correctly identifying species and testing sensitivity to antibacterial drugs to assure an adequate therapy.

Keywords: *Mycobacteria tuberculosis*; multidrug resistant; nontuberculous mycobacteria.

1. INTRODUCTION

Despite being one of the oldest infectious diseases, tuberculosis is still a major global health problem. Contributory factors include: social inequality, insufficient research aimed at developing new treatments and vaccines, population migration, disabled health systems, the high prevalence of multi-drug resistant cases, and an association with human immunodeficiency virus (HIV) infection [1].

Currently, tuberculosis affects approximately one-third of humanity; in 2012, there were an estimated 8.6 million new cases. Most cases (95%) occurred in middle to low-income countries. Each year, at least 1.3 million people die from tuberculosis; 12% of these cases are associated with the Aids epidemic [2,3].

Brazil has the largest number of cases in Latin America and is one of 22 nations responsible for 80% of all tuberculosis cases in the world [4]. It is estimated that one in four people are infected with the Koch's bacillus, and approximately 90,000 new cases are notified to the Health Ministry every year [5-7].

Human tuberculosis is caused by species of bacteria belonging to the *Mycobacterium* genus: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. pinnipedii*, and *M. canetti*, which together form the *M. tuberculosis* complex. *Mycobacterium* spp. can be transmitted through respiratory aerosols and initially locates in the lungs; it can then spread hematogenously to other body organs and cause extrapulmonary tuberculosis [8-10]. The combination of HIV infection and multidrug resistant *M. tuberculosis* (MDR-TB), which are, at a minimum resistant to isoniazid and rifampicin, represent a potential public health threat in many countries, particularly in those where AIDS is at epidemic levels and tuberculosis incidence is high. It is

therefore necessary to know the sensitivity pattern of *M. tuberculosis* lineages so that adequate treatment can be provided [11].

The aim of this study was to determine mycobacterial species affecting patients from the micro-region of Botucatu, SP, Brazil, and to evaluate the frequency of atypical *Mycobacteria* infection and their sensitivity to different drugs.

2. MATERIALS AND METHODS

Laboratory data were obtained from Clinical Laboratory Analysis records at Botucatu Medical School University Hospital, UNESP, between January 2008 and December 2010. All procedures were approved by the Research Ethics Committee of Botucatu Medical School. The following parameters were evaluated: number of patients with clinically suspected tuberculosis; type of biological sample; patient age, gender, and geographical origin; HIV serology; mortality; and result of species identification and sensitivity to drug treatments. One or more samples from a total of 87 patients were studied. All samples were processed according to standard isolation procedures from the 2008 Ministry of Health *Mycobacteria* Manual, which consist of staining smears by the Ziehl-Neelsen technique and seeding cultures in the Löwenstein-Jensen medium [12].

2.1 Samples Procedures

Samples were placed in 15 mL tapered tubes with an equal volume of NaOH 4%, agitated, and rested for 15 min. After adding the same volume of sterile distilled water, samples were centrifuged at 3,000 g for 15 min. The supernatant was discarded and Red phenol 0.4% was added to the sediment, followed by 1 N HCl for neutralization. Then, 0.2 ml of sediment was seeded on Löwenstein-Jensen tubes, which were incubated up to 60 days at 37°C.

Positive cultures were sent to the Adolfo Lutz Institute (IAL) in Sorocaba, Brazil, for species identification and drug susceptibility testing, using an automated Bactec MGIT 960 and modified Middlebrook 7H9 broth. When *Mycobacteria* identification was not possible using this technique, strains were sent to IAL in Sao Paulo, Brazil, for identification by polymerase chain reaction (PCR) using specific primers for *M. tuberculosis*. MNT identification was performed by PCR-restriction enzyme analysis (PRA)-hsp65 as previously described [13].

3. RESULTS AND DISCUSSION

Samples were isolated from sputum (70, 80.5%), bronchoalveolar lavage (12, 13.8%), pleural fluid (4, 4.6%), and cerebrospinal liquor (1, 1.1%). Fifty nine (67.0%) individuals were male. Ages ranged from 18 to 88 years; however, 41.7% of cases were between 35 and 45 years. Smears were evaluated in 87 cases and a total of 59 (67.8%) patients showed positive smears; 55 (75%) from 70 sputum samples and 4 (33.3%) from 12 bronchoalveolar lavage samples; no pleural fluid (4) or cerebrospinal liquor (1) samples showed positive smears. The most commonly identified strain was *M. tuberculosis* in 61 (91.0%) cases; followed by *M. avium* and *M. gordonae*2 (3.0%) cases each, and *M. abscessus* and *M. peregrinum*1 (1.5%) case each. *Mycobacteria* spp. were not identified in 20 patients (Table 1). From 87 patients, 60 had been treated at Botucatu Medical School University Hospital; 1 patient was not tested for HIV serology. In these cases there was a 16.7% mortality rate (10 cases). Six patients had positive serology for HIV; three of these died, two with *Mycobacterium* spp. and one with the *M. tuberculosis* strain. Only two strains of *M. tuberculosis* were multidrug resistant; one was resistant to isoniazid, rifampicin, and pyrazinamide. These two patients evolved to cure.

This study showed that patients investigated between 2008 and 2010 in the Botucatu region presented a high frequency of *Mycobacterium tuberculosis* strains corresponding to 92.4% of all identified samples; only 7.6% were nontuberculous *Mycobacteria* (NTM). These data are similar to those described in literature. In a study in Mozambique in 2008, Nunes et al. [14] reported that from 277 *Mycobacterium* spp. cultures, only three (1.1%) were NTM; of these, two were *Mycobacterium avium* and one *Mycobacterium simiae*. In a 2006 study at the

Clementino Fraga Filho University Hospital, Rio de Janeiro, Brazil, Senna et al. reported 15 cases of isolated NTM culture strains [15]. In a study by IAL, Sao Jose do Rio Preto, Brazil, between 1996 to 2005, there was an increased NTM rate corresponding to 34.3%. The increase in NTM frequency may in part have been due to the use of an automated liquid culture method which is more sensitive than solid media [16].

It should be pointed out that we use the traditional solid medium based methodology for *Mycobacteria* isolation. Additionally, concordance between positive smears and cultures was 67.8%, showing that culture is still the gold standard for laboratory diagnosis of *Mycobacterium* spp. However, recent studies have shown that PCR method is a valuable, cost-effective and alternative tool for quick diagnosis of active tuberculosis in different clinical specimens [17]. Our data were essential for corroborating a new protocol on biological sample processing. Due to the low percentage of positive pleural fluid and bronchoalveolar lavage smears (25%), cultures have been performed to eliminate false negative results, even when not requested by a patient's physician.

The isolated NTM species were *M. avium*, *M. gordonae*, *M. abscessus*, and *M. peregrinum*. Pedro et al. [16] observed a high incidence of *M. avium* complex, almost 50% of all isolated NTM samples; *M. gordonae* corresponded to 10% of all NTM. According to Fontana et al. [18], these NTMs are present in nature, and can be isolated from river water, the ground, house dust, and vegetation. Although NTMs are usually saprophytic, there are reports of them causing disease. Several authors have described the importance of identifying *Mycobacteria* to establish adequate therapy, as they have different drug resistance patterns and treatment periods may be long, ranging from 18 to 24 weeks [18,19].

Patient demographics revealed a 2:1 men-to-women ratio, which agrees with studies carried out in other regions of Brazil [20]. The age range with the highest frequency of tuberculosis was 35-45 years old, which accounted for 41% of all patients; this has also been observed by other authors [18,19,21]. Mortality and positive HIV serology rates were 16.6% and 10.1%, respectively. Taking these two variables together, from the 10 patients who died, three (30%) had HIV+ serology. As there were 6 HIV+ patients in all and three died, their death rate was high (50%).

Table 1. *Mycobacterium* spp. species identified in different biologic samples between 2008 and 2010

	<i>M. tuberculosis</i>	<i>M. gordonae</i>	<i>M. avium / M. colombiense</i>	<i>M. abscessus / M. boletii</i>	<i>M. peregrinum</i>	<i>Mycobacterium</i> spp.	Total
2008	24					9	33
2009	14	2		1	1	1	19
2010	23		2			10	35
Total	61	2	2	1	1	20	87

Several authors have considered multidrug resistance as a result of human error involved in prescribing or distributing patients' medicines [22]. According to the World Health Organization [23], drug resistance is a man-made amplification of the natural phenomenon of spontaneous mutations in *M. tuberculosis* genes. Another factor associated with drug resistance is non-adherence to therapy, especially in drug addicts, which is the most difficult to control. Furthermore, in a recent European research, it was observed that previous treatment for tuberculosis was the strongest risk factor for MDR-TB [24]. A WHO study conducted between 1994 and 1997 revealed that the global average for multidrug resistant tuberculosis cases was 2.2%; in more endemic regions, this could reach 5.0% [23]. Similar results were described by Dalcomo et al. [25] in a study conducted in Brazil between 1986 and 1989, where resistance to two or more drugs reached almost 3.0%. In a more recent study from Rio Grande do Sul, Brazil, in 2002, Boffo et al. [22] found 8.4% drug resistant strains from a total of 72 isolated *M. tuberculosis* strains, 2 of which were multidrug resistant, one to isoniazid and rifampicin and the other to isoniazid, rifampicin, and pyrazinamide. A limitation of the present study is the fact that we have evaluated only patients from a micro-region; therefore, our results cannot be generalized to the whole state or country.

4. CONCLUSION

Our study highlights a small but troubling percentage of multidrug resistant samples in the Botucatu region of Sao Paulo State, Brazil. It also revealed the occurrence of nontuberculous *Mycobacteria*, emphasizing the importance of correctly identifying species and testing sensitivity to antibacilar drugs to assure an adequate therapy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All procedures were approved by the Research Ethics Committee of Botucatu Medical School, Sao Paulo State University, UNESP (Proc. 4194-2012). All authors hereby declare that all experiments have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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