J Bras Pneumol. 2020;46(2):e20190184
https://dx.doi.org/10.36416/1806-3756/e20190184

ORIGINAL ARTICLE

# Nontuberculous mycobacteria in patients with suspected tuberculosis and the genetic diversity of *Mycobacterium avium* in the extreme south of Brazil

Caroline Busatto[1], Júlia Silveira Vianna[1], Ana Barbara Scholante Silva[1], Rossana Basso[2], Jussara Silveira[2], Andrea Von Groll[1], Ivy Bastos Ramis[1], Pedro Eduardo Almeida da Silva[1]

1. Núcleo de Pesquisa em Microbiologia Médica – NUPEMM – Faculdade de Medicina, Universidade Federal do Rio Grande, Rio Grande (RS) Brasil.
2. Faculdade de Medicina, Universidade Federal do Rio Grande, Rio Grande (RS) Brasil.

**Submitted:** 29 May 2019.
**Accepted:** 4 August 2019.

Study carried out in the Laboratório de Micobactérias, Universidade Federal do Rio Grande, Rio Grande (RS) Brasil.

## ABSTRACT

**Objective:** Nontuberculous mycobacteria (NTM) are a heterogeneous group of bacteria that are widely distributed in nature and associated with opportunistic infections in humans. The aims of this study were to identify NTM in patients with suspected tuberculosis who presented positive cultures and to evaluate the genetic diversity of strains identified as *Mycobacterium avium*. **Methods:** We studied pulmonary and extrapulmonary samples obtained from 1,248 patients. The samples that tested positive on culture and negative for the *M. tuberculosis* complex by molecular identification techniques were evaluated by detection of the *hsp65* and *rpoB* genes and sequencing of conserved fragments of these genes. All strains identified as *M. avium* were genotyped using the eight-locus mycobacterial interspersed repetitive unit–variable-number tandem-repeat method. **Results:** We found that NTM accounted for 25 (7.5%) of the 332 mycobacteria isolated. Of those 25, 18 (72%) were *M. avium*, 5 (20%) were *M. abscessus*, 1 (4%) was *M. gastri*, and 1 (4%) was *M. kansasii*. The 18 *M. avium* strains showed high diversity, only two strains being genetically related. **Conclusions:** These results highlight the need to consider the investigation of NTM in patients with suspected active tuberculosis who present with positive cultures, as well as to evaluate the genetic diversity of *M. avium* strains.

**Keywords:** Mycobacterium avium; Nontuberculous mycobacteria; HIV; Genotyping techniques.

## INTRODUCTION

The incidence of nontuberculous mycobacterial (NTM) infections has increased worldwide, attracting attention in routine diagnostic settings, particularly among patients with suspected tuberculosis. Recognized as true pathogens in humans, NTM are major causes of opportunistic infections in people living with HIV.[1] Infections with NTM often occur in the respiratory tract, and such infections can progress to severe lung disease, thus increasing morbidity and mortality.[2]

Among the most important and frequently isolated NTM species are members of the *Mycobacterium avium* complex (MAC), particularly *M. avium* and *M. intracellulare*, followed by *M. abscessus*.[3] Given the ubiquitous presence of MAC in the environment, it has been assumed that exposure to environmental conditions would be the most common form of transmission of these NTM to the host, although it is a huge challenge to prove transmission by an environmental source or directly from patient to patient. However, it has been suggested that there is transmission of *M. abscessus* from patient to patient,[4] and a recent study used a nematode model to determine whether *M. avium* can be transmitted from host to host.[5] The results of that study suggested that *M. avium* may be acquired from a living source, such as an infected patient with a chronic pulmonary disease, as well as from the outside environment.

The distribution of NTM species that cause infections differs by geographic region. Therefore, defining the epidemiology of infections caused by NTM in developing countries is more challenging than delineating that of tuberculosis, because, unlike what is the case for tuberculosis, there is no compulsory reporting of cases of NTM infection.[3,6] To obtain reliable epidemiological data and prescribe the appropriate therapy, it is important to accurately identify the NTM responsible. The identification of acid-fast bacilli or a positive culture does not allow mycobacterial species to be differentiated.[7,8]

The American Thoracic Society issued diagnostic criteria to assist in diagnosis of NTM disease cases.[7] Clinical, radiographic, and (mainly) microbiological data are required; three or more sputum specimens should be collected for microscopy and culture or specimens should be collected by bronchoscopy. Although the diagnosis of NTM disease is based on isolation of the organisms from the culture of diagnostic specimens, simply isolating an

**Correspondence to:**
Caroline Busatto. Núcleo de Pesquisa em Microbiologia Médica – NUPEMM – Faculdade de Medicina, Universidade Federal do Rio Grande, Rua General Osório, s/n, Centro, CEP 96200-400, Rio Grande, RS, Brasil.
Tel.: 55 51 99802-0024. E-mail: caroline-busatto@hotmail.com
Financial support: None.

© 2020 Sociedade Brasileira de Pneumologia e Tisiologia

NTM does not mean that disease is present. There are at least three factors that can help clinicians differentiate between disease and colonization[9]: the bacterial load; the species isolated; and whether or not there is clinical or radiographic progression of disease.

Although species identification can be carried out by biochemical methods, that approach is cumbersome and poorly reproducible. Molecular techniques, such as whole genome sequencing, restriction fragment analysis by PCR, line probe hybridization, and sequencing of *hsp65* and *rpoB* fragments, have been widely used and have a clear advantage over phenotypic methods.[10,11]

In addition to the identification of the species involved in the infectious process, molecular biology tools have allowed the genotyping and differentiation of isolates of the same species, thus allowing epidemiological links to be established. The use of RFLP with IS*1245* as the target, which is considered the gold-standard method for the genotyping of *M. avium* strains, has been changed by the introduction of the mycobacterial interspersed repetitive unit–variable-number tandem-repeat (MIRU-VNTR) method, which presents similar IS*1245* RFLP discriminatory power. The main advantages of the MIRU-VNTR method are its simplicity, the fact that it produces rapid results, and its reproducibility.[12,13]

The main objectives of this study were to determine the prevalence of NTM in patients with suspected tuberculosis who present with positive cultures and to evaluate the clonal diversity of *M. avium*.

## METHODS

### Study design

This was a retrospective cross-sectional study of pulmonary and extrapulmonary samples collected from 1,248 patients suspected of tuberculosis who were seen at the Dr. Miguel Riet Corrêa Junior University Hospital, in the city of Rio Grande, located in the southern Brazilian state of Rio Grande do Sul. The samples were received at the Mycobacteria Laboratory of the Federal University of Rio Grande between January of 2014 and December of 2016. The characteristics of the patients were collected from medical records and from the Mycobacteria Laboratory database. The study was approved by the Research Ethics Committee of the Federal University of Rio Grande (Reference no. 47/2017).

### Experimental procedures

For PCR, sequencing, and genotyping experiments, we used DNA extracted from samples testing positive in liquid culture in an automated culture system (BACTEC Mycobacteria Growth Indicator Tube; Becton Dickinson, Sparks, MD, USA). Samples that were not identified as *M. tuberculosis* by IS*6110* PCR were submitted to PCR for the detection of fragments of the *hsp65* and *rpoB* genes. Samples testing positive for both genes were sequenced for identification of the mycobacterial species. Subsequently, all strains identified as *M. avium* were genotyped by the eight-locus MIRU-VNTR method (Figure 1).

### DNA extraction

For DNA extraction, colonies of mycobacteria grown in liquid media were resuspended in 1× Tris-EDTA and incubated for 30 min at 80°C for inactivation of the bacteria. Subsequently, DNA was extracted by the cetyltrimethylammonium bromide/NaCl method, as described by van Soolingen et al.[14]

### PCR for hsp65 and rpoB

A fragment of the *hsp65* gene was detected by using the primers TB11 (5′-ACCAACGATGGTGTGTCAT-3′) and TB12 (5′-CTTGTCGAACCGCATACCCT-3′), which amplify a 441-bp fragment. For detection of the *hsp65* gene fragment, PCR was performed as described by Telenti et al.[15] In addition, a fragment of the *rpoB* gene was detected by using the primers MycoF (5′-GGCAAGGTCACCCCGAAGGG-3′) and MycoR (5′-AGCGGCTGCTGGGTGATCATC-3′), which amplify a 764-bp fragment. For detection of the *rpoB* gene fragment, PCR was performed as described by Adekambi et al.[16]

### Sequencing

Sequencing was performed in an automated sequencer (ABI 3500 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). The PCR products were labeled with 5 pmol of TB11 primer (5′-ACCAACGATGGTGTGTCAT-3′, for the *hsp65* gene) or with 5 pmol of MycoF primer (5′-GGCAAGGTCACCCCGAAGGG0-3′, for the *rpoB* gene), together with 1 µL of reagent (BigDye Terminator v3.1 Cycle Sequencing Kit; Applied Biosystems), for 4.5 µL of purified PCR product in a final volume of 10 µL. The labeling reactions were performed in a 96-well thermocycler (Veriti; Applied Biosystems) with denaturation at 96°C for 1 min, followed by 35 cycles at 96°C for 15 s, 50°C for 15 s, and 60°C for 4 min. After labeling, the samples were purified by ethanol/EDTA precipitation and analyzed in the automated sequencer. The sequences obtained were analyzed with the software Chromas, version 2.6 (Technelysium, Southport, Australia), and sequence alignment was performed on the National Center for Biotechnology Information Basic Local Alignment Search Tool site (http://blast.ncbi.nlm.nih.gov).

### Genotyping of M. avium

The MIRU-VNTR method was performed with the primers described by Thibault et al.[12] and using eight loci. The PCR was performed as described in the MAC-INMV database (http://mac-inmv.tours.inra.fr). The fragment sizes were determined by the number of tandem repeats at each locus. The PCR products were visualized by 3% agarose gel electrophoresis, involving staining with 0.001 mg/mL ethidium bromide and fluorescence visualization under a UV light source. A 50-bp DNA ladder and a 100-bp DNA ladder (Ludwig

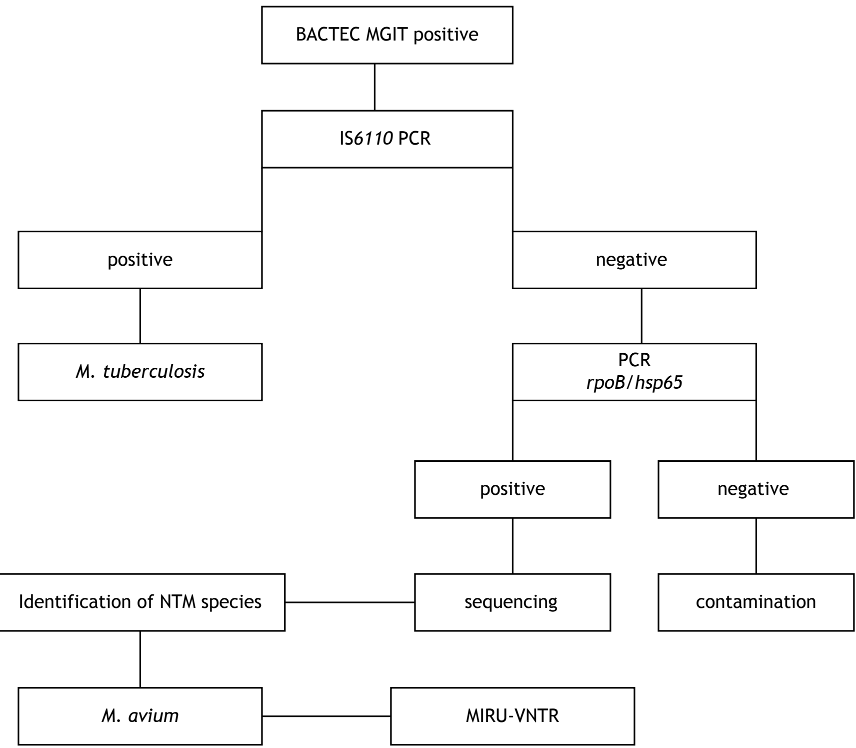


**Figure 1.** Flow chart of the experimental procedures. MGIT: Mycobacteria Growth Indicator Tube; *M.*: *Mycobacterium*; NTM: nontuberculous mycobacteria; and MIRU-VNTR: mycobacterial interspersed repetitive unit–variable-number tandem-repeat (method).

Biotec, Alvorada, Brazil) were used in order to define the size of the PCR products.

The allelic diversity of each MIRU-VNTR locus was calculated by the following equation:

$$h = 1 - \sum xi^2 \, [n / (n - 1)]$$

where $h$ is the heterozygosity at the locus, $xi$ is the allele frequency at the locus, and $n$ is the number of strains. According to the h, the discriminatory power of the loci was classified as high ($h > 0.6$), moderate ($h \leq 0.6$), or low ($h < 0.3$).[17]

## RESULTS

### Identification of NTM

Of the 1,248 patients suspected of having tuberculosis, 332 had positive mycobacterial cultures, 25 (7.5%) of whom were found to be infected with NTM. Of those 25 patients, 20 (80%) had undergone HIV testing and 13 (52%) were HIV positive. In addition, 18 (72%) were men, whereas only 7 (28%) were women, and the median age was 46 years (range, 26-78 years).

The NTM species were identified as *M. avium* in 18 (72%) of the 25 patients, as *M. abscessus* in 5 (20%), as *M. gastri* in 1 (4%), and as *M. kansasii* in 1 (4%). Of the 18 patients infected with *M. avium*, 10 (55.5%) were HIV positive, 5 (20.0%) were HIV negative, and 3 (16.7%) were of unknown HIV status. As can be seen in Table 1, 23 (92%) of the samples in which NTM species were identified were of pulmonary origin (sputum, bronchoalveolar lavage fluid, or tracheal aspirate).

### Genotyping of *M. avium*

Eighteen strains of *M. avium* were analyzed by eight-locus MIRU-VNTR, resulting in 16 (88.9%) being classified as orphan strains and 2 being grouped to form the only cluster (Figure 2). As detailed in Table 2, we identified 17 previously unknown INMV patterns and one known pattern (INMV 78).

The values of allelic diversity in the samples analyzed were calculated for each locus and are presented in Table 3. Loci X3, 25, 10, and 32 were highly discriminatory ($h \geq 0.6$); X3 and 10 were the most polymorphic, with eight different alleles each.

## DISCUSSION

The increase in the incidence of NTM infections in cases of suspected tuberculosis is a huge challenge in clinical practice. The possible explanations for the increase in the number of such cases include the improvement in the diagnostic capabilities of laboratories and greater awareness of such infections in clinical settings.[18] In the present study, NTM were identified in 7.5% of the positive cultures in patients with suspected tuberculosis. This result is consistent with those of other studies, in which NTM were identified in 4-10% of positive cultures in such patients.[19-21]

**Table 1.** Characteristics of patients infected with nontuberculous mycobacteria.

| Patient | Age | Gender | HIV status | CD4 count (cells/mm³) | Clinical specimen | Sequencing result |
|---|---|---|---|---|---|---|
| 1676 | 71 | F | Positive | 12 | Sputum | *M. abscessus* |
| 1871 | 67 | M | Negative | | Sputum | *M. avium* |
| 1895 | 41 | M | Positive | 56 | BALF | *M. avium* |
| 1896 | 58 | M | ND | | Sputum | *M. avium* |
| 1901 | 69 | M | ND | | Sputum | *M. abscessus* |
| 2006 | 46 | F | Negative | | Sputum | *M. abscessus* |
| 2091 | 26 | F | Positive | 54 | LB | *M. avium* |
| 3036 | 37 | F | Positive | 183 | BALF | *M. avium* |
| 3145 | 28 | F | Positive | 544 | Sputum | *M. avium* |
| 3168 | 46 | M | Positive | 152 | TA | *M. avium* |
| 3366 | 49 | M | Negative | | Sputum | *M. avium* |
| 3390 | 30 | F | Positive | ND | Sputum | *M. avium* |
| 3491 | 54 | M | Positive | 22 | Sputum | *M. gastri* |
| 3471 | 38 | M | Positive | 266 | Sputum | *M. avium* |
| 3594 | 58 | M | ND | | Sputum | *M. avium* |
| 3717 | 55 | M | ND | | Sputum | *M. avium* |
| 3811 | 32 | M | Positive | 290 | Sputum | *M. avium* |
| 3870 | 42 | M | Positive | 4 | Sputum | *M. avium* |
| 3913 | 36 | F | Negative | | Sputum | *M. avium* |
| 4111 | 78 | M | Negative | | Sputum | *M. avium* |
| 4127 | 59 | M | ND | | Sputum | *M. kansasii* |
| 4161 | 40 | M | Positive | 55 | Sputum | *M. abscessus* |
| 4307 | 45 | M | Negative | | BALF | *M. abscessus* |
| 4425 | 46 | M | Positive | 153 | CG | *M. avium* |
| 4695 | 37 | M | Negative | | Sputum | *M. avium* |

F: female; M: male; *M.*: *Mycobacterium*; BALF: BAL fluid; ND: no data; LB: liver biopsy; TA: tracheal aspirate; and CG: cervical ganglion.

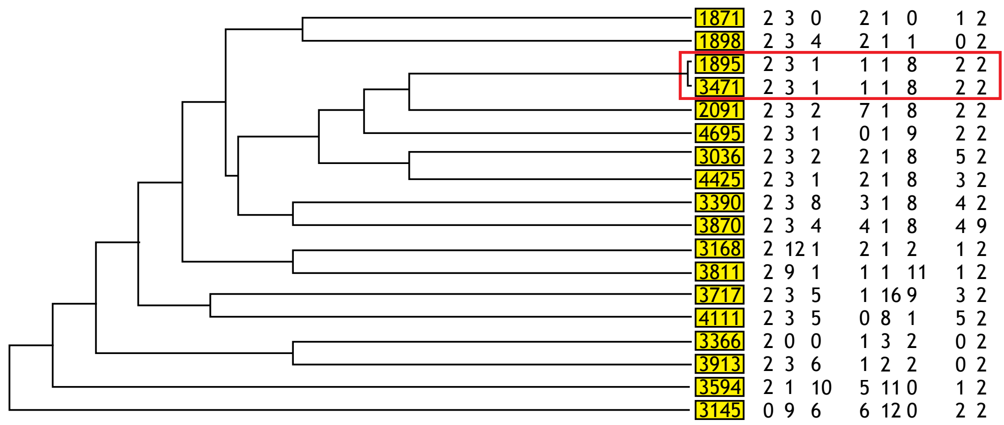


**Figure 2.** Epidemiological links between patients.

Other studies have shown that the prevalence of NTM is higher in men and in individuals over 40 years of age (72% and 68%, respectively).[22,23] In addition, 92% of NTM are isolated from respiratory specimens and individuals of advanced age could therefore be more susceptible to respiratory infections caused by NTM.[19]

It should be noted that the incidence of NTM infection can be 9.7-fold higher in patients with HIV infection, especially those with CD4 cell counts < 100 cells/mm³.[24] Recent studies conducted in Brazil have reported that *M. avium* is the NTM species most often isolated from respiratory specimens in HIV-infected patients.[25,26]

The present study was carried out at a referral hospital for HIV-infected patients, located in the Brazilian state of Rio Grande do Sul, where the prevalence of HIV infection is 38.3 cases/100,000 population, the second highest among all of the states in the country.[27] Among the patients infected with NTM, most were infected with *M. avium* (72.0%), and 55.5% of those patients were coinfected with HIV. Such infections

**Table 2.** Patterns of 18 *Mycobacterium avium* strains evaluated with the mycobacterial interspersed repetitive unit–variable-number tandem-repeat method.

| Patient | Loci of interest in the MIRU-VNTR method | | | | | | | | INMV pattern |
|---|---|---|---|---|---|---|---|---|---|
| | 292 | X3 | 25 | 47 | 3 | 7 | 10 | 32 | |
| | Number of tandem repeats | | | | | | | | |
| 1871 | 2 | 2 | 1 | 3 | 1 | 2 | 0 | 0 | Not yet listed |
| 1895 | 2 | 1 | 2 | 3 | 1 | 2 | 1 | 8 | Not yet listed |
| 1896 | 2 | 2 | 0 | 3 | 1 | 2 | 4 | 1 | Not yet listed |
| 2091 | 2 | 7 | 2 | 3 | 1 | 2 | 2 | 8 | Not yet listed |
| 3036 | 2 | 2 | 5 | 3 | 1 | 2 | 2 | 8 | Not yet listed |
| 3366 | 2 | 1 | 0 | 0 | 3 | 2 | 0 | 2 | Not yet listed |
| 3390 | 2 | 3 | 4 | 3 | 1 | 2 | 8 | 8 | Not yet listed |
| 3471 | 2 | 1 | 2 | 3 | 1 | 2 | 1 | 8 | Not yet listed |
| 3594 | 2 | 5 | 1 | 1 | 11 | 2 | 10 | 0 | Not yet listed |
| 3717 | 2 | 1 | 3 | 3 | 16 | 2 | 5 | 9 | Not yet listed |
| 3811 | 2 | 1 | 1 | 9 | 1 | 2 | 1 | 11 | Not yet listed |
| 3870 | 2 | 4 | 4 | 3 | 1 | 9 | 4 | 8 | Not yet listed |
| 3913 | 2 | 1 | 0 | 3 | 2 | 2 | 6 | 2 | Not yet listed |
| 4695 | 2 | 0 | 2 | 3 | 1 | 2 | 1 | 9 | Not yet listed |
| 4425 | 2 | 2 | 3 | 3 | 1 | 2 | 1 | 8 | 78* |
| 3145 | 0 | 6 | 2 | 9 | 12 | 2 | 6 | 0 | Not yet listed |
| 3168 | 2 | 2 | 1 | 12 | 1 | 2 | 1 | 2 | Not yet listed |
| 4111 | 2 | 0 | 5 | 3 | 8 | 2 | 5 | 1 | Not yet listed |

MIRU-VNTR: mycobacterial interspersed repetitive unit–variable-number tandem-repeat; INMV: MAC-*Institut National de la Recherche Agronomique* (French National Institute for Agricultural Research) Nouzilly MIRU-VNTR; and *M.*: *Mycobacterium*. **M. avium* subsp. *paratuberculosis*.

**Table 3.** Allelic diversity using the eight-locus mycobacterial interspersed repetitive unit–variable-number tandem-repeat method.

| N of alleles | MIRU 292 | MIRU X3 | MIRU 25 | MIRU 47 | MIRU 3 | MIRU 7 | MIRU 10 | MIRU 32 |
|---|---|---|---|---|---|---|---|---|
| 0 | 1 | 2 | 3 | 1 | | | 2 | 3 |
| 1 | | 6 | 4 | 1 | 12 | | 6 | 2 |
| 2 | 17 | 5 | 5 | | 1 | 17 | 2 | 3 |
| 3 | | 1 | 2 | 13 | 1 | | | |
| 4 | | 1 | 2 | | | | 2 | |
| 5 | | 1 | 2 | | | | 2 | |
| 6 | | 1 | | | | | 2 | |
| 7 | | 1 | | | | | | |
| 8 | | | | | 1 | | 1 | 7 |
| 9 | | | | 2 | | 1 | | 2 |
| 10 | | | | | | | 1 | |
| 11 | | | | | 1 | | | 1 |
| >12 | | | | 1 | 2 | | | |
| Measures of diversity | | | | | | | | |
| h | 0.104 | 0.783 | 0.808 | 0.456 | 0.540 | 0.104 | 0.820 | 0.765 |
| DP* | Low | High | High | Mod | Mod | Low | High | High |

MIRU: mycobacterial interspersed repetitive unit; h: heterozygosity; DP: discriminatory power; and Mod: moderate. *Discriminatory power is defined, according to the allelic diversity (heterozygosity), as high ($h > 0.6$), moderate ($h \leq 0.6$), or low ($h < 0.3$).

generate high morbidity and economic costs, because the current treatments have multiple side effects and an intention-to-treat cure rate < 50%.[28]

In Brazil, the available data suggest regional differences in the distribution of NTM species, especially in the relative proportions of MAC and *M. kansasii*. Carneiro et al.[25] also found MAC species to be the most common NTM species causing respiratory infection in the state of Rio Grande do Sul. However, at a referral center in the state of Rio de Janeiro, *M. kansasii* was found to account for a third of all NTM infections.[29] The fact that there are a greater number of HIV-infected patients in the state of Rio Grande do Sul than in the state of Rio de Janeiro is the most likely reason for

the higher prevalence of respiratory infection caused by MAC in the former.[29] It has been suggested that, in addition to the presence of cofactors such as HIV infection, host and environmental factors interact to influence the risk of disease and the geographic distribution of NTM infection.

Infection with *M. avium* can have clinical and radiological presentations indistinguishable from those of tuberculosis, making its differentiation and diagnosis difficult. The accurate identification of NTM species is critical because the management and treatment of infected patients, as well as the epidemiological control tools implemented, should reflect the specific mycobacterial species isolated and its sources.[30]

Studies involving the epidemiology of *M. avium* have been based on typing methods such as RFLP analysis using IS*1245* as a probe and are now based on typing methods such as MIRU-VNTR.[13] Thibault et al.[12] standardized the MIRU-VNTR method using eight loci to study variability in *M. avium* strains obtained from different hosts and from different geographic regions.

In the last five years, several genotypes of *M. avium* from diverse hosts (humans and animals) have been identified and registered in a web application known as the MAC-INMV database (http://mac-inmv.tours.inra.fr).[12] In the present study, we have described, for the first time, seventeen patterns that will later be included in the database. The only pattern that had previously been described was INMV 78 (*M. avium* subsp. *paratuberculosis*), which was previously isolated from a goat.[31] That pattern differs from those previously reported to be the most prevalent in different parts of the world (INMV 1 and 2).[12,32] However, caution is needed when using VNTR subtyping, given that it may overestimate or underestimate the relationship between strains because of the instability of some repetitive elements in the genome.[33]

Despite the wide acceptance of the MIRU-VNTR method, it is often difficult to make comparisons across studies reporting the results obtained with the method, because of the lack of standardization. Such studies have involved various hosts (such as cattle, goats, and sheep), loci (such as 7, 8, 16, and 20), and methodologies.[32,34,35] However, eight specific loci (292, X3, 25, 47, 3, 7, 10, and 32) are the most commonly used in the MIRU-VNTR method and have shown high discriminatory power.[12,36]

In the present study, we found only one strain cluster, comprising two strains of *M. avium*. We find it interesting that patients 1895 and 3471 occupied the same bed in the hospital within a short time period (30 days). Both were HIV positive and were immunocompromised, according to their CD4 count. The first patient to occupy the bed (patient 1895) reported having worked in fields and having had contact with birds. Although there are reports that humans and animals acquire *M. avium* infection from environmental sources,[37] direct transmission between animals and humans cannot be excluded, because the genetic profiles of strains isolated from both hosts are similar. In addition, soil, water, and biofilms can be important sources of transmission of *M. avium* because of its ability to survive for a long time (200-600 days) in those environments.[36-38]

In relation to allelic diversity, some loci were highly discriminatory and should be prioritized for rapid differentiation of *M. avium* strains. According to one previous study,[39] X3 is one of the most discriminatory loci, as are loci 3 and 10, although the last two were described as being less suitable for typing. The seven loci presented low allelic diversity (0.104), which is consistent with the findings of another study.[40]

In the present study, *M. avium* was the NTM most frequently identified among positive cultures in cases of suspected tuberculosis. In addition, HIV infection was the main condition predisposing patients to the development of NTM infectious diseases.

To our knowledge, this was the first study using the eight-locus MIRU-VNTR method as a tool to evaluate the clonal diversity of *M. avium* strains isolated from humans in the extreme south of Brazil. We observed high clonal diversity, with only one cluster (comprising two strains). It is noteworthy that the two strains in the cluster were obtained from patients who had an epidemiological link. Although we cannot affirm that there was a connection between those two cases, we also cannot rule it out.

Our study was limited by the small number of *M. avium* strains studied. However, our findings highlight the need to implement the rapid, accurate identification of NTM in positive cultures in patients with suspected tuberculosis, as well as to use molecular tools to monitor the clonal diversity of *M. avium* strains and establish possible epidemiologic links.

## REFERENCES

1. Varghese B, Memish Z, Abuljadayel N, Al-Hakeem R, Alrabiah F, Al-Hajoj SA. Emergence of clinically relevant Non-Tuberculous Mycobacterial infections in Saudi Arabia. PLoS Negl Trop Dis. 2013;7(5):e2234. https://doi.org/10.1371/journal.pntd.0002234
2. Stout JE, Koh WJ, Yew WW. Update on pulmonary disease due to non-tuberculous mycobacteria. Int J Infect Dis. 2016;45:123-134. https://doi.org/10.1016/j.ijid.2016.03.006
3. Prevots DR, Marras TK. Epidemiology of human pulmonary infection with nontuberculous mycobacteria: a review. Clin Chest Med. 2015;36(1):13-34. https://doi.org/10.1016/j.ccm.2014.10.002
4. Yan J, Kevat A, Martinez A, Teese N, Johnson K, Ranganatha S, et al. Investigating transmission of Mycobacterium abscessus amongst children in an Australian cystic fibrosis centre. J Cyst Fibros. 2019;S1569-1993(18)30918-4. https://doi.org/10.1016/j.jcf.2019.02.011
5. Bermudez LE, Rose SJ, Everman JL, Ziaie NR. Establishment of a Host-to-Host Transmission Model for Mycobacterium avium subsp. hominissuis Using Caenorhabditis elegans and Identification of Colonization-Associated Genes. Front Cell Infect Microbiol. 2018;8:123. https://doi.org/10.3389/fcimb.2018.00123
6. Nishiuchi Y, Iwamoto T, Maruyama F. Infection Sources of a Common Non-tuberculous Mycobacterial Pathogen, Mycobacterium

avium Complex. Front Med (Lausanne). 2017;4:27. https://doi.org/10.3389/fmed.2017.00027

7. 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 [published correction appears in Am J Respir Crit Care Med. 2007;175(7):744-5. Dosage error in article text]. Am J Respir Crit Care Med. 2007;175(4):367-416. https://doi.org/10.1164/rccm.200604-571ST
8. Ryu YJ, Koh WJ, Daley CL. Diagnosis and Treatment of Nontuberculous Mycobacterial Lung Disease: Clinicians' Perspectives. Tuberc Respir Dis (Seoul). 2016;79(2):74-84. https://doi.org/10.4046/trd.2016.79.2.74
9. Daley CL, Griffith DE. Pulmonary non-tuberculous mycobacterial infections. Int J Tuberc Lung Dis. 2010;14(6):665-671. PMID: 20487602
10. Brown-Elliott BA, Philley JV. Rapidly Growing Mycobacteria. Microbiol Spectr. 2017;5(1):10.1128/microbiolspec.TNMI7-0027-2016. https://doi.org/10.1128/microbiolspec.TNMI7-0027-2016
11. Tortoli E. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. Clin Microbiol Rev. 2003;16(2):319-354. https://doi.org/10.1128/CMR.16.2.319-354.2003
12. Thibault VC, Grayon M, Boschiroli ML, Hubbans C, Overduin P, Stevenson K, et al. New variable-number tandem-repeat markers for typing Mycobacterium avium subsp. paratuberculosis and M. avium strains: comparison with IS900 and IS1245 restriction fragment length polymorphism typing. J Clin Microbiol. 2007;45(8):2404-2410. https://doi.org/10.1128/JCM.00476-07
13. Guerrero C, Bernasconi C, Burki D, Bodmer T, Telenti A. A novel insertion element from Mycobacterium avium, IS1245, is a specific target for analysis of strain relatedness. J Clin Microbiol. 1995;33(2):304-307. PMCID: PMC227937.
14. van Soolingen D, de Haas PE, Hermans PW, van Embden JD. DNA fingerprinting of Mycobacterium tuberculosis. Methods Enzymol. 1994;235:196-205. https://doi.org/10.1016/0076-6879(94)35141-4
15. 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):175-178.
16. Adékambi T, Colson P, Drancourt M. rpoB-based identification of nonpigmented and late-pigmenting rapidly growing mycobacteria. J Clin Microbiol. 2003;41(12):5699-5708. https://doi.org/10.1128/JCM.41.12.5699-5708.2003
17. Selander RK, Caugant DA, Ochman H, Musser JM, Gilmour MN, Whittam TS. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl Environ Microbiol. 1986;51(5):873-884. https://doi.org/10.1128/AEM.51.5.873-884.1986
18. Busatto C, Vianna JS, da Silva LV Junior, Ramis IB, da Silva PEA. Mycobacterium avium: an overview. Tuberculosis (Edinb). 2019;114:127-134. https://doi.org/10.1016/j.tube.2018.12.004
19. Hoza AS, Mfinanga SG, Rodloff AC, Moser I, König B. Increased isolation of nontuberculous mycobacteria among TB suspects in Northeastern, Tanzania: public health and diagnostic implications for control programmes. BMC Res Notes. 2016;9:109. https://doi.org/10.1186/s13104-016-1928-3
20. Nasiri MJ, Dabiri H, Darban-Sarokhalil D, Hashemi Shahraki A. Prevalence of Non-Tuberculosis Mycobacterial Infections among Tuberculosis Suspects in Iran: Systematic Review and Meta-Analysis. PLoS One. 2015;10(6):e0129073. https://doi.org/10.1371/journal.pone.0129073
21. Nour-Neamatollahie A, Ebrahimzadeh N, Siadat SD, Vaziri F, Eslami M, Sepahi AA, et al. Distribution of non-tuberculosis mycobacteria strains from suspected tuberculosis patients by heat shock protein 65 PCR-RFLP. Saudi J Biol Sci. 2017;24(6):1380-1386. https://doi.org/10.1016/j.sjbs.2016.02.001
22. Antonenka U, Hofmann-Thiel S, Turaev L, Esenalieva A, Abdulloeva M, Sahalchyk E, et al. Comparison of Xpert MTB/RIF with ProbeTec ET DTB and COBAS TaqMan MTB for direct detection of M. tuberculosis complex in respiratory specimens. BMC Infect Dis. 2013;13:280. https://doi.org/10.1186/1471-2334-13-280
23. Dabó H, Santos V, Marinho A, Ramos A, Carvalho T, Ribeiro M, et al. Nontuberculous mycobacteria in respiratory specimens: clinical significance at a tertiary care hospital in the north of Portugal. J Bras Pneumol. 2015;41(3):292-294. https://doi.org/10.1590/S1806-37132015000000005
24. Procop GW. HIV and mycobacteria. Semin Diagn Pathol. 2017;34(4):332-339. https://doi.org/10.1053/j.semdp.2017.04.006
25. Carneiro MDS, Nunes LS, David SMM, Dias CF, Barth AL, Unis G. Nontuberculous mycobacterial lung disease in a high tuberculosis incidence setting in Brazil. J Bras Pneumol. 2018;44(2):106-111. https://doi.org/10.1590/s1806-37562017000000213
26. Pedro HSP, Coelho AGV, Mansur IM, Chiou AC, Pereira MIF, Belotti NCU, et al. Epidemiological and laboratorial profile of patients with isolation of nontuberculous mycobacteria. Int J Mycobacteriol. 2017;6(3):239-245. https://doi.org/10.4103/ijmy.ijmy_87_17
27. Brasil. Ministério da Saúde. Secretária de Vigilância à Saúde. Boletim Epidemiológico AIDS e DSTs. Brasília: Ministério da Saúde; 2017.
28. Thomson RM, Yew WW. When and how to treat pulmonary non-tuberculous mycobacterial diseases. Respirology. 2009;14(1):12-26. https://doi.org/10.1111/j.1440-1843.2008.01408.x
29. de Mello KG, Mello FC, Borga L, Rolla V, Duarte RS, Sampaio EP, et al. Clinical and therapeutic features of pulmonary nontuberculous mycobacterial disease, Brazil, 1993-2011. Emerg Infect Dis. 2013;19(3):393-399. https://doi.org/10.3201/eid1903.120735
30. Tortoli E. Microbiological features and clinical relevance of new species of the genus Mycobacterium. Clin Microbiol Rev. 2014;27(4):727-752. https://doi.org/10.1128/CMR.00035-14
31. Biet F, Sevilla IA, Cochard T, Lefrancois LH, Garrido JM, Heron I, Juste RA, et al. Inter- and intra-subtype genotypic differences that differentiate Mycobacterium avium subspecies paratuberculosis strains. BMC Microbiol. 2012;12:264. https://doi.org/10.1186/1471-2180-12-264
32. Gioffré A, Correa Muñoz M, Alvarado Pinedo MF, Vaca R, Morsella C, Fiorentino MA, et al. Molecular typing of Argentinian Mycobacterium avium subsp. paratuberculosis isolates by multiple-locus variable number-tandem repeat analysis. Braz J Microbiol. 2015;46(2):557-564. https://doi.org/10.1590/S1517-838246220140283
33. Ahlstrom C, Barkema HW, Stevenson K, Zadoks RN, Biek R, Kao R, et al. Limitations of variable number of tandem repeat typing identified through whole genome sequencing of Mycobacterium avium subsp. paratuberculosis on a national and herd level. BMC Genomics. 2015;16(1):161. https://doi.org/10.1186/s12864-015-1387-6
34. Inagaki T, Nishimori K, Yagi T, Ichikawa K, Moriyana M, Nakagawa T, et al. Comparison of a variable-number tandem-repeat (VNTR) method for typing Mycobacterium avium with mycobacterial interspersed repetitive-unit-VNTR and IS1245 restriction fragment length polymorphism typing. J Clin Microbiol. 2009;47(7):2156-2164. https://doi.org/10.1128/JCM.02373-08
35. Leão C, Canto A, Machado D, Sanches IS, Couto I, Viveiros M, et al. Relatedness of Mycobacterium avium subspecies hominissuis clinical isolates of human and porcine origins assessed by MLVA. Vet Microbiol. 2014;173(1-2):92-100. https://doi.org/10.1016/j.vetmic.2014.06.027
36. Castellanos E, de Juan L, Domínguez L, Aranaz A. Progress in molecular typing of Mycobacterium avium subspecies paratuberculosis. Res Vet Sci. 2012;92(2):169-179. https://doi.org/10.1016/j.rvsc.2011.05.017
37. Halstrom S, Price P, Thomson R. Review: Environmental mycobacteria as a cause of human infection. Int J Mycobacteriol. 2015;4(2):81-91. https://doi.org/10.1016/j.ijmyco.2015.03.002
38. Cook KL, Britt JS. Optimization of methods for detecting Mycobacterium avium subsp. paratuberculosis in environmental samples using quantitative, real-time PCR. J Microbiol Methods. 2007;69(1):154-160. https://doi.org/10.1016/j.mimet.2006.12.017
39. Radomski N, Thibault VC, Karoui C, Cruz K, Cochard T, Gutiérrez C, et al. Determination of genotypic diversity of Mycobacterium avium subspecies from human and animal origins by mycobacterial interspersed repetitive-unit-variable-number tandem-repeat and IS1311 restriction fragment length polymorphism typing methods. J Clin Microbiol. 2010;48(4):1026-1034. https://doi.org/10.1128/JCM.01869-09
40. Rindi L, Buzzigoli A, Medici C, Garzelli C. High phylogenetic proximity of isolates of Mycobacterium avium subsp. hominissuis over a two decades-period. Infect Genet Evol. 2013;16:99-102. https://doi.org/10.1016/j.meegid.2013.01.022