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. Infections with NTM often occur in the respiratory tract, and such infections can progress to severe lung disease, thus increasing morbidity and mortality.

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. 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, and a recent study used a nematode model to determine whether M. avium can be transmitted from host to host.

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. 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.

The American Thoracic Society issued diagnostic criteria to assist in diagnosis of NTM disease cases. 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
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NTM does not mean that disease is present. There are at least three factors that can help clinicians differentiate between disease and colonization(10): 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 IS1245 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 IS1245 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 IS6110 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 1x Tris-EDTA and incubated for 30 min at 80°C for inactivation of the bacteria. Subsequently, DNA was extracted by the ceteryltrimethylammonium 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′-ACCAACGATGGTGTTGTCAT-3′) and TB12 (5′-CTTGTCGAACCCGATACCCCT-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′-GGCAAGTGTCACCCGGAAG-3′) and MycoR (5′-AGCGGCTGCTGGGTGTACCATC-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′-ACCAACGATGGTGTTGTCAT-3′, for the *hsp65* gene) or with 5 pmol of MycoF primer (5′-GGCAAGTGTCACCCGGAAG-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
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:

\[ h = 1 - \sum x_i^2 \left[ \frac{n}{(n-1)} \right] \]

where \( h \) is the heterozygosity at the locus, \( x_i \) 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 (\( h < 0.3 \)).

**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.\(^{(19-21)}\)
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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). 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.

Recent studies conducted in Brazil have reported that *M. avium* is the NTM species most often isolated from respiratory specimens in HIV-infected patients. 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.

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 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.

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). 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.

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. Recent studies conducted in Brazil have reported that *M. avium* is the NTM species most often isolated from respiratory specimens in HIV-infected patients.

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. 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

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**Figure 2. Epidemiological links between patients.**
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 \(M.\) \textit{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, \(M.\) \textit{kansasii} was found to account for a third of all NTM infections.\(^{(29)}\)

### 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      | 0      | 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        |         |         |         | 2      |         |         |         |
| 8            | 1        |         |         |         | 1      | 7      |         |         |
| 9            | 2        | 1       |         |         | 2      |         |         |         |
| 10           | 1        |         |         |         |       |        |         |         |
| 11           |         |         |         |         |       |        | 1       | 1       |
| >12          |         |         |         |         |       |         | 1       | 2       |

**Measures of diversity**

| h | DP* |
|---|-----|
| 0.104 | Low |
| 0.783 | High |
| 0.808 | High |
| 0.456 | Mod |
| 0.540 | Mod |
| 0.104 | Low |
| 0.820 | High |
| 0.765 | High |

\(\text{MIRU-VNTR: mycobacterial interspersed repetitive unit–variable-number tandem-repeat; INMV: MAC-Institut National de la Recherche Agronomique (French National Institute for Agricultural Research) Nouzilly MIRU-VNTR; and } M.: \text{Mycobacterium.} \) \(^*\)Discriminatory power is defined, according to the allelic diversity (heterozygosity), as high (\(h > 0.6\)), moderate (\(h \leq 0.6\)), or low (\(h < 0.3\)).
the higher prevalence of respiratory infection caused by MAC in the former.\(^{(26)}\) 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 IS1245 as a probe and are now based on typing methods such as MIRU-VNTR.\(^{(12)}\) 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).\(^{(32)}\) 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.\(^{(12,34,35)}\) 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.

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