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Sequencing of *hsp65* Gene for Identification of *Mycobacterium* Species Isolated from Environmental and Clinical Sources in Rio de Janeiro, Brazil▽†

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This study evaluated the biodiversity of 28 clinical and 24 environmental *Mycobacterium* isolates from Rio de Janeiro, Brazil, by using *hsp65* sequences, with the aim of contributing to a better understanding of the genetic diversity and usefulness of this marker. An extensive phylogenetic analysis was performed. The nucleotide diversity was similar between clinical (0.06508) and environmental (0.06221) isolates.

Nontuberculous mycobacteria (NTM) are ubiquitous environmental microorganisms that can be found in a variety of ecosystems (2). The genus *Mycobacterium* comprises a wide range of organisms, including obligate parasites which cause serious human and animal diseases, opportunistic pathogens, and saprophytic species (1). Human activities likely influence the distribution and prevalence of mycobacteria. Mycobacteria are capable of inducing biofilm formation, which helps them to persist in a flowing system in spite of their slow growth. Biofilms may be important sources of NTM and may be responsible for pseudo-infections and pseudo-outbreaks as well as diseases and disease outbreaks. The rapid detection of pseudo-infections and diseases due to NTM is important and requires the use of molecular techniques. Telenti et al. (15) developed a rapid method based on evaluation of the gene encoding the 65-kDa heat shock protein by PCR. The 65-kDa protein contains epitopes that are common to various species of mycobacteria (13). Therefore, we assessed the feasibility of using *hsp65* sequencing to identify mycobacteria and to analyze their genetic variability. In this study, we report a phylogenetic analysis of clinical mycobacteriology laboratory and environmental isolates.

The clinical isolates used in this study were provided by the Laboratory of Mycobacteria, Federal University of Rio de Janeiro, and were isolated from different places. Fifty-two isolates were investigated to determine the species (28 clinical isolates and 24 environmental isolates) (Table 1). Isolates were cultured in solid Lowenstein-Jensen medium, and conventional identification procedures were carried out according to the methods of Kent and Kubicae (4). DNA samples were extracted according to the cetyltrimethylammonium bromide protocol of Van Embden and colleagues (17), and PCR assays were performed according to the method of Telenti et al. (15). Samples were purified with MicroSpin S-400 columns (Amersham Biosciences) and a QIAquick PCR purification kit (Qiagen). Sequencing was performed with a DYEnamic ET dye terminator kit (MegaBace; Amersham Biosciences) and read with a MegaBace1000 (Amersham Biosciences) automated system. All chromatograms were checked using the CHROMAS 1.45 program, and the sequences were aligned using Clustal_X 1.83 (16), with manual adjustments using the BioEdit 7.0.9 program. The substitution model used for phylogenetic reconstructions was estimated with Modeltest 3.7 (11), using the minimum theoretical information criterion and the Bayesian information criterion, as suggested by Posada and Buckley (10). Isolates were identified by comparing unknown sequences to reference databases by a FASTA BLAST search (see the supplemental material). Genetic diversity parameters, such as haplotype and nucleotide diversity (6), were estimated employing DnaSP 4.0 software (Table 2). Phylogenetic trees were reconstructed by the maximum likelihood (ML) and neighbor-joining methods, using the program PAUP* 4.0b10 (D. Swofford, Sunderland, MA). The branch confidence values were estimated using 1,000 bootstrap replicates. We inferred ML trees with a heuristic nearest-neighbor interchange search option. The neighbor-joining analysis used the ML distance in the evolutionary model selected by the model test. *Nocardia* sp. and *Corynebacterium* sp. were used as outgroup species, and *Mycobacterium tuberculosis* was used as a more closely related species (Fig. 1).

The clinical isolates are from Rio de Janeiro University Hospital, a reference hospital for mycobacterial detection, with a great number coming from long-term human immunodeficiency virus-positive patients. No incidences of *hsp65* gene

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TABLE 1. Environmental and clinical strains

Strain	GenBank accession no.
Environmental strains	
Swine source 255	EU343669
Swine source 259	EU343670
Swine source 260	EU343671
Water 262	EU343672
Water 263	EU343673
Water 264	EU343674
Water 269	EU343675
Soil 299	EU343676
Soil 301	EU343677
Bovine feces 308	EU343678
Bovine feces 314	EU343679
Bovine feces 315	EU343680
Bovine feces 318	EU343681
Bovine feces 328b	EU343682
Bovine feces 328c	EU343683
Bovine feces 345	EU343684
Bovine feces 346	EU343685
Swine source 358	EU343686
Swine source 359	EU343687
Swine source 369	EU343688
Swine source 370	EU343689
Swine source 371	EU343690
Soil 395	EU343691
Bovine feces 417	EU343692
Clinical strains[a]	
423	EU343693
424	EU343694
427	EU343695
428	EU343696
430	EU343697
432	EU343698
434	EU343699
436	EU343700
438	EU343701
440	EU343702
442	EU343703
445	EU343704
446	EU343705
447	EU343706
448	EU343707
450	EU343708
451	EU343709
452	EU343710
454	EU343711
456	EU343712
457	EU343713
458	EU343714
462	EU343715
463	EU343716
465	EU343717
466	EU343718
467	EU343719
468	EU343720

[a] From induced sputum.

amplification, sequencing failure, or interprimer sequence variation were encountered in the 368 sites studied. The genetic variations of the *hsp65* gene sequences between clinical and environmental samples were similar, which suggests an increasing relationship among some species from the environment and those infecting humans. Clinical sample 451 was clearly identified as *M. gordonae*, being grouped with 100% bootstrap support with *M. gordonae* ATCC 14470. Another clinical sample (438) was identified as *M. shottsii*, since it presented 100% identity with *M. shottsii* ATCC 700981. Samples used in the present study showed great variability, since we found many slow-growing isolates and also rapidly growing isolates in clinical and ambient samples. In general, the great majority of the samples showed slow growth. Many isolates were grouped with *M. avium*, *M. intracellulare*, *M. scrofulaceum* ATCC 19981, and the *M. tuberculosis* complex. A possible explanation for this fact is that environmental mycobacteria are normal inhabitants of a wide variety of environmental reservoirs, including natural and municipal water, soil aerosol, protozoans, animals, and humans. Environmental mycobacteria also have extraordinary starvation survival, persisting in tap water despite low nutrient levels (8, 12, 14).

Although water does not represent the only source of *M. avium* complex in humans, it is possible that it might be the primary source (3). Human activities probably have a great influence on the distribution and prevalence of mycobacteria. The treatment of drinking water supplies with chlorine or other disinfectants (e.g., ozone) leads to selection for environmental mycobacteria (7). This fact could explain why many clinical and environmental species in the present study grouped with *M. avium.* In this investigation, two clinical isolates grouped with *M. scrofulaceum*, maybe because of implementation of a clean water method, similar to the one that occurred in the United States in 1975, when increased chlorination rates may have led to a strong reduction of *M. scrofulaceum* in the water. Additionally, the epidemiology of infection by environmental mycobacteria is poorly understood due to a lack of data regarding the primary reservoirs of different mycobacterial species (5, 12).

A great number of the environmental isolates collected were identified by slow growth and were grouped with clinical isolates. This result could be related to the previously discussed question of adaptive value. There are many situations in which human and mycobacterial distributions can overlap geographically and environmentally, resulting in human exposure and in an impact on mycobacterial ecology. Humans are exposed to mycobacteria in water through drinking, swimming, and bathing. Contamination has been facilitated mainly in hospitals, where patients with reduced immunity are more exposed. Pre-

TABLE 2. Genetic diversity parameters

Sample group	No. of sequences	No. of variable sites	Total no. of mutations	No. of haplotypes	Haplotype diversity value	Nucleotide diversity (P_i)	Avg no. of nucleotide differences (K)
Clinical strains	28	73	91	19	0.947	0.06508	23.88360
Environmental strains	24	85	106	17	0.920	0.06221	22.82971
Clinical and environmental strains	52	95	128	34	0.963	0.06590	24.18627

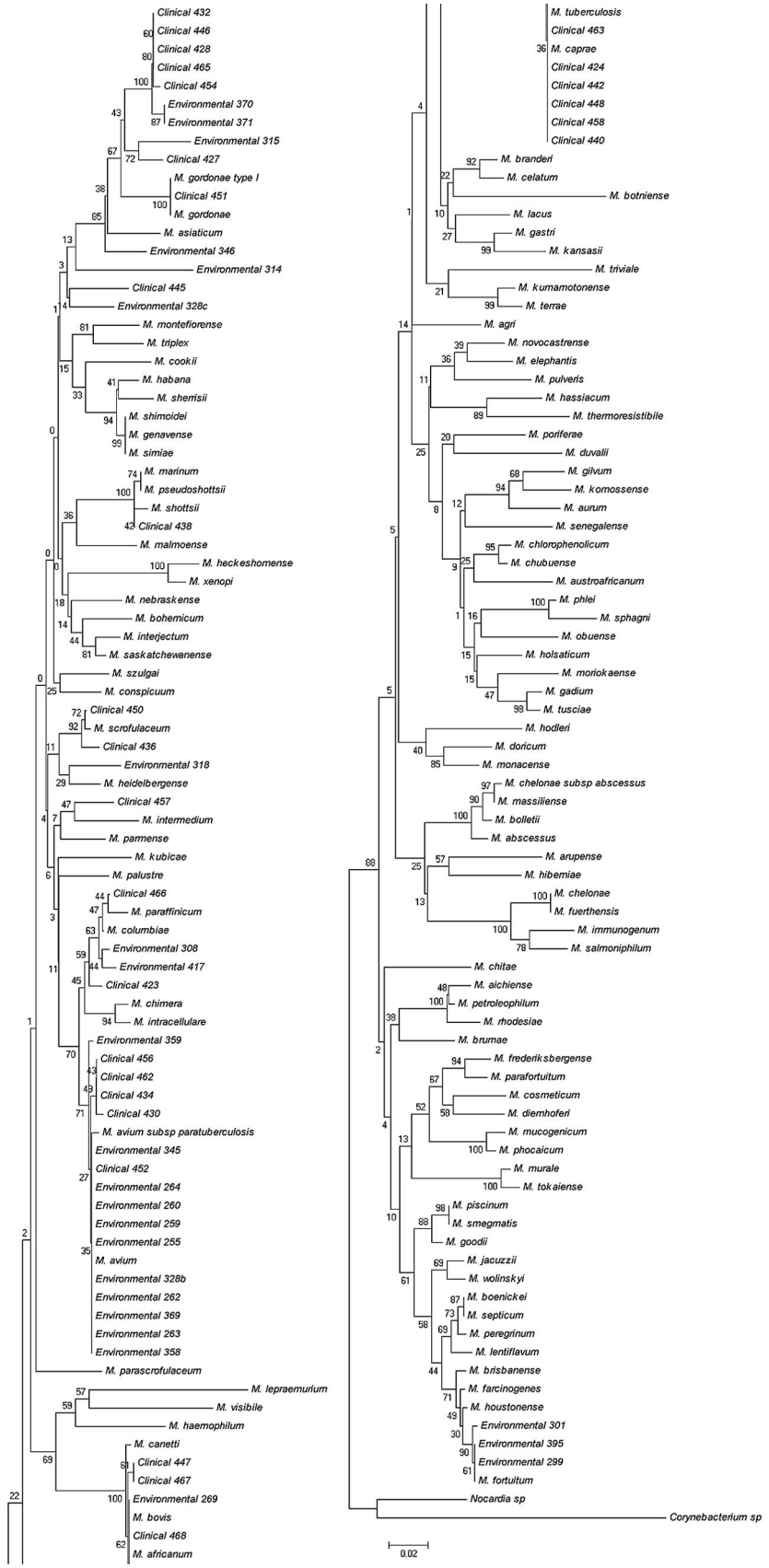

FIG. 1. Consensus tree for 200 bootstraps showing the phylogenetic relationships among environmental and clinical isolates from several places in the region surrounding Rio de Janeiro, Brazil, and sequences of known mycobacterial species. The tree is based on a comparison of a 368-bp sequence from the mycobacterial *hsp65* gene, using the neighbor-joining method. Bootstrap values of >70% are indicated. The distance between two strains is the sum of the branch lengths between them.

vious studies which correlate environmental parameters with the isolation of environmental mycobacteria were performed with acidic, organic, rich material and stagnant water reservoirs (1). However, we could see that infection by NTM can almost exclusively be associated with environmental mycobacteria that have adapted to humans. Our investigation provides evidence that *hsp65* sequencing has the potential of being an accurate, reliable, and effective means for identifying clinical and environmental *Mycobacterium* isolates. It has the advantages over biochemical test profiles of being rapid and trustworthy. Moreover, the results of sequencing can be used to correlate the specimens between themselves and to give support in their identification.

Our results show that the *hsp65* sequences from reference strains of mycobacteria provide a basis for determining systematic phylogenetic relationships. The phylogenetic analysis suggests that slow growth evolved recently in mycobacteria and, as discussed above, possibly has a great adaptive value (9). This study also shows the possibility that species correlate with each other and, moreover, the possible entry ports of mycobacteria in the artificial human environment. With regard to the information about DNA polymorphism obtained with the clinical and environmental isolates individually, we observed that it was greater in clinical than in environmental samples. This could be explained by an adaptation of the environmental species to the artificial human environment, probably through biofilms. It could also help us to understand why so many infections caused by mycobacteria have been reported recently. Currently, it is very important to understand associations between species of mycobacteria in Brazil because infections by these microorganisms have been increasing and causing outbreaks in hospitals, where the port of entry for infection is mainly surgery patients, and are becoming a serious and delicate problem for public health.

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