Original Article

Utilization of Molecular and Conventional Methods for the Identification of Nontuberculous Mycobacteria Isolated from Different Water Sources

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Abstract

Background: The environment is the nontuberculous mycobacteria (NTM) reservoir, opportunistic pathogens of great diversity and ubiquity, which is observed in the constant description of new species capable of causing infection. Since its introduction, molecular methods are essential for species identification. Most comparative studies between molecular and conventional methods, have used isolated strains from clinical samples. **Methods:** The aim of this study was to evaluate the usefulness of molecular methods, especially the *hsp65*-PRA (PCR-Restriction Enzyme Analysis), and biochemical tests in the identification of NTM recovered from water of different origins, using the sequencing of 16S rRNA and *hsp*65 genes as assessment methods of the previous ones. Species identification was performed for all 56 NTM isolates what were recovered from 32 (42.1%) positive water samples, using conventional phenotypic methods, *hsp65*-PRA, partial sequencing of 16S rRNA and sequencing of *hsp*65 genes. **Results:** Phenotypic evaluation and *hsp65*-PRA were concordant with 23 (41.1%) isolates. Also, the PRA was concordant with 16 (28.6%) and 27 (48.2%) isolates, with the partial sequencing of 16S rRNA and sequencing of *hsp*65 genes, respectively. It is considered that the 19.6% (n = 11) could not be identified. **Conclusion:** Identification of NTM environmental isolates to the species level, especially when they are pigmented and fast-growing, both the analysis of the restriction patterns obtained by PRA and the sequencing of the 16S rRNA and *hsp*65 genes are insufficient by themselves. Although they are demanding and time-consuming, biochemical tests are very useful to support data obtained by molecular methods.

Keywords: 16S rRNA sequencing, hsp65 sequencing, nontuberculous mycobacteria, restriction enzyme analysis-hsp65, phenotypic methods

Introduction

The infections caused by *Mycobacterium* species called environmental mycobacteria or nontuberculous mycobacteria (NTM) are considered to be emerging diseases worldwide.[1,2] In Argentina, although precise information is limited, infections are described in both immunocompromised and immunocompetent patients,[3,4] with pulmonary and extrapulmonary cases.[5] They also affect different species of farm and wild animals.[1]

NTM are characterized by being part of the environmental microflora, not being transmitted from person to person, and being resistant to common disinfectants, such as those used to make water drinkable.[1] Some NTM are of global distribution and other species possess geographically delimited habitats, depending on their ability to survive in different environmental conditions.[6] In our country, there are few studies about possible *Mycobacteria* species identified in the environment.[7] The ability of these microorganisms to form biofilm and to grow under oligotrophic conditions could explain their adaptation to new ecosystems closely related to humans, such as drinking water distribution systems.[1] Mycobacteria may aerosolize more readily than other bacteria as they have highly hydrophobic cell walls and inhalation of aerosols appears to be its primary transmission route. This

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usually occurs in artificial water environments, in which aerosolization increases the concentration of NTM in the air.[8]

There is currently a tendency to replace the biochemical typing of NTM by new faster and less complex molecular techniques,[9] of which several comparative studies are known,[10-12] made mainly with mycobacteria recovered from clinical samples. The sequencing of the 16S rRNA gene is universally considered to be the method of first choice and the sequencing of the *hsp*65 gene as the second best alternative,[13] although these are limited to specialized laboratories. Another molecular method is the polymerase chain reaction (PCR)-restriction enzyme analysis (PRA) that can be implemented in low-complexity laboratories.[14] Strains of the same species of NTM may have variants in restriction patterns or sequences not previously described.[15] In addition, the intra-species genetic variability is manifested in their virulence factors, the morphological variation of the colonies, and also whether it is recovered from the environment or from a host.[1] It is not less important that after the introduction of molecular methods, the number of NTM has been expanded and still some remain unclassified.[16] In this study, we expect to compare the phenotypic and molecular methods to be able to establish a work protocol that is more convenient for the identification of the different NTM species in our laboratory. We have identified NTM isolated from tap water, public fountains in General Pico (La Pampa, Argentina), and wetlands in its influence zone, by phenotypic testing and PRA, using the sequencing of the 16S rRNA and *hsp*65 genes as methods of assessment of the previous ones.

Methods

Sampling and decontamination of water samples

A total of 76 water samples were collected from General Pico city (65,438 inhabitants, 2012), from three different types of sources, namely, 32 from drinking water distribution systems, 32 from wetlands, and 12 from public fountains, from different sites representing each source. According to the standard methods, 500 ml of each sample was collected in sterile bottles; $Na_2S_2O_3$ was added to the tap water at a final concentration of 18 mg/l to neutralize up to 5 mg/l of residual chlorine.

Different decontamination methods were used considering the origin of the water sample, for samples of wetlands with high microorganism load, the method described in Fujimora Leite *et al*.,[17] and for the tap water and water from public fountains processing, the one of Engel.[18] It was inoculated in duplicate onto Löwenstein–Jensen, Stonebrink, and Herrold with mycobactin media,[19] and they were incubated at 25°C, 32°C, 37°C, and 42°C for 3 months.

Phenotypic characterization

The following phenotypic tests were performed on all acid-fast colonies according to the methodology described:[19] evaluation of pigment production and determination of development temperatures; growth in the presence of hydroxylamine; 5% NaCl; semi-quantitative catalase; catalase at 68°C, reduction of nitrates; urease; pyrazinamidase (at 4 days); arylsulfatase (3 days and 2 weeks); β-galactosidase, hydrolysis of Tween 80 (at 5 days and 10 days), iron uptake, tellurite reduction (in 7 days and 9 days), use of mannitol and citrate.

Polymerase chain reaction-restriction enzyme analysis

The methodology described by Telenti *et al*.[20] was used. One loopful of bacterial growth was suspended in 200 µl of sterile apyrogenic water contained in a capped tube of 1.5 ml and then incubated at 95°C in thermoblock (with agitation) for 40 min. It was then centrifuged 5 min at 12,000 rpm. Five microliters of the bacterial lysate obtained was added to each reaction tube. The mixture for *hsp*65 amplification consisted of buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, and 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmols of each primer, Tb11 (5'-ACCAACGATGGTGTGTCCAT) and Tb12 (5'-CTTGTCGAACCGCATACCCT), 5 µl annealing, and 1.25 U Taq Polymerase (Go Taq®, Promega Corp., USA) in a final volume of 50 µl. The reaction was subjected to an initial denaturation of 96°C for 3 min, followed by 45 cycles of amplification (1 min at 96°C, 1 min at 60°C, and 1 min at 72°C) and 7 min extension at 72°C. A PTC-100 thermocycler (MJ Research, Inc USA) was used. Subsequently, 10 µl of the PCR product was digested with each of the restriction enzymes *BstE*II and *Hae*III separately, in a reaction volume of 20 µl. Digestion with the *BstE*II enzyme was performed at 60°C while with *Hae*III at 37°C for 12 h. Separation of the resulting fragments from the enzymatic restriction was done in 4% agarose (Agarose 1000, Invitrogen Life Technologies) in 1X Buffer TAE, with ethidium bromide (0.5 µl/ml). Electrophoresis was performed at 100 volts for 4 h. As a molecular weight marker, Cincuenta Marker (Biodynamics, Argentina) was used. Visualization and photographic record were performed by exposing the gel in a UV-light transilluminator (Gel Doc, Bio-Rad). The size of the restriction fragments was determined using the BioNumerics program (Applied Maths, Belgium). Using the algorithm (http://app.chuv.ch/prasite/index.html),[20,21] we determined the mycobacterial species corresponding to the restriction patterns.

Sequencing of the *hsp*65 and 16S rRNA genes

Sequencing of *hsp*65 gene was performed using the same oligonucleotides described for PRA[20] (Tb11 and Tb12). For the sequencing of the 16S rRNA gene, a 1037 bp fragment was amplified using oligonucleotides 285 (5'GAGAGTTTGATCCTGGCTCAG3') and 264 (5'TGCACACAGGCCACAAGGGA3').[22] The reaction mixture was identical to that described for PRA and the amplification was performed on a PTC-100 thermocycler (MJ Research, Inc USA) with the following program: initial denaturation at 96°C for 3 min followed by 35 cycles of amplification (1 min at 96°C, 1 min at 55°C, and 2 min at 72°C) and 10 min extension at 72°C. For the sequencing, oligonucleotides 271 (5'CTTAACACATGCAAGTCGAAC3') and 259 (5'TTTCACGAACAACGCGACAA3')[22] were used. The obtained PCR products were purified using the "Illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare,

UK" kit, according to the manufacturer's specifications. The purified PCR products were quantified in a spectrophotometer at a wavelength of 260 nm (NanoDrop 2000, Thermo Scientific, USA). Sequencing was performed on a 16 capillary ABI3130xl sequencer (Applied Biosystems), using "Big Dye Terminator v3.1" (Cycle Sequencing Kit). The sequences obtained were compared with those deposited in the basic local alignment search tool database (http://blast. ncbi.nlm. nih.gov/Blast.cgi).

Statistical analysis used

Taking into account the partial sequencing of the 16S rRNA gene as a reference method, the data obtained with the other typing methods were classified as identified and unidentified. From there, Cohen's Kappa coefficient (k) was proposed as a measure of agreement. To measure the random error between two methods, we used the correlation coefficient (ρ). Systematic error was analyzed through marginal proportions. These proportions were compared by the McNemar test.

Results

A total of 56 NTM isolates were recovered from 32 (42.1%) positive water samples; 34 isolates corresponded to samples from wetlands ($n = 17$, 53.1% positive samples), 11 from public fountains ($n = 4$, 33.3% positive samples), and 11 from drinking water distribution systems ($n = 11$, 34.4% positive samples). All the isolates were studied using biochemical tests.[18,23] Initially, slow-growing isolates ($n = 13$, 33.9%) and/or nonpigmented ($n = 16$, 28.6%) were identified but not most fast-growing and pigmented colonies ($n = 30$, 53.6%). Further, the data obtained by conventional methods for 26.8% ($n = 15$) of the strains did not coincide with any species proposed by the molecular techniques performed. The identification of the isolates by PRA began with the analysis of the restriction patterns, followed by the comparison of results obtained by biochemical and phenotype analysis. The independent use of each method did not allow for the accurate identification of species. The same species with both methods was identified in 41.1% ($n = 23$) of the strains recovered from the environmental samples. The Kappa coefficient (k) obtained indicates a weak degree of agreement between both methods ($k = 0.374$). The correlation coefficient (ρ) was 0.448. The difference is significant according to McNemar test ($P < 0.001$). The biochemical tests showed a higher frequency of strains identified with respect to PRA. The results obtained from the partial sequencing of the 16S rRNA gene are shown in Table 1. 58.93% ($n = 33$) of the NTM had a sequence homology range of 99%–100%, which were identified at species level since they corresponded with some of the other methods used. In the case of strains 76, 78E, 87B, and 88B, although they had a lower percentage of identity (between 97 at <99%), they could be identified with the use of two or more methods. However, isolates 5, 77B, 77C, 78A, 80A, 80C, 89B, and 89C, with homology between 99% and 100%, did not show any coincidence with the other methods used.

The percentages of identity of *hsp*65 gene sequences were lower than those obtained by 16S rRNA gene sequencing [Table 1]; a considerable number of isolates ($n = 25$) showed ≤97% homology although this did not mean any identification at the species level. In the particular case of *Mycobacterium vaccae* strains, values as low as 93%–94% of similarity were found.

Compared with the other molecular methods, PRA shows a higher percentage of results consistent with the *hsp*65 gene sequencing (48.2%) [Table 2]. A value of 0.307 was obtained for Cohen's Kappa coefficient indicating low agreement between these two methods and value of $\rho = 0.343$ was obtained. The McNemar test showed that this result was significantly different ($P < 0.01$). The *hsp*65-sequencing method had a higher frequency of identified strains. When analyzing the data obtained by biochemical tests with the molecular data, there is a greater coincidence with the sequencing methods than with the PRA. When the concordance between the phenotypic methods and the sequencing of the *hsp*65 gene was evaluated, a value of 0.475 was obtained for Cohen's Kappa coefficient, indicating a moderate agreement among them. In this case, the correlation coefficient was $\rho = 0.484$. The McNemar test showed that the results of conventional methods were not significantly different from the results obtained by *hsp*65 sequencing ($P = 0.267$) with 95% confidence.

The use of different molecular techniques for NTM typing would show the possible presence of nondescribed restriction patterns in the prasite that would belong to new allelic variants within the *hsp*65 gene (data not shown).

Biodiversity found in tap water, wetlands, and public fountains is shown in Figures 1-3. It is considered that 19.6% of isolated strains could not be identified since there was no concordance between any of the methods used. At the same time, 46.9% ($n = 15$) of the pigmented and fast-growing strains studied did not present definite results in their identification by the methods used.

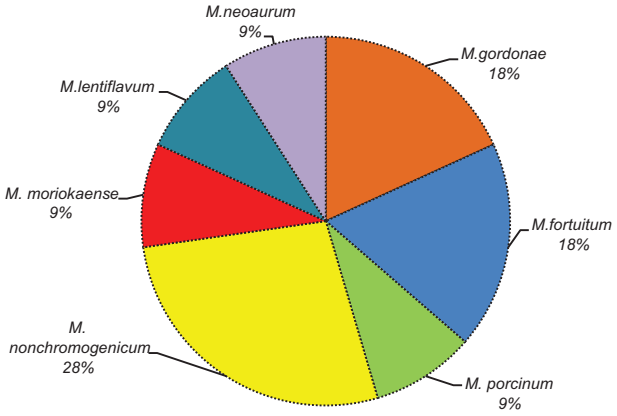

Figure 1: Species distribution of mycobacteria isolates in tap water samples based on16S rRNA sequencing and/or *hsp65*-sequencing

Table 1: Identification nontuberculous mycobacteria isolated from tap water, wetlands and public fountains by polymerase chain reaction-restriction enzyme analysis, 16S rRNA sequencing, *hsp65* sequencing and biochemical test

Source	ID number	hsp65 PRA	16S rRNA sequencing (percentage similarity)	*hsp65* sequencing (percentage similarity)	Biochemical tests
Tap water	1	Not amplified	*M. fortuitum* (100)	SPQ	*M. fortuitum*
	5	*M. neoaurum* Type 1/*M. parafortuitum* Type 2	*M. fluoranthenivorans* (99)	*M. lacticola*/*M. neoaurum* (96)	*M. neoaurum*
	6	*M. florentinum* Type 1/*M. lentiflavum* Type 1	*M. lentiflavum* (99)	*M. lentiflavum* (99)	*Mycobacterium spp.*
	54	*M. gordonae* Type 2	*M. gordonae* (100)	*M. gordonae* (100)	*M. gordonae*
	34	*M. gordonae* Type 9	SPQ	*M. gordonae* (100)	*M. gordonae*
	69	*M. fortuitum* Type 2	*M. nonchromogenicum* (100)	*M. terrae*/*M. nonchromogenicum* (99/97)	*M. nonchromogenicum*
	73		*M. nonchromogenicum* (99)		
	76		*M. nonchromogenicum* (98)		
	71	*M. porcinum* Type 1/*M. peregrinum* Type 2/*M. septicum* Type 1	*M. porcinum*/*M. fortuitum* (100)	*M. brisbanense*/*M. peregrinum*/*M. septicum*/*M. porcinum*/*M. fortuitum* (98)	*M. porcinum*
	74				*M. fortuitum*/*M. peregrinum*
	75	*M. brumae* Type 1	*M. moriokaense* (100)	*M. moriokaense* (97)	*Mycobacterium spp.*
PF	87A	*M. peregrinum* Type 1	*M. peregrinum*/*M. septicum* (100)	*M. peregrinum*/*M. porcinum*/*M. septicum* (99)	*M. peregrinum*
	87B	*M. confluentis* Type 1	*M. parafortuitum* (97)	*M. iranicum*/*M. parafortuitum* (97/95)	*M. parafortuitum*
	88A	*M. moriokaense* Type 1	*M. aurum* (99)	SPQ	*M. aurum*
	88B	*M. kumamotonense* Type 1	*M. arupense* (97)	*M. arupense* (100)	*M. arupense*
	88C		*M. arupense* (100)	*M. arupense* (99)	
	88D	*M. asiaticum* Type 1	*M. asiaticum* (99)	*M. asiaticum* (95)	*M. asiaticum*
	89A	*M. peregrinum* Type 3	*M. peregrinum*/*M. septicum* (100)	*M. peregrinum* (99)	*M. peregrinum*
	89B	*M. parascrofulaceum* Type 3	*M. austroafricanum* (99)	*M. rutilum*/*M. chubuense*/*M. novocastrense* (96)	*M. chubuense*/*M. clorophenolicum*
	89C				
	90A	*M. bohemicum* Type 1	*M. gordonae* (100)	*M. gordonae* (98)	*M. gordonae*
	90B	*M. fortuitum* Type 2	*M. fortuitum* (99)	*M. setense*/*M. fortuitum* (99/98)	*M. fortuitum*
AR	29A	*M. vaccae* Type 1	*M. vaccae* (99)	SPQ	*M. vaccae*
	29B	*M. komassense* Type 1/*M. parafortuitum* Type 1	*M. farcinogenes*/*M. senegalense*/*M. mucogenicum* (98)	SPQ	*Mycobacterium* spp.*
	83C			*Mycobacterium* spp. (98)	
	83D				
	86				
	30	*M. vaccae* Tipo 1	*M. vaccae* (99)	*M. aurum* (96)	*M. vaccae*
	44A	*M. gordonae* Type 3	*M. gordonae* (100)	*M. gordonae* (98)	*M. gordonae*
	44B	*M. gordonae* Type 9	*M. gordonae* (100)	SPQ	*M. gordonae*
	47A	*M. flavescens* Type 3/*M. vaccae* Type 1	*M. vaccae* (99)	*M. aurum*/*M. flavescens*; *M. vaccae* (96/94)	*M. vaccae*
	47B				
	83A	*M. aurum* Type 2	*M. aurum* (100)	*M. aurum* (99)	*M. aurum*
RRU	24	*M. duvalii* Type 1	*M. duvalii* (100)	*M. duvalii* (99)	*M. duvalii*
	28	*M. poriferae* Type 1	SPQ	*M. poriferae* (95)	*M. poriferae*
	48B	*M. vaccae* Type 1	SPQ	*M. gilvum*/*M. vacae* (96/93)	*M. vaccae*
	48C	*M. gilvum* Type 1	*M. vaccae* (100)	SPQ	*M. vaccae*
	52B	*M. vaccae* Type 1	*M. aurum* (100)	*M. aurum* (96)	*M. aurum*
	53A	*M. senegalense* Type 2	*M. duvalii* (100)	*M. duvalii* (99)	*M. duvalii*
	53B	*M. vaccae* Type 1	*M. aurum* (100)	SPQ	*M. aurum*
	77A	*M. vaccae* Type 1	*M. aurum* (100)	*M. aurum*; *M. komossense*/*M. vaccae* (97/94)	*M. aurum*
	77B	*M. parafortuitum* Type 1/*M. komossense* Type 1	*M. duvalii* (99)	*M. komossense*/*M. vaccae*/*M. novocastrense* (96)	*Mycobacterium* spp.*
	77C				
	78A				
	80C				

Contd...

Table 1: Contd...

Source	ID number	hsp65 PRA	16S rRNA sequencing (percentage similarity)	*hsp65* sequencing (percentage similarity)	Biochemical tests
	77D	*M. vaccae* Type 1	SPQ	SPQ	*M. aurum*
	78B 78G	*M. simiae* Type 2	*M. vaccae*/*M. flavescens*/*M. duvalii*/*M. acapulcensis* (98)	*M. austroafricanum* (96)	*Mycobacterium* spp.*
	78C	*M. gilvum* Type 1	*M. duvalii* (100)	*M. duvalii* (99)	*Mycobacterium* spp.
	78E	*M. arupense* Type 1	*M. arupense* (98)	*M. arupense* (100)	*M. arupense*
	78F		*M. terrae* (low identity) (84)		
	79A 80B	*M. novocastrense* Type 1	*M. vaccae* (100)	*M. vaccae* (93)	*M. vaccae*
	79B	*M. porcinum* Type 1/*M. peregrinum* Type 2/*M. septicum* Type 1	*M. fortuitum* (100)	*M. peregrinum*/*M. porcinum*/*M. septicum* (98)	*M. fortuitum*/*M. peregrinum*
	80A	*M. lentiflavum* Type 1/*M. florentinum* Type 1/*M. simiae* Type 5	*M. intracellulare*/*M. colombiense* (99)	*M. lentiflavum* (97)	*Mycobacterium* spp.
	80D	*M. gordonae* Type 4	SPQ	*M. gordonae* (96)	*M. gordonae*

Sequencing was repeated two or three times. *All yielding identical phenotypical. The strains with equal numbers (ID number) mean that were recovered from a same sample of water. *M. neoaurum*: *Mycobacterium neoaurum*, *M. parafortuitum*: *Mycobacterium parafortuitum*, *M. fluoranthenivorans*: *Mycobacterium fluoranthenivorans*, *M. fortuitum*: *Mycobacterium fortuitum*, *M. lacticola*: *Mycobacterium lacticola*, *M. florentinum*: *Mycobacterium florentinum*, *M. lentiflavum*: *Mycobacterium lentiflavum*, *M. gordonae*: *Mycobacterium gordonae*, *M. nonchromogenicum*: *Mycobacterium nonchromogenicum*, *M. terrae*: *Mycobacterium terrae*, *M. porcinum*: *Mycobacterium porcinum*, *M. peregrinum*: *Mycobacterium peregrinum*, *M. septicum*: *Mycobacterium septicum*, *M. brisbanense*: *Mycobacterium brisbanense*, *M. brumae*: *Mycobacterium brumae*, *M. moriokaense*: *Mycobacterium moriokaense*, *M. confluentis*: *Mycobacterium confluentis*, *M. iranicum*: *Mycobacterium iranicum*, *M. aurum*: *Mycobacterium aurum*, *M. kumamotonense*: *Mycobacterium kumamotonense*, *M. arupense*: *Mycobacterium arupense*, *M. asiaticum*: *Mycobacterium asiaticum*, *M. parascrofulaceum*: *Mycobacterium parascrofulaceum*, *M. austroafricanum*: *Mycobacterium austroafricanum*, *M. rutilum*: *Mycobacterium rutilum*, *M. chubuense*: *Mycobacterium chubuense*, *M. clorophenolicum*: *Mycobacterium clorophenolicum*, *M. bohemicum*: *Mycobacterium bohemicum*, *M. vaccae*: *Mycobacterium vaccae*, *M. komassense*: *Mycobacterium komassense*, *M. farcinogenes*: *Mycobacterium farcinogenes*, *M. senegalense*: *Mycobacterium senegalense*, *M. setense*: *Mycobacterium setense*, *M. mucogenicum*: *Mycobacterium mucogenicum*, *M. flavescens*: *Mycobacterium flavescens*, *M. duvalii*: *Mycobacterium duvalii*, *M. poriferae*: *Mycobacterium poriferae*, *M. gilvum*: *Mycobacterium gilvum*, *M. komossense*: *Mycobacterium komossense*, *M. novocastrense*: *Mycobacterium novocastrense*, *M. simiae*: *Mycobacterium simiae*, *M. acapulcensis*: *Mycobacterium acapulcensis*, *M. intracellulare*: *Mycobacterium intracellulare*, *M. colombiense*: *Mycobacterium colombiense*, SPQ: Sequence poor quality, RRU: Wetland "recycling urban waste", AR: Wetland "The Arocena", PF: public funtains

Table 2: Summary of concordance among species identification results obtained by sequencing methods, polymerase chain reaction-restriction enzyme analysis-*hsp65* and phenotypic tests

Method	*n*	Phenotypic methods		*hsp65* PRA	
		Concordant (%)	Discordant	Concordant (%)	Discordant
16S rRNA sequencing					
Identified	50	31	19	16	34
Not identified	6	-	6	-	6
Total	56	31 (55.3)	25	16 (28.6)	40
hsp65 sequencing					
Identified	45	33	12	26	19
Not identified	11	4	7	1	10
Total	56	37 (66.1)	19	27 (48.2)	29

PRA: PCR-restriction enzyme analysis, PCR: Polymerase chain reaction

Discussion

The differentiation of the species of the genus *Mycobacterium* has conventionally been done through the use of biochemical test profiles, being a very laborious methodology that requires a considerable time to be able to emit a result.[21] According to the included studies in the distribution of NTM species from environmental and clinical samples in the Middle East over the last 30 years, identification of NTM by conventional techniques was the most frequently used method.[2] As described by other authors,[21,23] standard biochemical identification schemes can provide both ambiguous and erroneous results as some of the tests used are not highly reproducible. On the other hand, the phenotype of a species may exhibit remarkable variability depending on the origin of the sample either clinical and/or environmental.[1] It should also be considered that the available documented data of the phenotypic characteristics are limited to the common species, whereas in other less frequent species, all the determinations are not standardized, making the precise identification through this method not possible due to the increasing number of *Mycobacterium* species with overlapping phenotypic characteristics.[21,24] We observed some

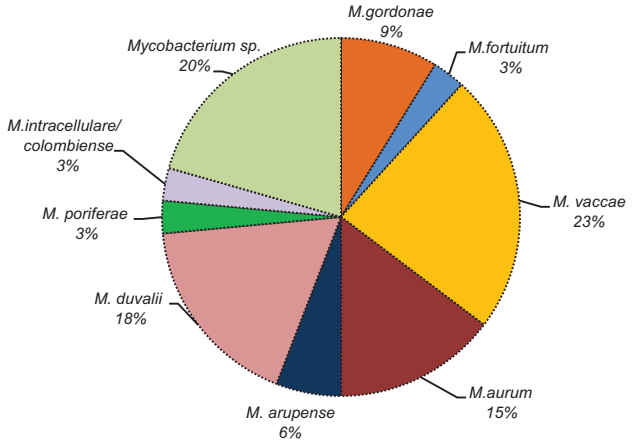

Figure 2: Species distribution of mycobacteria isolated in wetlands waters based on 16S rRNA sequencing and/or *hsp65*-sequencing

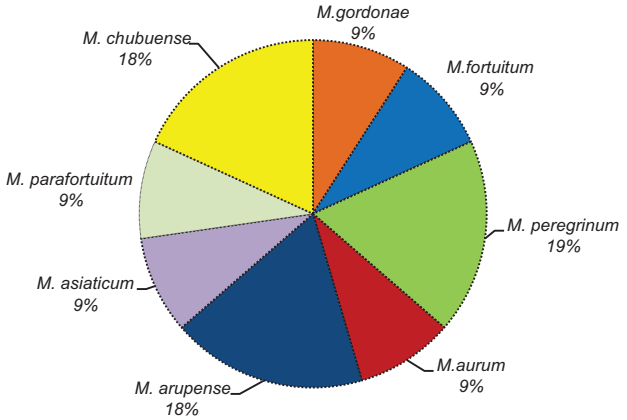

Figure 3: Species distribution of mycobacteria isolated in water of public fountains based on 16S rRNA sequencing and/or *hsp65*-sequencing

intrinsic variables of the culture that may influence the results of biochemical typing such as inoculum size, incubation time, temperature, and composition of the medium of culture. The biochemical tests must be carried out meticulously and strictly according to the described methodology.

Considering the known difficulties of conventional techniques and the increased incidence of mycobacteriosis over the last decades, nucleic acid sequence identification procedures and commercially available systems have been developed, such as AccuProbe System (Gen-Probe, San Diego, CA) and INNO LiPA Mycobacteria v2 (Fujirebio Europe, Ghent, Belgium). These are relevant methods for laboratory diagnosis, with the disadvantage of characterizing a limited number of species, some probes being nonspecific, several cross-reactions being observed.[25] Other commercial DNA kits were not valid options to solve the problem of specimens that elude NTM species identification in Argentina.[26] The PRA is a rapid method that constitutes a valuable diagnostic tool considered as a test of orientation and support to the identification in the laboratory of mycobacteria.[27] However, its sensitivity and specificity are influenced by a large number of variables or critical points, such as the quality of the agarose for gel production, conditions of electrophoresis, estimation of molecular weights of restriction fragments, and interpretation of patterns.[28] When comparing the results obtained by the PRA method and the biochemical tests, we determined a concordance of 41.1%, while other studies showed a concordance of 74%,[10] 82%,[11] and 95.3%,[27] all cases dealing with strains isolated from clinical samples. On the other hand, the PRA showed higher concordant results with the sequencing of the *hsp*65 gene (48.2%) than with the partial sequencing of 16S rRNA (28.6%). This is reasonable since PRA and *hsp*65-sequencing are based on the same gene. Different authors obtained better results with the PRA method since they correctly identified 90.3%[10] and 96%[11] of the clinical isolates. Other researchers analyzed NTM isolated from 192 patients, and only 30% of NTM strains were correctly identified by the PRA compared to the sequencing of the *hsp*65 gene, although the suggested inclusion of an additional restriction enzyme (Sm1I) increased resolution in approximately 90%.[9]

At the same time, 55.3% and 66.1% of the isolates were identified with the biochemical tests in correspondence with the sequencing of the 16S rRNA gene and the *hsp*65-sequencing, respectively; these values being higher than those obtained with the PRA. These data differ from those described by other authors, as although they described that biochemical tests identified 77.9%[10] and 92%[11] of the studied strains, these percentages were relatively lower than those of PRA.

Studies conducted by da Silva Rocha *et al*.[15] in clinical isolates from 16 Brazilian states determined the presence of 10% of allelic variants not previously described. They included new patterns for some species such as *Mycobacterium scrofulaceum*, *Mycobacterium intracellulare*, *Mycobacterium flavescens*, *Mycobacterium fortuitum*, *Mycobacterium gordonae*, and *Mycobacterium terrae*, while other strains could not be identified, demonstrating the great diversity and biogeographic distribution of mycobacterial genotypes. Other researchers who studied NTM isolated from natural and treated waters from a zoological garden in São Paulo found only 19% of the isolates with defined PRA patterns.[29]

We observed that even when there is a 100% homology with the 16S rRNA gene sequence in the database, the correct identification of the strains with the PRA is low (36.8%). Since these strains are isolated from the environment and from geographic locations that have never been studied before, the presence of already known species with new PRA pattern is possible and even of new species.

There is some background where suspected strains with possible new patterns of PRA were actually species of the genus *Nocardia*.[30] The PRA technique is not used exclusively to differentiate *Mycobacterium* species, and this possible interference is due to the fact that both the identification of *Mycobacterium* and *Nocardia* species by PRA use the same primers described by Telenti *et al*.[20] for amplification of the *hsp*65 gene. As *Nocardia* lacks the BstEII restriction site, this

feature can be used in the presumptive identification of the genus.[31] In our study, all the studied isolates corresponded to the genus *Mycobacterium*.

According to da Silva Rocha *et al*.,[15] the consequences of using PRA as a unique identification procedure will depend on how much the frequencies of NTM genotypes are known in the region of interest and an additional molecular and phenotypic method will be required for each case. Some researchers[28,32] agree that the PRA technique is not suitable for identifying new or rarely observed species and it is necessary to resort to 16S rRNA sequencing and to the analysis of mycolic acids by HPLC for definitive identification. It should be borne in mind that sequencing of the 16S rRNA gene is useful for the identification of all species, except to differentiate the species officially recognized as distinct which are characterized by genetic similarities >99% with one or more species of the genus.[14] In some of these cases, the PRA is useful for its differentiation.[22]

On the other hand, the nucleotide sequences obtained from the *hsp*65 gene had a lower percentage of similarity than those found in the 16S rRNA sequenced fragments, even though the results were concordant. A similar problem has been reported previously.[16,33] In addition, in a study using clinical isolates, the percent similarities ranged from 96.57% to 100% for the 16S rRNA gene, 89.27% to 100% for *hsp*65, and 92.71% to 100% for *rpo*B.[34] We observed that while the probability of correct identification is low when the percentages of similarity are <99%,[12] the simultaneously use of several molecular techniques as the conventional methods increase this probability. Other researchers say that multiple, but not single, gene analysis is the approach of choice; however, this does not guarantee identification to the species level in every case.[8,26,34]

Biochemical tests correctly identified 87.50% of the isolates when the degree of identity in the partial sequence of the 16S rRNA gene was 100%. However, it is important that the phenotypic typing required the complementarity of this technique for the correct identification.

As for the diversity of NTM in the drinking water network, fountains of the General Pico city and in the wetlands of the influence area, it was similar to the one described in the bibliography.[1,9,14,35,36]

Conclusion

Both the analysis of the restriction patterns obtained by PRA and the sequencing of the 16S rRNA and/or *hsp*65 genes alone are insufficient for the precise identification of NTM, specifically when it is desired to identify mycobacteria from the environment, even more so when they are pigmented fast-growing isolates. Biochemical tests are very useful to support the data obtained by molecular techniques. The results of this work suggest that for the correct typification of NTM isolated from environmental samples, the PRA technique must be accompanied by sequencing methods and phenotypic methods. The diversity of these highly ubiquitous microorganisms leads to the constant evaluation of the usefulness of the typing methods in different contexts.

Some of the species recovered from the water samples studied have been described in cases of mycobacteriosis in Argentina, such as *M. gordonae*, *M. intracellulare*, *M. fortuitum*, *M. vaccae*, *Mycobacterium lentiflavum*, and *Mycobacterium nonchromogenicum*. These results suggest that water is a great source of NTM, which means that continuous monitoring should take place, to study the potential risk that the identified species would mean for human health.

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Conflicts of interest

There are no conflicts of interest.

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