Original Article

Prevalence and Species Diversity of Nontuberculous Mycobacteria in Drinking Water Supply System of Bahía Blanca City, Argentina

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Abstract

Background: There is evidence that tap water is the vehicle through which nontuberculous mycobacteria (NTM) infect or colonize the human body. The objective of this study was to determine the presence and diversity of NTM in the water distribution system of Bahía Blanca city, Argentina (sites S2/S3) and in the dike that supplies water to it (S1). **Methods:** Culture-dependent method, biochemical tests, and molecular method (16S rRNA sequencing gene) were combined to detect and identify NTM. **Results:** NTM were isolated in 51.6% (64/124) of all the samples analyzed. *Mycobacterium gordonae* was the most frequently isolated organism (15/64) in all samples analyzed, followed by *Mycobacterium peregrinum* and *Mycobacterium frederiksbergense*. Significant differences were found in the residual chlorine values between sampling S2 and S3. In both sites, maximum counts were recorded but they did not correlate with low chlorine values. A concentration higher than 500 colony-forming unit/L of NTM was never found, which can be attributed to the negative effect caused by decontamination methods being a point to consider for the recovery of NTM. In 46.9% (30/64) of samples, both methods coincided in the identification, and the obtained sequences presented ≥99% identity. Identification at the species level was achieved in 50% (32/64) of the isolates. Nearly 17.2% (11/64) of the isolates showed a similarity <99%. **Conclusions:** It should be taken into account that sequencing of the 16S rRNA gene and biochemical tests are useful for the identification of several species, but it is necessary to incorporate other genes (*hsp*65 and *rpo*B) to obtain accurate identification.

Keywords: Drinking water distribution system, identification methods, isolation methods, nontuberculous mycobacteria

Introduction

Nontuberculous mycobacteria (NTM) are common saprophytes in all natural ecosystems, including water, soil, food, dust, and aerosols. Under certain circumstances, for example, immune dysfunctions and chronic diseases, these NTM may cause disease.[1-3] There is evidence which suggests that tap water is the vehicle by which NTM infect or colonize the human body.[4-6] The NTM are considered as normal inhabitants of the water distribution systems and not as contaminants of them because they can survive, grow, and persist there.[5] According to numerous authors,[7-9] NTM are oligotrophs, and effective competitors in environments with low nutrient content and disinfected, such as drinking water. Mycobacteria are hydrophobic and may be important agents accelerating biofilm formation,[10,11] so they are not washed out, in spite of their slow growth. NTM are considered the dominant group within the biofilms of drinking water pipes, probably selected for the presence of a residual disinfectant.[12] For the recovery of NTM from the environment, it is necessary to use decontamination methods that reduce the microbiota that accompanies it. The complex wall of the mycobacteria allows them to resist alkalis and strong acids, which allows the use of these to decontaminate the sample. There are several proposed methods for the decontamination of samples,[13] but

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while they improve the recovery of NTM, they also reduce their number by approximately 30%.[14]

The identification of species of the genus *Mycobacterium* through biochemical tests is an old and laborious method, which in many cases leads to ambiguous results, and the database is limited to the species most commonly found. The tendency now is to replace biochemical identification with molecular methods. The molecular reference method is the sequencing of a fragment of the 16S ribosomal RNA gene (16S rRNA). It has variable sequences, whose changes have phylogenetic significance and are specific at the species level.[15] Other used targets are *hsp*65 and 16S-23S internal transcribed spacer, *rpo*B, gyrA/B, *dna*J, *sod*A, *sec*A1, and *rec*A.[16,17]

The aim of this study was to determine the presence and diversity of NTM in water distribution system of Bahía Blanca city, Argentina, and in the dike that supplies water to it.

Methods

Sampling sites

Three sampling sites were established. S1: Paso de las Piedras dike, which is the main source of water supply for the city. This artificial lake comprises an area of 4000 hectares, and its maximum depth is 28 m. S2: Area near the water treatment plant (0.5 km), the only chlorination site in the city, and S3: Distant area of the plant (7 km). The collection of the samples in S2 and S3 was carried out in private houses located in the same block. Samples S2 and S3 were collected monthly during the extreme climatic seasons, summer and winter (summer 2012/2013 to winter 2015). In S1, three sampling points were established to obtain information in different areas of the dike. Samples were collected during summer 2013/2014 and winter 2016.

Recollection and decontamination methods of water samples

A total of 124 samples were analyzed: 60 from S1, 32 from S2, and 32 from S3.

Samples from tap water were decontaminated according to Engel method recommended by Kamala *et al*.[13] One liter of water was passed through a membrane filter (pore size: 0.45 μm; Millipore). The filter was placed in a container with 5 mL of sterile distilled water (SDW) and 5-mm diameter glass beads and incubated on a rotary shaker at 37°C and 200 rpm (New Brunswick Scientific Co., Inc., Endfield, CT, United States) for 1 h. Subsequently, decontamination was carried out using equal amounts of 1% NaOH and 3% sodium dodecyl sulfate for 10 min at room temperature. The contents were then transferred to a conical tube, centrifuged at 3500 rpm for 15 min, the supernatant was discarded, and the same volume was replaced by SDW and washed three times.

The samples from the dike were decontaminated according to the modified Schulze–Röbbecke technique.[18] Cetylpyridinium chloride (CPC) was added to the samples to give a final concentration of 0.05% (wt/vol), and the suspensions were shaken for 30 s. After an exposure time of 30 min, the samples were filtered (cellulose acetate membrane filters diameter, 50 mm; pore size, 0.45 mm) and rinsed with 100 mL of SDW to remove residual CPC. The filter was scraped off with 3 ml of SDW, centrifuged, and the supernatant was discarded.

Nontuberculous mycobacteria isolation

After decontamination, the samples were seeded in Löwenstein–Jensen culture media with and without mycobactin, Stonebrink, Middlebrook 7H10, and Herrold's egg yolk medium with and without mycobactin. Herrold's egg yolk medium with antibiotics (amphotericin B, vancomycin, and nalidixic acid) was used only in the samples from the dike. All tubes were incubated at 25°C, 32°C, and 42°C in the presence of light and in darkness for 3 months.[19] The colonies compatible with NTM were stained using the Ziehl–Neelsen technique.[20]

Identification of nontuberculous mycobacteria

Phenotypic characterization

The following biochemical and phenotypic tests were performed on the isolated strains according to the methodology described by the Mycobacteria Diagnostic Manual of importance in Veterinary Medicine (Scientific Commission of Mycobacteria,[21] and in the Manual of Procedures from SENASA):[22] growth temperature (25°C, 32°C, and 42°C); pigment production; growth in the presence of isoniazid (1 mg/L) and hydroxylamine (500 mg/L), growth on 5% NaCl, qualitative and semi-quantitative catalase, catalase at 68°C, nitrate reduction, urea hydrolysis, pyrazinamidase, arylsulfatase activity (3, 7, and 10 days), β-galactosidase, Tween 80 hydrolysis in 5 and 10 days, iron uptake, tellurite reduction (3 and 9 days), and utilization of carbon sources namely mannitol, citrate, and inositol. To complete the biochemical typing, they were incorporated resistance to doxycycline (16 mg/mL), growth in xylose and sorbitol, and growth in MacConkey agar at 28°C and 37°C, as described in Volume V of Bergey's Manual of Systematic Bacteriology 2nd edition.[23]

Sequencing of a 16S rRNA gene

The methodology described by Kirschner and Böttger[24] was used.

DNA extraction by rapid lysis: An annealed bacterium was suspended from colonies isolated by culture, in 200 μL of sterile apyrogenic water free of DNases contained in a 1.5-mL capped tube, incubated at 95°C in thermoblock (with agitation) for 40 min. Then, it was centrifuged for 5 min at 12,000 rpm, and 10 μL of the supernatant was used as template for polymerase chain reaction (PCR).

Amplification of the 16S rRNA gene: A 1037 bp fragment was amplified using primers 285 (5'GAGAGTTTGATCCTGGCTCAG3') and 264 (5'TGCACACAGGCCACAAGGGA 3').[24] The reaction

mixture consisted of the following: 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 pmol of each primer, 10 µL of template, and 1.25 U of Taq Polymerase (Go Taq®, Promega Corp., USA), in a final volume of 50 µL. The amplification was carried out in a PTC-100 thermal cycler (MJ Research, Inc., USA) with the following program: initial denaturation of 96°C 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. The amplification was evaluated by electrophoresis in 1% agarose gel.

Purification of the PCR products: The obtained PCR products were purified by using the "Illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare, UK" kit, following the manufacturer's specifications.

Quantification: The purified PCR products were quantified in a spectrophotometer at a wavelength of 260 nm (Nanodrop 2000, Thermo Scientific™, Thermo Fisher Scientific, USA).

Sequencing: Oligonucleotides 271 (5'CTTAACAC ATGCAAGTCGAAC 3') and 259 (5'TTTCACGAA CAACGCGACAA 3') were used for sequencing.[24] It was performed in a 16-capillary sequencer ABI3130xl (Applied Biosystems, Thermo Fisher Scientific, USA), using "Big Dye Terminator v3.1" (Cycle Sequencing Kit), in the Genomics Unit of the Institute of Biotechnology, of Institute of Agrobiotechnology and Molecular Biology, of CONICE(INTA). Sequence analysis: The sequences obtained were compared with those included in the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

Sequencing of a secA1 gene

The methodology described by Zelazny *et al*.[25] was used. Briefly, mycobacterial DNA was extracted as described in sequencing of a 16S rRNA gene section. PCR was performed using the following primers: secA1 – forward (5′-GACAGYGAGTGGATGGGYCGSGTGCACCG-3′) and secA1 – reverse (5′-ACCACGCCCAGC TTGTAGATCTCGTGCAGCTC-3′). The PCR thermocycling program consisted of 10 min at 95°C and then 49 cycles of 1 min at 95°C, 1 min at 65°C, 1 min at 72°C, and a final incubation step of 10 min at 72°C. A negative control of ultrapure water was included with every amplification reaction mixture.

Physicochemical and microbiological parameters of water quality

All water samples were assayed bacteriologically for: *Pseudomonas aeruginosa* (membrane- filtration method), total viable count (poured plate counting method) and total coliform (most probable number). Similarly, pH and temperature *in situ* were recorded. In addition, residual-free chlorine was determined in drinking water samples.

Statistical analysis

Two-way ANOVA to determine the chlorine differences between the sampling sites and seasons was used. To determine differences between NTM counts between sites S2 and S3, one-way ANOVA was used.[26]

InfoStat version 2017, Grupo InfoStat, FCA, National University of Córdoba, Argentina, was used for statistical analysis. P <0.05 was used in data analysis.

Results

NTM were isolated in 51.6% of all samples analyzed (64/124). The recovery of NTM in S1, water from the dike, was 25% (15/60); in S2, near to the water treatment plant, 71.9% (23/32); and in S3, far from the plant, 81.25% (26/32). Of the total isolates, 61% corresponded to fast-growing species (39/64) and 39% to slow-growing species (25/64); 65.6% (42/64) were scotochromogenic, 31.3% (20/64) were nonchromogenic, and 3.1% (2/64) were photochromogenic species.

Figure 1 shows species diversity of NTM isolated in tap water and in the dike (raw source water). The results of S1 include the three points sampled from that area. *Mycobacterium gordonae* was the most frequently isolated organism (15/64) in all the analyzed samples, followed by *Mycobacterium peregrinum* and *Mycobacterium frederiksbergense*. Most of the isolated species were harmless saprophytes, but *Mycobacterium fortuitum* is an opportunistic pathogen that is causing increasing concern.[27,28] All tap water samples met the microbiological requirements established by the argentine food code (AFC) for drinking water. The pH values of the water ranged between 6.9 and 7.0, and the residual chlorine values varied according to the sampling site. The mean recorded in S2 was 1.06 mg/L with a minimum value of 0.3 and a maximum of 2.3; the mean in S3 was 0.68 mg/L with minimum and maximum values of 0.1 and 1.5 mg/L, respectively. A single sample had a chlorine value lower than that established by the AFC, which is 0.2 mg/L. The statistical analyses found significant differences in residual chlorine values between sampling sites S2 and S3 (P = 0.0006), but not between the seasons (P = 0.1488).

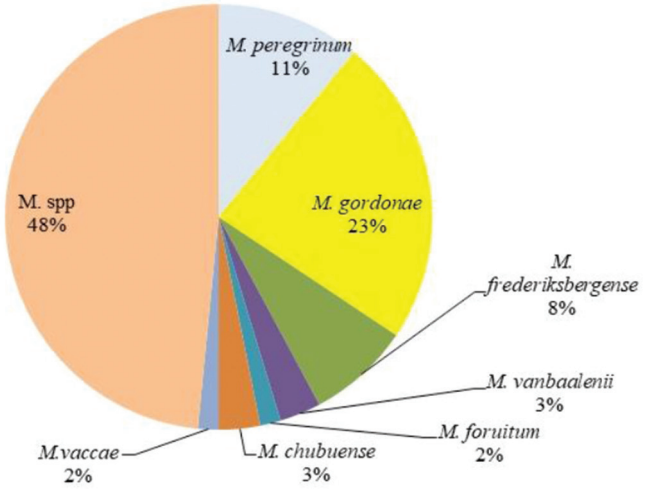

Figure 1: Diversity of nontuberculous mycobacteria isolated in tap water and dike of Bahía Blanca city, Argentina

Although ANOVA found significant differences between the log of the NTM count in S2 and S3 ($P = 0.048$), the difference between the means of the counts in each site did not have microbiological significance (34 colony-forming unit [CFU]/L and 58 CFU/L). In both sites, maximum counts of 430 CFU/L in S2 and 330 CFU/L in S3 were recorded. These counts did not correlate with low chlorine values. As is known, NTM are resistant to chlorine whether they are in planktonic growth or in the form of biofilms.[29,30]

The mean NTM counts of the water in S1 (8 CFU/L) were lower than those found in water distribution system. This low recovery could be caused by overgrowth of fast-growing bacteria because in S1, the number of heterotrophic bacteria was elevated (mean: 1600 CFU/mL). Furthermore, the decontamination methods employed for treated water and raw water were different.

Our results are consistent with those shown by Le Dantec *et al*.[31] in a water distribution system in Paris. In those studies, the frequency of recovery of NTM was 72% and in 78% of the samples, a concentration between 1 and 50 NTM/L was found.

In our study, the species assignment was performed considering ≥99% similarity by 16S rRNA sequencing and the concordance with biochemical and phenotypic tests. Identification at the species level was not achieved in 48% (31/64) of the isolates. They are shown as *Mycobacterium* spp. in Figure 1. In two isolates (3.1%), corresponding to *Mycobacterium chubuense,* the identification was confirmed by sequencing of the *sec*A1 gene.[32]

Table 1 shows the results of two identification approaches (16S rRNA gene sequencing and phenotypic/biochemical test) for NTM isolate from tap water and dike from Bahía Blanca city. In 46.9% (30/64) of samples, both methods coincided in the identification and the obtained sequences presented ≥99% identity, reaching identification at the species level.

The identification by 16S rRNA gene sequence of strains 22 and 23 led to more than one possible species with the 99% of identity (*M. fortuitum*/*M. peregrinum*/*Mycobacterium alvei* for strain 22 *and M. fortuitum*/*M. peregrinum* for strain 23). However *M. peregrinum* can grow in the presence of doxycycline 16 mg/L, whereas *M. fortuitum* is sensitive to that antibiotic. In addition, *M. fortuitum* and *M. peregrinum* grow on MacConkey agar (without crystal violet) at 28°C, whereas *M. alvei* does not grow. In strain 58, 16S rRNA sequencing showed 99% identity for both *M. porcinum* and *M. fortuitum*. The species is phenotypically similar to *M. fortuitum* but lacks nitrate reductase. This test was negative, indicating the possible presence of *M. porcinum*, although it is known that these data are insufficient for confirmation at the species level.

Strain 19 presented 100% identity for *M. vanbaalenii* and *M. austroafricanum*. According to the description in the Bergey's Manual,[23] both species are closely related phylogenetically. However, in dot-blot DNA-DNA hybridization studies, the organisms showed <40% hybridization.[33] According to the phenotypic characteristics, *M. vanbaalenii* develops at 42°C, whereas *M. austroafricanum* does not. In this case, the culture developed at 42°C.

The analysis of the sequence corresponding to strain 39 led to two possible species: *M. houstonense* (99%) and *M. mucogenicum* (98%). In this case, the growth temperature is useful but not sufficient to identify at the species level.

Discussion

The prevalence of NTM in Bahía Blanca is high despite the high values of chlorine. It should be taken into account that the choice of a particular decontamination method will select certain species. It is likely that hard decontamination procedures to isolate NTM from environmental sources will lead to the loss of number and diversity of species.[31] A concentration higher than 500 CFU/L of NTM was never found. The low count can be attributed to the negative effect caused by decontamination methods. The results of previous studies[34] were taken into account for the choice of decontamination methods. In these studies, it was observed that the Engel method[35] recovered more mycobacteria than the other proposed methods, due to reduced only 1 log the NTM count and 4 log the fast-growing microbiota. It was also shown that the method for the highest NTM recovery of the dike water was that proposed by Schulze-Röbbecke *et al*.[18]

In a study conducted by Peters *et al*.[36] in hospital water, the highest isolation number of NTM was obtained using CPC as a decontaminant, rather than a combination of NaOH and N-acetyl-L-cysteine. Neumann *et al*.[37] also proposed CPC as a decontaminating agent for surface water because it manages to inactivate bacterial spores and fungi present, achieving more significant effects if the exposure time agent is prolonged.

Even though biochemical and phenotypic tests were useful to approach the isolated species, another gene (e.g., *hsp*65 and *rpo*B) would be useful to ensure the correct identification. Due to the close links among mycobacteria, sequencing of a single DNA target does not always guarantee species discrimination.[38] The sequencing of other genes was not possible when the work was done.

It is necessary to emphasize that the biochemical protocols used for the identification of mycobacteria can give erroneous and ambiguous results,[39,40] not being highly reproducible methods, but they are supportive in the identification.

Molecular methods also have limitations, requiring the analysis of multiple genes to obtain accurate results, and sometimes they do not guarantee correct identification. Therefore, it is important to keep the biochemical and phenotypic tests within the identification protocol.

According to Tortone *et al*.,[41] the NTM-pigmented and fast-growing strains presented difficulty for identification by the methods used, whereas the slow-pigmented strains were

Table 1: Identification of nontuberculous mycobacteria isolated from tap water and surface water (dike) from Bahía Blanca city (Argentina) by 16S rRNA sequencing, biochemical and phenotypic features

Source of water sites sampled	Growth rate/ pigmentation	Reference numbers	16S rRNA sequencing (percentage similarity)	Biochemical tests	Proposed species
Tap water					
S2	R/S	[1]	*M. chubuense*/*M. gilvum*/*M. duvalii*/*M. sphagni*/*M. parafortuitum* (99)	*M. chubuense*	*M. chubuense*
S2	R/N	[3]	*M. conceptionense* (98)	*M. conceptionense*	*Mycobacterium* spp.
S2	R/S	[4]	*M. chubuense*/*M. gilvum*/*M. sphagni* (99)	*M. chubuense*	*M. chubuense*
S3	R/N	[7]	*M. peregrinum* (99)	*M. peregrinum*	*M. peregrinum*
S3	R/N	[8]	*M. peregrinum* (99)	*M. peregrinum*	*M. peregrinum*
S2	R/N	[9]	*M. peregrinum* (100)	*M. peregrinum*	*M. peregrinum*
S3	R/S	[10]	*M. celeriflavum* (98)	*M. celeriflavum*/*M. flavescens*	*Mycobacterium* spp.
S3	S/S	[11]	*M. gordonae* (100)	*M. gordonae*	*M. gordonae*
S2	R/N	[14]	*M. peregrinum* (100)	*M. peregrinum*	*M. peregrinum*
S2	R/S	[15]	*M. frederiksbergense* (99)	*M. frederiksbergense*	*M. frederiksbergense*
S2	S/S	[16]	*M. gordonae* (100)	*M. gordonae*	*M. gordonae*
S2	R/S	[17]	*M. vanbaalenii* (99)	*M. vanbaalenii*	*M. vanbaalenii*
S3	R/S	[18]	*M. frederiksbergense* (99)	*M. frederiksbergense*	*M. frederiksbergense*
S3	R/S	[19]	*M. vanbaalenii*/*M. austroafricanum* (100)	*M. vanbaalenii*	*M. vanbaalenii*
S3	S/S	[20]	*M. gordonae* (99)	*M. gordonae*	*M. gordonae*
S2	R/S	[21]	*M. frederiksbergense* (99)	*M. frederiksbergense*	*M. frederiksbergense*
S2	S/S	[25]	*M. gordonae* (98)	*M. gordonae*	*Mycobacterium* spp.
S3	S/S	[26]	*M. gordonae* (100)	*M. gordonae*	*M. gordonae*
S3	R/S	[27]	*M. frederiksbergense* (98)	*M. frederiksbergense*	*Mycobacterium* spp.
S2	R/N	[28]	*M. chelonae* (98)	*M. mucogenicum*	*Mycobacterium* spp.
S2	S/S	[29]	*M. gordonae* (98)	*M. gordonae*	*Mycobacterium* spp.
S2	S/S	[30]	*M. gordonae* (99)	*M. gordonae*	*M. gordonae*
S3	R/S	[31]	*M. frederiksbergense* (99)	*Mycobacterium* spp.	*Mycobacterium* spp.
S2	S/S	[33]	*M. asiaticum*/*M. marinum* (95)	*Mycobacterium* spp.	*Mycobacterium* spp.
S3	S/S	[34]	*M. gordona* (100)	*M. gordonae*	*M. gordonae*
S3	S/S	[35]	*M. gordonae* (99)	*M. gordonae*	*M. gordonae*
S3	S/S	[36]	*M. gordonae* (100)	*M. gordonae*	*M. gordonae*
S3	S/S	[32]	*M. gordonae* (100)	*M. gordonae*	*M. gordonae*
S3	R/N	[37]	*M. houstonense* (98)	*M. houstonense*	*Mycobacterium* spp.
S2	R/N	[38]	*M. houstonense* (98)	*M. houstonense*	*Mycobacterium* spp.
S3	R/N	[39]	*M. houstonense* (99) *M. mucogenicum* (98)	*M. mucogenicum*	*Mycobacterium* spp.
S3	S/S	[40]	*M. gordonae* (99)	*M. gordonae*	*M. gordonae*
S3	R/N	[41]	*M. abscessus* (100)	*M. massiliense*	*Mycobacterium spp.*
S2	R/S	[42]	*M. frederiksbergense* (99)	*M. frederiksbergense*	*M. frederiksbergense*
S3	S/S	[43]	*M. gordonae* (99)	*M. gordonae*	*M. gordonae*
S3	R/N	[44]	*M. fortuitum* (99)	*M. fortuitum*	*M. fortuitum*
S2	S/S	[45]	Seq. poor quality	*M. gordonae*	*Mycobacterium* spp.
S3	S/S	[46]	Not amplify	*Mycobacterium* spp.	*Mycobacterium* spp.
S3	S/S	[47]	Seq. poor quality	*M. gordonae*	*Mycobacterium* spp.
S3	R/S	[51]	*M. alvei* (100)	*M. septicum*	*Mycobacterium* spp.
S2	R/N	[53]	*M. peregrinum* (100)	*M. peregrinum*	*M. peregrinum*
S3	S/S	[54]	*M. gordonae* (99)	*M. gordonae*	*M. gordonae*
S2	R/N	[55]	*M. peregrinum* (99)	*M. peregrinum*	*M. peregrinum*
S2	R/S	[56]	No sequence	*M. poriferae*	*Mycobacterium* spp.
S2	R/N	[58]	*M. porcinum*/*M. fortuitum* (99)	*M. porcinum*	*Mycobacterium* spp.
S2	R/S	[59]	No sequence	*Mycobacterium* spp.	*Mycobacterium* spp.
S2	S/N	[60]	No sequence	*Mycobacterium* spp.	*Mycobacterium* spp.
S2	R/P	[61]	No sequence	*M. vaccae*	*Mycobacterium* spp.
S3	R/N	[64]	No sequence	*Mycobacterium* spp.	*Mycobacterium* spp.

Contd...

Table 1: Contd...

Source of water sites sampled	Growth rate/ pigmentation	Reference numbers	16S rRNA sequencing (percentage similarity)	Biochemical tests	Proposed species
Dike					
S1	R/N	[5]	*M. peregrinum* (99)	*M. peregrinum*	*M. peregrinum*
S1	R/S	[6]	Seq. poor quality	*Mycobacterium* spp.	*Mycobacterium* spp.
S1	S/S	[12]	*M. gordonae* (100)	*M. gordonae*	*M. gordonae*
S1	R/S	[13]	No sequence	*Mycobacterium* spp.	*Mycobacterium* spp.
S1	R/N	[22]	*M. alvei*/*M. fortuitum*/*M. peregrinum* (99)	*M. peregrinum*	*Mycobacterium* spp.
S1	R/N	[23]	*M. peregrinum*/*M. fortuitum* (99)	*M. peregrinum*	*Mycobacterium* spp.
S1	R/S	[24]	*M. frederiksbergense* (100)	*M. neoaurum*/*M. cosmeticum*	*Mycobacterium* spp.
S1	S/S	[48]	*M. gordonae* (100)	*M. gordonae*	*M. gordonae*
S1	R/S	[49]	*M. frederiksbergense* (100)	*M. frederiksbergense*	*M. frederiksbergense*
S1	S/S	[50]	*M. gordonae* (100)	*M. gordonae*	*M. gordonae*
S1	R/S	[52]	Seq. poor quality	*M. neoaurum*	*Mycobacterium* spp.
S1	R/P	[57]	*M. vaccae* (100)	*M. vaccae*	*M. vaccae*
S1	S/N	[62]	No sequence	*Mycobacterium* spp.	*Mycobacterium* spp.
S1	S/S	[63]	No sequence	*Mycobacterium* spp.	*Mycobacterium* spp.
S1	S/S	[65]	No sequence	*M. gordonae*	*Mycobacterium* spp.

[a]Species confirmed using sequencing of the *sec*A1 gene. S1: Dike Paso de las Piedras, S2 and S3: Site near and far from the water treatment plant, respectively, S/S: Slowly growing/scotochromogenic, R/S: Rapidly growing/scotochromogenic, R/N: Rapidly growing/nonchromogenic, R/P: Rapidly growing/photochromogenic, S/N: Slowly growing/nonchromogenic, Seq. poor quality: Sequence poor quality, *M. chubuense: Mycobacterium chubuense, M. conceptionense: Mycobacterium conceptionense, M. peregrinum: Mycobacterium peregrinum, M. celeriflavum: Mycobacterium celeriflavum, M. gordonae: Mycobacterium gordonae, M. frederiksbergense: Mycobacterium frederiksbergense, M. vanbaalenii: Mycobacterium vanbaalenii, M. austroafricanum: Mycobacterium austroafricanum, M. chelonae: Mycobacterium chelonae, M. asiaticum: Mycobacterium asiaticum, M. marinum: Mycobacterium marinum, M. houstonense: Mycobacterium houstonense, M. mucogenicum : Mycobacterium mucogenicum, M. alvei: Mycobacterium alvei, M. abscessus: Mycobacterium abscessus, M. fortuitum: Mycobacterium fortuitum, M. vaccae: Mycobacterium vaccae, M. flavescens: Mycobacterium flavescens, M. massiliense: Mycobacterium massiliense, M. neoaurum: Mycobacterium neoaurum, M. cosmeticum: Mycobacterium cosmeticum, M. septicum: Mycobacterium septicum, M. poriferae: Mycobacterium poriferae, M. gilvum: Mycobacterium gilvum, M. duvalii: Mycobacterium duvalii, M. sphagni: Mycobacterium sphagni, M. parafortuitum: Mycobacterium parafortuitum, M. porcinum: Mycobacterium porcinum*

identified with a high percentage of identity, and the majority of the biochemical tests coincided with those established by the Bergey`s Manual.[23]

Conclusions

This study suggests the NTM occurrence in Bahía Blanca water distribution system, as well as the main source of water supply for the city (Paso de las Piedras dike); most are saprophytes such as *M. gordonae, M. frederiksbergense, M. peregrinum,* and *M. chubuense,* but others (e.g. *M. fortuitum*) are potential pathogens that cause more problems.[28,29]

It is evident that the values of free chlorine specified by the AFC are not adequate for the control of the NTM of the network water. Because of this, drinking water distribution systems select NTM, allowing its persistence in them. Although the sites located within the city's water distribution system showed highly significant differences in free chlorine, no relationship was found between the NTM count and the concentration of the disinfectant.

The decontamination of the samples is a point of interest for the recovery of NTM. It is important to consider the different decontamination methods according to the matrix to be analyzed, as well as the reduction of the number of mycobacteria that cause them. It is important to correct the values of NTM counts in environmental samples and not to exclude certain environments as possible mycobacterial habitats when these are not recovered by conventional methods because they may be below the detection limit value. The ideal decontamination method should be one that minimizes the accompanying microbiota without modifying or minimally the number of mycobacteria present in the sample. The CPC method would seem to be the method of choice for the recovery of NTM from water from the dike because in other studies, CPC proved to result in higher mycobacterial yields than decontamination methods using NaOH without preincubation of the sample.[18] The comparison of NTM prevalence results is difficult because several protocols for decontamination have been described, but a standard protocol for the isolation from drinking water is not available. According to Makovcova *et al*.,[42] general rules to guide the isolation cannot be formulated due to differences in the types of samples, culture media for primary isolates, and differences in the geographic distribution of mycobacteria. In addition, mycobacteria differ in their susceptibility to decontamination procedures.[43]

It should be taken into account that sequencing of the 16S rRNA gene is useful for the identification of most species, but a similarity of 99% is believed to be a minimum requirement for bacterial identification using this gene.[43] In our study, 17.2% (11/64) of the isolates showed a similarity <99% [Table 1]. This point is the main limitation

of this study, and an additional evaluation is necessary. Some authors suggest the use of other genes such as *rpo*B and *hsp*65 when the isolates cannot be identified at the species level by the analysis of the 16S rRNA gene because they present the same percentage of similarity.[44,45]

In agreement with Tortone *et al*.,[41] it is suggested that, for the correct identification of NTM isolated from environmental samples, the molecular methods used should be complemented with phenotypic ones. In this work, examples were mentioned that demonstrate their utility.

It is considered that the continuous improvement of the quality of drinking water constitutes one of the main preventive actions available in the field of public health. Optimizing water quality is a permanent challenge that should not be neglected.

Finally, it would be interesting to be able to establish a relationship between the species isolated from water and those obtained from clinical samples,[45,46] in order to elucidate the risk of disease by NTM in this city, as well as identify critical environmental reservoirs and routes of transmission of these microorganisms.

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Conflicts of interest

There are no conflicts of interest.

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