Ann Ist Super Sanità 2010 | Vol. 46, No. 3: 254-258
DOI: 10.4415/ANN_10_03_05

Non-tuberculous mycobacteria and microbial populations in drinking water distribution systems

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Summary. Data on the occurrence of non-tuberculous mycobacteria (NTM), in parallel with those obtained for bacterial indicators and amoebae, are presented with the aim to collect information on the spread of NTM in drinking water distribution systems in Italy. Samples were collected from taps of hospitals and households in Central and Southern Italy. The concentration values obtained for the more traditional microbial parameters complied with the mandatory requirements for drinking water. Conversely, moderate-to-high microbial loads (till 300 CFU/L) were observed for the NTM. Positive samples were obtained from 62% of the investigated water samples. Analogous results were observed for amoebae showing a higher percentage of positive samples (76%). In terms of public health, the presence of mycobacteria in water distribution systems may represent a potential risk especially for vulnerable people such as children, the elderly or immunocompromised individuals.

Key words: amoebae, drinking water, atypical mycobacteria, water supply.

Riassunto *(Micobatteri non tubercolari e popolazioni microbiche in reti idriche di distribuzione)*. Per la prima volta in Italia, acqua potabile distribuita in rete è stata analizzata per verificare l'eventuale presenza di micobatteri non tubercolari (NTM). I microrganismi sono stati ricercati in acque di rubinetto prelevate da ospedali e da abitazioni del Centro e del Sud Italia; parallelamente, oltre ai tradizionali indicatori microbici di contaminazione e di qualità dell'acqua, i campioni sono stati esaminati per la presenza di amebe. Con specifico riferimento agli indicatori, quali parametri di monitoraggio delle acque, le analisi hanno evidenziato la rispondenza ai requisiti di qualità stabiliti per le acque potabili. Diversamente, per i micobatteri sono state rilevate densità variabili, anche piuttosto elevate (fino a 300 UFC/L), con una percentuale di positività del 62% nei campioni di acqua esaminati; valori di positività più elevati (76%) sono risultati per le amebe. In termini di salute pubblica, la presenza di NTM nelle reti di distribuzione idrica può costituire una condizione di rischio, soprattutto per le fasce di popolazione più suscettibili (immunodepressi, bambini e anziani).

Parole chiave: acque potabili, amebe, micobatteri atipici, reti idriche.

INTRODUCTION

Since the 1980s clinic and laboratory-based studies have shown an increased prevalence of persons with non-tuberculous mycobacterial (NTM) pulmonary disease, while during the same period a continued decline in the incidence and prevalence of tuberculosis has been reported [1].

Disseminated infections with NTM organisms were first recognized in immunocompromised patients, subsequently identified as a common complication of advanced AIDS and as one of the leading cause of death amongst HIV positive populations [1].

NTM have been reported as opportunistic pathogens causing cutaneous and pulmonary infections, immune dysfunctions and chronic and disseminated diseases. Differently from *Mycobacterium tuberculosis* and *M. leprae*, most of the mycobacterial species today described are generally not considered as obligate human pathogens. Although NTM are not transmissible, the diseases they cause may greatly affect public health and medical care resources.

NTM comprise a multispecies group of environmental organisms, living in soil as well as in treated and untreated water sources. In the USA and in some European countries, these bacteria have been recovered from freshwater, brackish/sea water, and wastewater, sometimes at high densities [1]. NTM have also been recovered in drinking-water systems before and after treatment, from the distribution system and from raw source waters. Water treatment itself is likely to be ineffective due to the high

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resistance of mycobacteria to disinfection; in fact, it has been well-documented that these microorganisms are resistant to ozone and chlorine-based disinfectants [2]. Mycobacterial numbers seem to be higher in drinking water distribution systems (average 25 000-fold) than in those collected just after treatment, suggesting that they are able to multiply along distribution. Some environmental mycobacteria have been shown to grow within amoebae [3, 4] and ciliates [3] and it has been suggested that this condition may provide an helpful haven when environmental conditions deteriorate. While environmental transmission within amoebae is a possibility, it remains unclear whether amoebae, or any other protozoa, play a role in the pathogenesis or epidemiology of any mycobacterial diseases.

In recent years, there has been an increase in the number of potentially pathogenic mycobacterial species, whose transmission route is associated with water. Some species, also potentially causative agents of various diseases, can colonize cold water distributions systems *(M. kansasii)* whilst *M. xenop*i and *M. avium* are more commonly associated with hot water systems. *M. marinum*, often recovered in swimming pools or aquaria, can cause infection of abraded skin. Contamination of injected liquids can cause abscesses *(M. chelonae, M. fortuitum)*, and other infections are linked to contaminated endoscope washers and renal dialysis fluid. Mycobacterial infections linked to contaminated hospital tap water have been recognized for many years [5], and environmental mycobacteria have been isolated from hospital waters, particularly hot water systems [6].

NTM infections result from diverse and likely undetectable environmental and nosocomial exposures. However, the epidemiological link between presence of environmental mycobacteria in drinking water and disease has not been effectively made. Investigating the waterborne transmission of mycobacteria may be difficult, since infections are generally sporadic and there are a variety of sources of exposure other than water; in addition the typing schemes that are routinely applied are not sufficiently discriminating, to confidently identify whether isolates from the environment are the same as those from associated patients.

Till now, in Italy, the absence of epidemiological evidence associates with the lack of adequate reference methodologies of sampling and analysis, has delayed monitoring studies on the potential risk for human health, due to the presence of NTM in water. Therefore, it is now imperative to start this control and to evaluate the dimension of the problem in our country.

Data on the occurrence of NTM, in parallel with those obtained for bacterial indicators and amoebae, are presented with the aim to collect information on the occurrence and spread of mycobacteria in various drinking water distribution systems. Water samples were collected from taps of hospitals and households in Central and Southern Italy.

MATERIALS AND METHODS

A total of 42 water samples (34 in the Latium region and 8 in the Calabria region) were collected and analysed. In particular, 16 and 4 samples were from households of Latium and Calabria, while 18 and 4 samples from hospitals, respectively.

Microbiological analysis

- *Heterotrophic plate count at 22 °C.* Membrane filtration technique (0.45 µm pore size, Millipore) on WPC agar (Oxoid). Incubation at 22 ± 1 °C for 5 days. All colonies were counted [7].
- *Coliform bacteria at 37 °C and Escherichia coli.* Most probable number technique with Colilert 18 Quanti-Tray. Incubation at 36 ± 1 °C. Yellow and fluorescent micro-wells were counted, respectively [7].
- *Pseudomonas aeruginosa.* Membrane filtration technique (0.45 µm pore size, Millipore) on *Pseudomonas* agar/CN supplemented with *Pseudomonas* CN selective supplement (Oxoid). Incubation at 37 °C for 3 days. Green-blue and fluorescent colonies were counted [7].
- *Mycobacterium* spp. Decontamination with cetylpyridinium chloride (0.04% wt/vol), membrane filtration technique (0.45 µm pore size, millipore) on Middlebrook 7H10 (Difco). Incubation at 36 ± 1 °C up to 6-10 weeks [8]. Subculture of presumptive colonies on Lowestein Jensen Medium (Biolife). Incubation at 36 ± 1 °C up to 6-10 weeks. Ziehl-Neelsen staining of re-grown colonies to identify acid-fast organisms. PCR to confirm mycobacteria to genera level and restriction analysis to identify to species level.
- *Free-living amoebae.* Membrane filtration technique (1.2 µm pore size, Sartorius AG-37070) on Non Nutrient Agar (NNA pH 7.5) seeded with *E. coli* ATCC 8739. Incubation at 30 °C for 5-7 days and direct presence/absence analysis at 40× inverted microscope [9].

Molecular analysis

- *Polymerase chain reaction (PCR).* A 439 bp fragment between positions 398 and 836 of the 65 kDa heat shock protein, was amplified with primers common to all mycobacteria, according to Telenti *et al.* [10]. Bacteria from Middlebrook 7H10 and Lowenstein-Jensen medium were lysed by heating-cooling treatment. PCR mixture (50 µL): 5 µL lysate, 200 µM dNTPmix (Promega), 1 µM Primer Tb11(5'ACCAACGATGGTGTGT CCAT), 1µM Primer Tb12(5'CTTGTCGAACC GCATACCCT), Buffer Taq (Promega), "nucleases free" water (Promega) and *Taq* Polymerase (5U/µL) (GoTaq DNA Polymerase Promega). The reaction conditions were: denaturation at 94 °C for 2 min, 45 cycles of amplification (1 min at 94 °C, 1 min at 64 °C, 1 min at 72 °C); final extension: 10 min at 72 °C.
- *Restriction analysis.* Identification of *Mycobacterium* species was performed by Restriction Enzyme Analysis of PCR products digested with BstEII

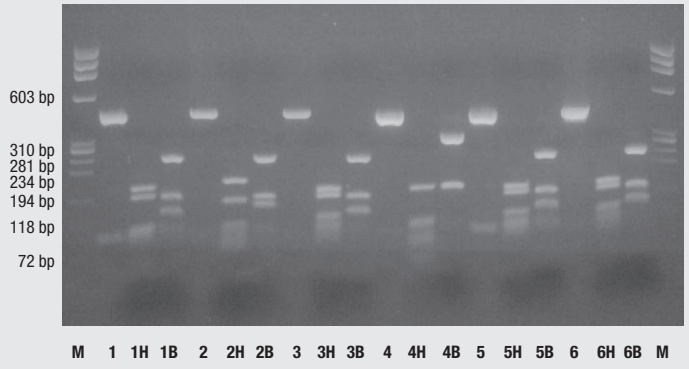


Fig. 1 | *PCR-Restriction enzyme pattern analysis of mycobacteria isolated from drinking water samples. M (marker): ΦX174 DNA/HaeIII marker (72 bp-1353 bp); 1:* M. fortuitum *ATCC; 1H:* M. fortuitum *ATCC digested with HaeIII; 1B:* M. fortuitum *ATCC digested with BstEII; 2:* Mycobacterium *sp; 2H:* Mycobacterium *sp digested with HaeIII; 2B:* Mycobacterium *sp digested with BstEII; 3:* M. intracellulare*; 3H:* M. intracellulare *digested with HaeIII; 3B:* M. intracellulare *digested with BstEII; 4:* M. genavense*; 4H:* M. genavense *digested with HaeIII; 4B:* M. genavense *digested with BsteII; 5:* M. intracellulare*; 5H:* M. intracellulare *digested with HaeIII; 5B:* M. intracellulare *digested with BstEII; 6:* M. intracellulare*; 6H:* M. intracellulare *digested with HaeIII; 6B:* M. intracellulare *digested with BstEII.*

and HaeIII restriction enzymes *(Figure 1)* according to Telenti *et al*. [10]. Ten µL of PCR product were added to a mixture (25 µL) containing BstEII enzyme (5U) (Promega), Buffer C (Promega), BSA (0.1 mg/mL). The mixture was incubated for 60 min at 60 °C. Similarly, an aliquot of 10 µL of product was digested at 37 °C in a solution containing HaeIII enzyme (5U) (Promega), Buffer D (Promega), BSA (0.1 mg/mL). Ten µL of digestion mixtures were run onto 2% agarose gel and fragments visualized by ethidium bromide staining and UV light.

Physical and chemical analysis

Analyses of temperature, pH and free chlorine residual were performed according to the procedures described in Rapporti Istisan, 07/31 [7].

Statistical analysis

Possible reciprocal correlations of the bacterial faecal indicators and the heterotrophic bacteria at 22 °C with the presence of environmental mycobacteria were performed using Spearman's test (SPSS, v. 11).

RESULTS

The largest part of the drinking water samples (98%) complied with the microbial requirements established by the Italian Decree n. 31/2001 on drinking water quality if *E. coli* was considered; only 14% of the samples exceeded limits for coliforms at 37 °C *(Table 1)*.

Pseudomonas spp. was detected in 21% of the analyzed samples, with densities that in some cases reached 300 CFU/100 mL *(Table 1)*.

Moderately high microbial loads, ranging from 0 to 300 CFU/L, were calculated for mycobacteria. Positive samples were obtained from 62% of the water samples. Analogous results were observed for amoebae, showing a high percentage of positive samples (76%) *(Table 1)*.

As far as the physical and chemical characteristics, temperature and pH ranged from 21 to 25 °C and from 6.0 to 6.7, respectively. Only 5% of the samples complied with the limit provided for free chlorine residual in water distribution systems (0.2 mg/L) *(Table 1)*.

The numbers of NTM in private house and hospital drinking water collected in Latium and Calabria are reported in *Table 1*. In tap water collected in private houses, 60% of the samples were positive for mycobacteria, while a higher percentage (73%) was detected in hospital water samples.

All the samples collected in Calabria, both from houses and hospitals, were positive for the presence of environmental mycobacteria, whilst the corresponding samples collected in Latium showed lower positive percentage (50% and 67%, respectively).

Figure 1 shows the 439 bp PCR products and the restriction enzyme pattern analysis of some mycobacteria isolated from drinking water samples. The more frequently isolated Mycobacterium species were *M. intracellulare, M. genavens*e; in addition, *M. haemophilum*, a species associated with disseminated infections in HIV infected patients, was often recovered [1].

DISCUSSION AND CONCLUSIONS

Environmental mycobacteria are a frequent cause of opportunistic infection in human beings and livestock. There is a growing recognition that water is an important vehicle of their transmission and this is based on the fact that in recent years contaminated water supplies have been responsible of several hospital and community outbreaks of mycobacterial infections.

The present investigation represents the first Italian study on the occurrence of environmental mycobacteria in drinking water. Results show a wide-spread prevalence of NTM in the analyzed samples: 50% and 100% of water samples collected from private houses were positive in Latium and Calabria, respectively; similar values, 67% and 100% have been

Table 1 | *Microbial and physical-chemical parameters in the investigated drinking water samples*

Bacterial indicators						
Samples	***E. coli***		**Coliforms at 37 °C**		**HPC at 22 °C**	
N°	% positive	Concentration (range) CFU/100mL	% positive	Concentration (range) CFU/100mL	% positive	Concentration (range) CFU/mL
42	2	0-1	14	0-300	74	0-300
Environmental microrganisms						
	P. aeruginosa		***Mycobacterium***		**Free living amoebae**	
	% positive	Concentration (range) CFU/100mL	% positive	Concentration (range) CFU/L	% positive	
42	21	0-300	62	0-300	76	
NTM in household water						
	Latium (% positive)		**Calabria (% positive)**		**% total positive samples**	
20	50		100		60	
NTM in hospital water						
	Latium (% positive)		**Calabria (% positive)**		**% total positive samples**	
22	67		100		73	
Physical-chemical parameters						
	Temperature range (°C)		**pH** (range)		**Free residual chlorine** range (mg/L)	
42	21-25 °C		6.0-7.67		0.02-0.54	

HPC: heterotrophic plate counts; NTM: non-tuberculous mycobacteria.

observed in samples collected from hospital water samples in the same areas.

However, no reciprocal correlation between NTM and faecal bacterial indicators has been found ($p > 0.05$, Spearman) while heterotrophic bacteria at 22 °C positively correlated with them ($p < 0.05$, Spearman). A lack of significant correlation between mycobacteria and bacteriological indicators was widely recognized [1, 12]. It suggests that traditional indicators are not able to warn for the presence of these microorganisms.

In 76% of the analyzed water samples, free-living amoebae were also observed. Even if the study does not speculate on intracellular survival of NTM in amoebae, their simultaneous presence in drinking water samples, supports what already underlined by several authors about their association in aquatic environments [4].

All the species identified in the present study show a variable virulence and each species can be responsible of a certain variety of diseases. It seems to be difficult to avoid contact or exposure with NTM as they are ubiquitous microorganisms in water and biofilm and are resistant to disinfecting agents and to environmental adverse conditions. Complete eradication of them from distribution systems seems not possible, but a number of measures are known to decrease the mycobacterial concentration and might prevent nosocomial infections in hospitals [1]. It has been also suggested that exposure to tap water mycobacteria could be reduced by using the recommendations for preventing *Legionella* infection [13]. In contrast to many other waterborne pathogens, for which re-growth within the distribution system is minimal or does not occur, point-of-use protection (standard cleaning and disinfection protocols used during main repairs) would appear to be the most important critical control point for these bacteria.

There is a limited understanding of the social, environmental and personal risk factors responsible for initiating disease caused by most of the environmental mycobacteria. This is also due to the limited use of typing and environmental testing in investigating individual cases of infection. Greater effort needs to be put into sensitive typing methods and their use in elucidating the epidemiology of these diseases. Sequencing the genomes of further environmental mycobacteria will undoubtedly benefit our long-term understanding of the impact of environmental exposure to these organisms on human health.

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

Submitted on invitation.
Accepted on 22 April 2010.

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