Nontuberculous mycobacteria in hospital water systems: application of HPLC for identification of environmental mycobacteria

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ABSTRACT

Nontuberculous mycobacteria (NTM), ubiquitous in water environments, are increasingly recognized as nosocomial pathogens. Our study reports a one-year survey of the water system of two hospitals, A and B, in a small town near Florence, Italy. NTM were found throughout the study period in both settings, but B showed a significantly higher mycobacterial load. *Mycobacterium gordonae* and *Mycobacterium fortuitum* were the most frequent species isolated. Identification was carried out by conventional techniques and by high performance liquid chromatography (HPLC) analysis of cell wall mycolic acids. HPLC profiling could be used as a first-choice method for identification of environmental mycobacteria.

Key words | hospital, HPLC, nontuberculous mycobacteria, water

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INTRODUCTION

Nontuberculous mycobacteria (NTM) are widespread in nature. With some exceptions NTM are free-living saprophytes of many environments, but water, both natural and conducted, is their main reservoir. Owing to their intrinsic resistance, NTM can withstand the conventional treatments for potable water. Moreover, the formation of biofilm increases the probability of recovering NTM from water pipes. Although saprophytic, NTM can represent a threat to humans. There is an increasing awareness of their public health relevance, especially as nosocomial pathogens. Since person-to-person transmission does not seem to play an important role, infections are probably environmental and hospital water systems should be under observation (Pelletier *et al.* 1988; Falkinham 1996; Covert *et al.* 1999; Iivanainen *et al.* 1999; Stickler 1999).

In a survey carried out two years ago in Florence we did not isolate mycobacteria in the water system of local hospitals (unpublished observations). The aim of our study was to investigate the water systems of two health care settings in the vicinity of Florence. These derive their water supply from different waterworks that do not use full treatment as they receive spring water of good microbiological quality. This study also evaluated the usefulness of high performance liquid chromatography (HPLC) analysis of cell wall mycolic acids profiles for the identification of environmental mycobacteria.

MATERIALS AND METHODS

Hospital settings

The two hospitals, A and B, are located in a small town in Tuscany (approximately 45,000 inhabitants) about 30 km west of Florence. A, a 93-bed hospital, is some 100 years old, and is located in the centre of the town; B, a 193-bed hospital, was built more recently, around 1970, in the outskirts. Water in the town is supplied by various springs spread all over the area, each one with its own treatment plant; raw groundwater is characterized by the absence of faecal pollution markers.

Water samples

Over a one-year period the water arrival point and tap water from all the wards of the two hospital settings were sampled at two-month intervals. One litre samples were collected in sterile bottles containing 0.8 ml 3% Na thiosulphate (Sigma, Germany), as suggested by standard procedures. The samples were filtered through a 0.45 μm membrane (Millipore, USA); the membranes were placed in 4.5 ml sterile distilled water and agitated for 10 min at room temperature. Decontamination was carried out by adding 0.5 ml 1% dodecyl di-(beta-oxyethyl)-benzylammonium chloride (Rusch Hospital, Italy) and incubating for 2 h at 37°C.

Isolation

Four Löwenstein–Jensen slants (Becton Dickinson Microbiology Systems, USA) were inoculated with 0.2 ml from each sample, two tubes were incubated at 37°C and two at 28°C. Cultures were inspected weekly; final readings were carried out at eight weeks. Acid-fast presumptive colonies were subcultured on 7H11 medium (Difco, USA) to continue with conventional identification in addition to HPLC.

Conventional identification

The tests were carried out according to standard methods (Wayne & Kubica 1986; Metchock *et al.* 1999) and included growth characteristics (growth rate and preferred growth temperature, pigmentation and photoreactivity, colony morphology) and biochemical tests:

- urease
- arylsulphatase
- niacin production
- Tween 80 hydrolysis
- catalase (drop method catalase after heating cells at 68°C, semiquantitative)
- nitrate and tellurite reduction
- tolerance to 5% NaCl
- susceptibility to isoniazide, thiophene-2-carboxylic acid hydrazide, hydroxylamine, p-nitrobenzoic acid, thiacetazone and oleic acid
- growth on McConkey agar without crystal violet.

The results were computer-evaluated according to Tortoli *et al.* (1992) with the Mycob ID program.

HPLC

A loopful of bacteria from 7H11 cultures was processed for the cell wall mycolic acid preparation as previously described (Tortoli & Bartoloni 1996). The derivatized UV-absorbing bromophenacyl esters were suspended in chloroform, spiked with the high-MW standard and injected into the HPLC apparatus (HP series 1050) equipped with a reverse phase C-18 ultrasphere-XL column (Beckman, USA) and a HP 1040 M UV detector set at 260 nm. HPLC was carried out according to the procedure developed at the Centers for Disease Control and Prevention (CDC) (Butler *et al.* 1991), as described by Tortoli & Bartoloni (1996). For pattern analysis, fractions eluted within the first 4 min and peaks less than 2% of the total peak height were not considered; valid peaks were identified by their relative retention time (RRT) determined with an internal standard according to the CDC scheme. The profiles were compared visually to our collection of profiles.

RESULTS AND DISCUSSION

NTM were found in the water systems of both hospitals. 51 of the total 97 samples were acid-fast bacilli (AFB)

Table 1 | Isolation of acid fast bacilli (AFB) and identification of the 107 NTM isolates from the water systems of the two hospital settings

	Hospital A	Hospital B	Total
AFB pos/N (%)	13/35 (37.1)	38/62 (61.3)	51/97 (52.6)
Isolates	26	81	107
Species N (%)			
M. gordonae	22 (84.6)	47 (58.0)	69 (64.5)
M. fortuitum	2 (7.8)	19 (23.5)	21 (19.7)
M. smegmatis	0	8 (9.9)	8 (7.5)
M. chelonae	1 (3.8)	6 (7.4)	7 (6.5)
M. mucogenicum	1 (3.8)	0	1 (0.9)
M. gordonae-like	0	1 (1.2)	1 (0.9)

positive; the prevalence was significantly higher in hospital B. Of the 107 total isolates, 26 were from hospital A and 81 from hospital B; *M. gordonae* and *M. fortuitum* were the most frequent species (Table 1). Unlike Kubalek & Komenda (1995), we did not find any seasonality with respect to isolation rate or species found. The bacterial load varied from none (less than 10 CFU l^{-1}) to more than 2000 CFU l^{-1} (the maximum count level of the method employed). The highest concentrations of NTM were found in hospital B. The outlets of the nursery and the operating rooms were the most heavily colonized and, unlike in hospital A, the water arrival point was also positive, although not heavily (Table 2).

The two hospitals are supplied by different springs with similar physicochemical and microbiological characteristics and similar treatment. Nevertheless, a difference in the quality of water at the respective arrival points was noted: NTM were found at the arrival point of B but not of A. Also, the type and the condition of the two plumbing systems differed. The plumbing system of hospital B was larger and possibly had more dead ends, which are known to favour bacterial settlement and proliferation. The NTM probably originate from the influent water, as shown in hospital B where NTM were found at the arrival point and, most notably, the species recovered were also found in the samples taken from the wards.

The results presented here are at variance with those obtained in our unpublished observations for Florence, where two hospitals were studied. Six wards and the arrival point from one hospital, and four wards and the arrival point from the other were monitored for one year. None of the 48 samples yielded NTM. This is probably related to the quality of water and hence to the treatments carried out by the two waterworks. The Florentine central water treatment plant is supplied with surface water which, because of the poor microbiological characteristics of the raw water, is subjected to three oxidation–disinfection steps: chlorine dioxide, ozone and, for the final disinfection step, chlorine dioxide to ensure a high chlorine residual in the distribution system. For the two hospitals in this study, each waterworks is supplied with groundwater of good microbiological quality, but with Fe and Mn at relatively high concentrations, 4.5 mg l^{-1} and 1.5 mg l^{-1}, respectively. Therefore, less harsh treatment is carried out: water undergoes oxidation by air and sand filtration followed by addition of chlorine dioxide to ensure adequate amounts of free chlorine in the conduits (Berchielli *et al.* 2001). Thus, not surprisingly, mycobacteria were recovered from distributed potable water.

We found complete agreement between identification by conventional techniques and by HPLC. All but one of our isolates, isolate #84, could be readily identified by HPLC profiling in comparison to our reference library. Figure 1 shows the HPLC profiles of the most frequently isolated species. Isolate #84 was a slow-growing AFB with a double-cluster HPLC profile, which did not match any of the more than 60 profiles of our reference library (Tortoli *et al.* 1995; Tortoli & Bartoloni 1996), though there were some similarities to *M. gordonae* type 2. Biochemical and cultural tests on isolate #84 also gave inconclusive results. Figure 2 and Table 3 show the HPLC profile and the conventional identification, respectively. 16S rRNA sequencing for this isolate indicated *M. gordonae* as the most similar species (Tortoli *et al.* 2001).

From a methodological point of view, we recommend the use of HPLC for mycobacterial identification. The great

Table 2 | NTM isolation during the one-year survey of the water systems from the various sampling points of the two hospital settings

Sampling points	AFBpos/N	CFU l^{-1}	Species (number of isolates)
Hospital A			
Arrival	0/5		
Surgery wards	6/12	20–780	*M. gordonae* (11)*, *M. fortuitum* (1), *M. chelonae* (1), *M. mucogenicum* (1)
Surgery operating rooms	4/6	10–800	*M. gordonae* (8)*, *M. fortuitum* (1)
Orthopaedic ward	1/6	20	*M. gordonae* (1)
Dialysis ward	2/6	20	*M. gordonae* (2)*
Total	13/35	10–800	(26)
Hospital B			
Arrival	3/5	20	*M. gordonae* (1), *M. fortuitum* (1), *M. smegmatis* (1)
Nursery	7/11	20–>2000	*M. gordonae* (9)*, *M. fortuitum* (5), *M. chelonae* (3)
Eye ward	2/6	20–300	*M. gordonae* (3), *M. fortuitum* (1)
Eye operating rooms	4/5	40–>2000	*M. gordonae* (8), *M. fortuitum* (2)
Gynaecology ward	4/5	20–160	*M. gordonae* (5)*, *M. smegmatis* (2)
Gynaecology operating rooms	4/6	20–>2000	*M. gordonae* (2), *M. fortuitum* (2), *M. smegmatis* (1), *M. chelonae* (1)
Intensive care ward	5/6	20–800	*M. gordonae* (7)*, *M. fortuitum* (3), *M. smegmatis* (1), *M. chelonae* (1)
Medicine wards	7/12	20–160	*M. gordonae* (9)*, *M. fortuitum* (3), *M. smegmatis* (3), *M. chelonae* (1), *M. gordonae*-like (1)
Cardiology ward	2/6	20–80	*M. gordonae* (3)*, *M. fortuitum* (2)
Total	38/62	20–>2000	(81)

*Strains isolated on at least two samplings.

number and variety of environmental mycobacteria necessitates the use of rapid and easy to run identification strategies. HPLC profiling of cell wall mycolic acids fulfils these requirements because of its rapidity, use of low-cost reagents and good correlation with genome-based identification methods such as 16S rRNA sequencing. According to our results, HPLC profiling shortens the time required for mycobacteria identification and, as an open-ended method, allows the prompt recognition of unusual non-matching profiles which require further investigation (Butler *et al.* 1991; Thibert & Lapierre 1993; Tortoli *et al.* 1995; Tortoli & Bartoloni 1996; Tortoli *et al.* 1996, 1999, 2001).

CONCLUSIONS

Water represents the main reservoir for environmental mycobacteria. Potable water treatment can lower the

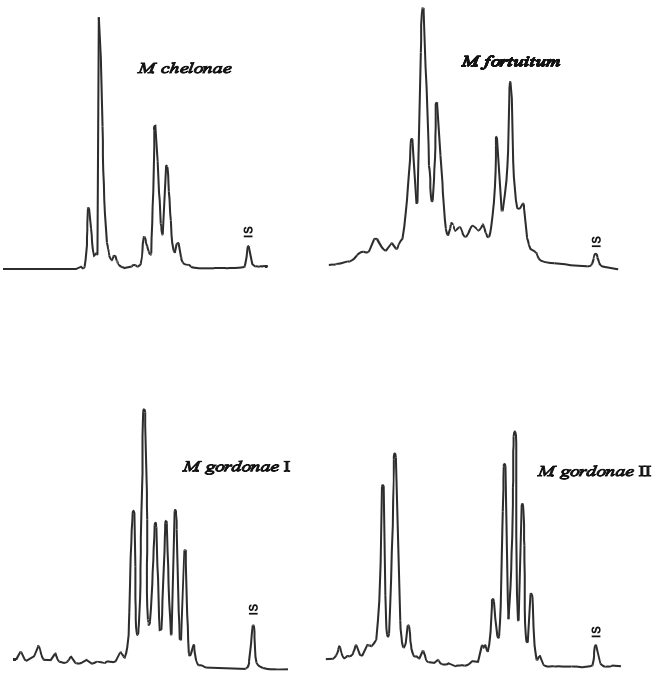

Figure 1 | Mycolic acid profiles in HPLC of NTM most frequently isolated in the two hospitals, together with the profile of *M. gordonae* type 2.

bacterial load, but minimal treatments, such as when the influent water has good microbiological quality, may not be sufficient against mycobacteria because of their intrinsic hardiness. Additionally, the formation of biofilm influences the persistence of bacteria inside the pipes.

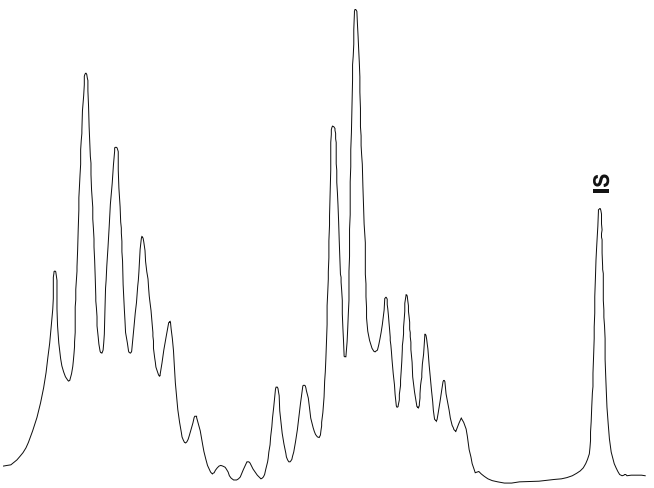

Figure 2 | Mycolic acid profile of isolate #84.

Table 3 | Conventional test results on isolate #84

Scotochromogenicity	+
Photochromogenicity	+
Colony morphology	R
Growth at 25°C	+
Growth at 45°C	–
Growth on McConkey agar	–
Urease	+
Arylsulphatase	–
Niacin	–
Tween 80 hydrolysis	+
Catalase at 68°C	+
Catalase, height >45 mm	+
Nitrate reduction	+
Tellurite reduction	–
NaCl	–
Isoniazide	+
TCH*	+
Hydroxylamine	–
p-nitrobenzoic acid	+
Thiacetazone	+
Oleic acid	+

*TCH=tiophene-2-carboxylic acid hydrazide.

Compared with free-living microorganisms, bacteria associated with biofilm are more resistant to inactivation because of the barrier represented by the amorphous matrix itself. Biofilm development is facilitated by the type and condition of the water pipes, but dead ends also play an important role (Pedersen 1990; Wallace *et al.* 1998;

Iivanainen *et al.* 1999; Stickler 1999; Norton & LeChevallier 2000; Berchielli *et al.* 2001; Hallam *et al.* 2001; Phillips & von Reyn 2001).

The pathogenic potential of NTM may still be debatable, since it has not always been possible to demonstrate causation in human diseases. Nevertheless, defects of the body's defence mechanisms enhance the susceptibility of the host to infections. With advances in modern medicine, the population at risk increases and the presence of severely immunocompromized patients has to be taken into consideration (Phillips & von Reyn 2001). Therefore, the occurrence of mycobacteria in hospital water systems is disturbing. As potential nosocomial pathogens they can cause infections and outbreaks in health care settings (Wallace *et al.* 1998; Squier *et al.* 2000), often through contamination of medical devices (Uttley & Simpson 1994; Cooke *et al.* 1998; Pang *et al.* 2002). The role of temperature in NTM survival and proliferation has been raised and recommendations have been proposed to reduce the risk of infections (Sniadack *et al.* 1993).

Our study confirmed the widespread colonization of NTM in distributed water. Most of the species we isolated are considered mainly saprophytic, but the high NTM concentration from sites such as the nursery room and the operating theatres is disturbing in view of the serious risk for nosocomially acquired infections. Because of the high number recovered, it is also likely that the mycobacteria were not transient colonizers of the water pipes but once introduced, the environment was suitable for a permanent colonization. In this respect, the presence and significance of biofilms for the persistence of mycobacteria have to be recognized in any control measures. Finally, HPLC analysis of cell wall mycolic acids profiles could well be used as a first-choice method for identification of environmental mycobacteria.

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