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Prevalence and Concentration of Non-tuberculous Mycobacteria in Cooling Towers by Means of Quantitative PCR: A Prospective Study

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Abstract There is an increasing level of interest in non-tuberculous mycobacteria (NTM) due to the increasing reported rates of diseases caused by them. Although it is well known that NTM are widely distributed in the environment it is necessary to identify its reservoirs to prevent possible infections. In this study, we aimed to investigate the occurrence and levels of NTM in cooling towers to provide evidences for considering these settings as possible sources of respiratory infections. In the current study, we detected and quantified the presence of NTM by means of a rapid method in water samples taken from 53 cooling towers of an urban area (Barcelona, Spain). A genus-specific quantitative PCR (Q-PCR) assay with a quantification limit (QL) of 500 cells l^{-1} was used. 56% (30) of samples were positive with a concentration range from 4.6×10^3 to 1.79×10^6 cells l^{-1}. In some cases (9/30), samples were positive but with levels below the QL. The colonization rate confirmed that cooling towers could be considered as a potential reservoir for NTM. This study also evaluated Q-PCR as a useful method to detect and quantify NTM in samples coming from environmental sources.

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Introduction

Non-tuberculous mycobacteria (NTM) have ubiquitous distribution [9]. They are found in soil and water, including both natural and treated water sources. Many species have also been isolated from human pathological samples where they cause opportunistic infections. Recent retrospective studies have highlighted increasing reported rates of NTM diseases worldwide [7, 11, 13, 15–17].

The most common clinical manifestation of NTM infection is pulmonary disease [10, 12] due possibly to inhalation of aerosols containing microorganisms. The hydrophobic nature of mycobacteria makes them especially prone to be concentrated in the micro-droplets making up aerosols, where they remain viable [21]. Moreover, the ability of NTM to grow in biofilms may lead to dissemination into the bulk water, constituting a risk for consumers by drinking or through the inhalation of aerosols from showers, swimming pools, spas and other water systems [6, 8, 24].

Non-tuberculous mycobacteria (NTM) infections are frequently acquired by inhalation or inoculation from contaminated water samples. Despite their omnipresence in environmental sources, the actual transmission to humans, with subsequent clinical disease, has rarely been proven [29]. Recent studies suggest cooling towers as a possibility [18]. Such environments not only harbour a variety of

microorganisms but they also disseminate them in aerosols. People living in urban areas are continuously exposed to these aerosols generated by cooling towers.

Few evidences exist, suggesting the presence of mycobacteria in cooling towers: Black and Berk [4] isolated by culturing slow-growing species and identified them by conventional biochemical methods. Recently, Pagnier et al. [19] using a co-culture method detected the presence of rapid-growing amoeba-associated mycobacteria species in three cooling towers. They further identified the isolated mycobacteria by molecular methods. Despite of these evidences no prospective studies have been carried out in order to evaluate the real prevalence and concentration of NTM in these settings.

Previous studies have also proved that culture protocols currently in use can underestimate the presence of mycobacteria in biofilms and aerosols [1]. Although molecular techniques such as quantitative PCR (Q-PCR) have been applied in several settings for improving the detection and quantification of mycobacteria [5, 14], it has never been proven in cooling towers.

In this prospective study, we observed a considerable prevalence and in some cases, a high concentration of NTM in cooling towers by means of a Q-PCR assay. According to the results, cooling towers are a likely reservoir of NTM and consequently they should be considered as feasible infection source in future epidemiological studies.

Materials and Methods

Mycobacterial Strains

PCR primers used in this study were previously tested [20], although their specificity was re-checked against multiple additional mycobacteria and few non-mycobacteria species.

Twenty-two different strains of fast and slow-growing mycobacteria, listed in Table 1, were tested as positive controls. They were obtained from American Type Culture Collection (ATCC) or German Collection of Microorganisms and Cell Cultures (DSMZ), with the exception of three clinical isolates: *Mycobacterium abcessus* (obtained from the strains collection of the Mycobacteriology Laboratory.

Table 1 Melting temperature for all the strains analysed

Species	Source	Amplification	Melting temperature (°C)
Mycobacterium abscessus	Clinical isolate	+	83.92
Mycobacterium aichiense	ATCC 27280	+	87.43
Mycobacterium alvei	ATCC 51304^T	+	86.09
Mycobacterium aurum	ATCC 23366	+	86.65
Mycobacterium chubuense	ATCC 27278	+	86.02
Mycobacterium fortuitum	ATCC 35754	+	86.54
Mycobacterium gilvum	DSM 43547	+	85.84
Mycobacterium goodii	ATCC 700504	+	86.95
Mycobacterium gordonae	ATCC 14470	+	85.73
Mycobacterium intracellulare	ATCC 13950^T	+	86.27
Mycobacterium kansasii	ATCC 35775	+	86.06
Mycobacterium mucogenicum	ATCC 49649	+	86.86
Mycobacterium obuense	ATCC 27023	+	85.78
Mycobacterium palustre	FI 16795	+	87.58
Mycobacterium parafortuitum	ATCC 19686	+	86.00
Mycobacterium septicum	DSMZ 44393	+	86.93
Mycobacterium scrofulaceum	ATCC 19981^T	+	87.20
Mycobacterium smegmatis mc^{2}155	ATCC 700084	+	87.43
Mycobacterium triplex	DSMZ 44626	+	86.20
Mycobacterium vaccae	ATCC 15483^T	+	86.54
Mycobacterium wolinski	ATCC 700010	+	86.98
Mycobacterium xenopi	Clinical isolate	+	87.71
Escherichia coli O157:H7	ATCC 43895	–	n.a.
Pseudomonas aeruginosa	ATCC 6872	+	76.67
Legionella pneumophila	NCTC 11984	+	76.33
Enterococcus faecalis	ATCC 23655	+	76.56

ATCC American type culture collection, DSMZ German collection of microorganisms and cell cultures, NCTC national collection of type cultures, *Tm* melting point temperature (°C) obtained after the run, *n.a.* not applicable

Universitat Autònoma de Barcelona, Barcelona, Spain), *Mycobacterium palustre* (provided by Dr. E. Tortoli. Regional Mycobacteria Reference Centre, Florence, Italy) [28], and *Mycobacterium xenopi* (provided by Dra. Nuria Martin-Casabona. Vall d'Hebrón Hospital, Barcelona, Spain). All strains were cultured on Middlebrook 7H10 agar (Difco Laboratories), supplemented with 10% oleic-albumin-dextrose-catalase (OADC) enrichment media.

Four strains of different microorganisms (*Escherichia coli 0157:H7*, *Pseudomonas aeruginosa*, *Legionella pneumophila* and *Enterococcus faecalis*) were also tested, as negative controls.

Collection of Water Samples

Water samples from 53 different cooling towers, taken from 25 different industries in the urban area around Barcelona city (NE Spain), were collected during a 6-month period. All towers are located near (<0.5 km) or in residence areas. From each cooling tower 2 l of water were taken in sterile plastic containers supplemented with 2 ml of sodium thiosulphate (3%). The criteria for sampling selection was the wherever practicable point located on the return line, water was sampled in the tower basin below the filing material. Samples were transported at <8°C; they were received by the laboratory in less than 5 h, and stored at 2–5°C until they were processed, always within 18 h after reception.

DNA Extraction

One litre of water from each cooling tower was concentrated by filtration, using a nylon membrane (0.45 µm porous diameters, Millipore). Filtered cells were then resuspended in 10 ml sterile saline solution (0.85% NaCl), by vigorous vortexing for 60 s into a tube containing 15 glass beads (diameter, 5 mm), followed by sonication for 3 min (Selecta, Ultrasons 6L 150 W). Finally, a cell suspension (2 ml) was concentrated by centrifugation (10,000×*g*, 5 min) and resuspended to a final volume of 150 µl prior to DNA extraction using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. Additionally, if samples were suspected to be highly contaminated, the DNA extraction was made directly from 150 µl of the first concentration volume of the sample.

Mycobacteria cells were scraped from the 7H10-OADC culture plates, resuspended in 1.5 ml of sterile saline solution, concentrated by centrifugation (10,000×*g,* 5 min) and resuspended again in 200 µl of phosphate buffer saline (PBS). To ensure a correct DNA extraction, samples were exposed to two cycles of frozen (15 min, −20°C) and heating (10 min, 99°C), prior to DNA extraction with the same kit.

Quantitative PCR

Genus-specific mycobacterial primers (Internal Transcribed Spacer (ITS)-F, 16S rRNA, 5′-TGGATCCGACGA AGTCGTAACAAGG-3′, and Mycom2, ITS region, 5′-AT GCTCGCAACCACTATCCA-3′) were used as previously described [20], but adapted to a Q-PCR assay.

The Q-PCR assay was performed using Quantitec SYBR Green PCR mix (Qiagen) in a Roche LightCycler 1.5 with a 0.5 µM final primer concentration. Ten µl of each sample were analysed by duplicate, in a final volume of 20 µl in glass capillaries. The PCR conditions, previously optimized (data not shown), were: initial activation step at 95°C for 15 min followed by 45 cycles at 94°C for 15 s of denaturing step, 60°C for 30 s of annealing step and 72°C for 30 s of elongation step. Melting curves were obtained by initial denaturation at 95°C, immediately cooling to 65°C for 15 s and heating to 95°C at 0.10°C/s with continuous fluorescence measurement.

In order to semi-quantify the amplification results, serial logarithmic dilutions of *M. vaccae* from 10^1 to 10^5 cells/reaction were used as standard. As was previously described, 10 mg/ml of *M. vaccae* cells is equivalent to approximately 1×10^{10} cells/ml [26]. This ratio was used to estimate the number of cells in the Q-PCR assay. Efficiency of amplification was calculated according to the slope from the calibration dilution curve [22]. The estimated quantification limit (QL) of this technique taking into account the detection limit of the equipment, volume of filtered water and the standard curve calculated, was 500 cells $l^{-1.}$

Samples were considered as negative when non-amplification was observed or when there was amplification but the melting temperature was out of the range of the temperatures observed in previous amplifications using the reference strains (Table 1). Samples were considered as positive when the amplification of at least one of the replicas was produced and the melting temperature was inside the mentioned range. Finally, samples were considered positive but non-quantifiable when the amplification was produced with a melting temperature inside the considered range, but with a Ct higher than 40.

Identification of Mycobacteria by Intergenic Region Sequencing

Eight of the amplified samples were recovered from the LightCycler capillaries and then run on a 2% E-gel agarose (Invitrogen) using the E-gel iBase system (Invitrogen). As positive controls, *Mycobacterium gordonae*, *Mycobacterium kansasii* and *M. vaccae* were run in parallel to the eight samples. The negative control capillary (purified PCR-grade water) was also recovered and included as a

negative control. In addition, samples were purified using Cycle-Pure kit (Omega Biotek) and sequenced by SECUGEN (www.secugen.es) company, using Mycom2 primer.

DNA sequences were analyzed using the basic local alignment search tool (BLAST) [2] at the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

In the adapted Q-PCR assay, we used a well-known set of primers extending the intergenic region between the 16S and 23S rRNA genes (so called ITS) and specific for the *Mycobacterium* genus [20]. As additional verification for primer specificity 22 reference *Mycobacterium* strains were tested. As expected, all of them were positive. The range of melting temperatures obtained in the NTM studied were similar to those obtained by Richardson et al. [23] even though these authors used different set of primers and amplicon. The non-mycobacterium species (*E. coli*, *P. aeruginosa*, *L. pneumophila* and *E. faecalis*) presented no amplification or amplification but with melting temperatures out of the range accepted. This result also confirmed primer specificity (data not shown).

Fifty-three samples from different cooling towers were analyzed. Fifty-six percent of the samples (30 samples) were positive for *Mycobacterium* (see Table 2) according to the criterion described in 'Materials and Methods' section. Our positive results showed melting temperatures ranging from 83.32 to 87.51°C, which are in complete agreement to the values found before.

As an additional verification of correct amplification, eight of the positive amplified samples were evaluated by sequencing. These randomly selected samples together with *M. gordonae*, *M. kansassii* and *M. vaccae* reference strains as positive controls, were run in a 2% agarose gel and further sequenced. The cooling tower samples showed good amplification results with expected amplified band ranging from 250 to 400 bp, which were in good agreement with the amplification product obtained previously [20] (data not shown). Subsequently, the amplified samples were subjected to sequencing. As shown in Table 3, the sequencing results of the positive controls displayed a 100% identity to the corresponding *Mycobacterium* species. The sequences from the cooling tower samples also match to different *Mycobacterium* strains. Two samples were identified as close homologues of *M. gordonae*, *M. kansasii*, *M. fortuitum* and the rest were identified as different *Mycobacterium* sp. (Table 3).

Twenty-one out of 30 positive samples were quantifiable as described in the 'Materials and Methods' section. The lowest concentration found was 4.6×10^3 cells per litre and the highest was 1.79×10^6 cells per litre, with a QL

Table 2 Real-time PCR amplifications with cooling tower samples

No	Source	Amplification (cells/l)	Tm (°C)
1	A1	–	76.93
2	B1	+	85.34
3	C1	–	77.30
4	D1	+	86.00
5	E1	–	77.04
6	F1	–	77.36
7	F2	+	86.46
8	F3	–	77.19
9	F4	–	76.60
10	G1	–	77.42
11	G2	6.85×10^3	86.69
12	G3	–	76.70
13	G4	4.63×10^3	87.00
14	G5	+	87.51
15	H1	–	n.d
16	I1	–	77.20
17	I2	+	85.47
18	I3	9.46×10^3	85.09
19	I4	–	76.22
20	I5	–	76.68
21	J6	+	83.32
22	J7	–	77.72
23	K1	–	77.03
24	L1	2.05×10^4	87.12
25	L2	+	85.45
26	L3	3.48×10^4	86.16
27	L4	+	86.18
28	M1	–	77.51
29	M2	1.31×10^5	85.35
30	M3	6.79×10^4	86.06
31	M4	–	77.01
32	N1	–	n.d
33	O1	5.57×10^4	87.34
34	P1	9.20×10^4	86.58
35	Q1	+	87.44
36	R1	–	n.d
37	R2	3.14×10^4	85.85
38	R3	–	75.98
39	S1	2.02×10^4	86.12
40	T1	–	77.60
41	U1	1.91×10^4	87.06
42	U2	–	n.d
43	V1	4.42×10^4	86.67
44	W1	4.60×10^3	86.08
45	W2	1.48×10^4	86.13
46	W3	–	77.17
47	W4	1.52×10^4	85.65
48	W5	1.14×10^5	86.03

Table 2 continued

No	Source	Amplification (cells/l)	Tm (°C)
49	X1	5.57×10^4	86.89
50	X2	8.08×10^5	85.18
51	X3	1.79×10^6	85.44
52	X4	–	76.77
53	Y1	3.29×10^5	85.30

Source cooling tower water samples from 25 different locations (each location with a *different letter* code), *Tm* melting point temperature (°C) obtained after the run, *n.d.* non detected; + positive but non quantifiable, if quantifiable, results are expressed in cells/l, – not detected

estimated for this technique of 500 cells l^{-1}. Only nine out of 30 positive samples were considered non-quantifiable.

Discussion

This is a first survey, and further and more exhaustive studies should be carried out, but we confirmed and quantified the presence of NTM in cooling towers, which is the basis to support the hypothesis about the spread of NTM by theses settings.

Our results are in agreement with those obtained previously [4, 19], who also detected NTM in cooling towers but by culturing methods. The proposed alternative method is more rapid and sensitive and allows a reliable quantification of NTM concentration in samples. Considering that only a pair of primers for *Mycobacterium* sp. has been used, the quantification is not precise. If an accurate quantification would be necessary, specific primers for each *Mycobacterium* should be used. Taking into account that some legionellosis outbreaks from cooling towers had been produced with levels (by culture) of at least 10^5 CFU/l [3], the concentrations found in this work are high enough to consider that the analyzed cooling towers could show a risk to Public Health.

Little information is found in the literature to support the hypothesis of NTM disease transmission from cooling tower aerosols. Since the epidemiology of NTM diseases is not obligatory to report, the information regarding the occurrence of outbreaks is likely to be underestimated. Recent reviews [11, 27] describe the need to identify the microbial agents responsible for lung diseases in the workplace and also due to household exposure. In this line, some evidence arises when Shelton et al. [25] investigating the hypersensitivity pneumonitis in three machine workers, obtained data suggesting the aerosolized NTM as a possible cause.

Different questions have to be considered in order to assess the microbial health risk. First, consideration must be made of the physiological characteristics of mycobacteria that are consistent with transmission through aerosols (although little information exists on aerosolization of the environmental opportunistic mycobacteria) [8], added to the own nature of cooling tower, that is widely known to

Table 3 Identification of sequence samples after blast analysis

Lane	Source	Description	Acc. Num	*E* value	Max identity (%)
1	M2	*Mycobacterium gordonae* 23S ribosomal RNA (23S rRNA) gene and 16S ribosomal RNA (16S rRNA) gene	L42260.1	3e-96	99
2	M3	*Mycobacterium gordonae* strain Tropicalis-2 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	EU497913.1	2e-82	96
4	V1	*Mycobacterium* sp. THO100 16S ribosomal RNA gene and 16S-23S intergenic spacer, partial sequence	AY642533.1	2e-39	82
5	X3	*Mycobacterium* sp. Thai4 genes for 16S rRNA, internal transcribed spacer, 23S rRNA, partial and complete sequence	AB178778.1	1e-90	99
6	W1	*Mycobacterium kansasii* partial 16S rRNA gene, partial 23S rRNA gene and ITS1, isolate K7377-03	AM709725.1	3e-75	77
7	W2	*Mycobacterium fortuitum* internal transcribed spacer 1 (ITS1), strain S358	AJ291593.1	6e-110	95
8	X2	Uncultured *Mycobacterium* sp. clone NSG3Q1i16 16S-23S internal transcribed spacer, partial sequence	EU697033.1	1e-93	99
Mv	*M. vaccae*	*Mycobacterium vaccae* 16S-23S intergenic spacer region, complete sequence	AF163814	2e-148	100
Mg	*M. gordonae*	*Mycobacterium gordonae* 16S ribosomal RNA gene, partial sequence; internal transcribed spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	AJ604571.1	1e-107	100
Mk	*M. kansasii*	*Mycobacterium kansasii* isolate 1635 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic spacer, partial sequence	AM709725.1	2e-98	100

disseminate the bacteria in the form of aerosols [4]. Second, the high abundance of *Mycobacterium* spp. rRNA gene sequences in the clone libraries from air samples, show strong evidence to validate aerosol partitioning as a mechanism for acquisition of pulmonary disease in this environment [1]. Third, *Mycobacterium* species are usually resistant to the commonly used disinfectant treatments for cooling tower maintenance and, as a consequence, its selective growth can be favoured [8], increasing the risk of transmission. Finally, the increasing reported rates of NTM disease must also be taken into account [13]. As a consequence, the sanitary risk associated with the presence of NTM in cooling towers could be significant, especially if opportunistic pathogens can be found (as *M. kansasii* and *M. fortuitum* were found in some of the samples), even though additional epidemiological surveys would be required to confirm this hypothesis.

In conclusion, our work presents the first prospective study about presence of NTM in cooling towers, using Q-PCR. The method presented is rapid and sensitive and could be used as a screening, alleviating the need of culturing and then providing a substantial advantage in a sanitary crisis.

Our results show the presence of NTM in cooling towers; this represents the confirmation of the hypothesis suggested from other authors. That means that cooling towers could be considered potential reservoirs for NTM. Further analysis should be carried out, with specific primers and an exhaustive epidemiologic design, to accurately define the risk.

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