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Occurrence of Nontuberculous Mycobacteria in Environmental Samples

TERRY C. COVERT,* MARK R. RODGERS, ANTOLIN L. REYES, AND GERARD N. STELMA, JR.

National Exposure Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268

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Nontuberculous mycobacteria (NTM) are a major cause of opportunistic infection in immunocompromised hosts. Because there is no evidence of person-to-person transmission and NTM have been found in drinking water, the environment is considered a likely source of infection. In this study the widespread occurrence of NTM was examined in drinking water, bottled water, and ice samples. A total of 139 samples were examined for NTM by a membrane filtration culture technique followed by PCR amplification and 16S rRNA sequence determination to identify the isolates. NTM were not detected in bottled water or cisterns but were detected in 54% of the ice samples and 35% of the public drinking-water samples from 21 states. The most frequently occurring isolate was *M. mucogenicum* (formerly referred to as an *M. chelonae*-like organism).

Attitudes toward the genus *Mycobacterium* have long been dominated by the belief that *Mycobacterium tuberculosis* was the only clinically significant species, beginning in 1882 when Koch first described the "tuberkelbazillus." This organism was so important in public health that for the next 70 or 80 years other acid-fast bacilli found in humans, animals, or the environment were usually dismissed as saprophytes of little consequence (4, 26). Changes in attitudes toward species of mycobacteria other than *M. tuberculosis* were stimulated by numerous reports in the 1950s and 1960s that acid-fast bacilli had been cultured from pathological materials under circumstances that led some to believe that these organisms may be clinically significant. Searches for mycobacteria in the environment were renewed. Water did not at first assume much importance among the many sources of what became known as "atypical," "anonymous," "opportunist," "tuberculoid," or "nontuberculous" mycobacteria. Recently, however, there has been increasing evidence that water may be the vehicle by which these organisms infect or colonize the human body.

The nontuberculous mycobacteria (NTM) include those *Mycobacterium* species that are not members of the *Mycobacterium tuberculosis* complex. In recent years NTM have emerged as a major cause of opportunistic infections in those who have AIDS. NTM disease in AIDS is caused primarily by *M. avium* and is second only to AIDS wasting syndrome as the most common cause of death (11). There have been numerous reports showing that NTM can survive, persist, grow, and colonize in drinking water supply systems (6, 7, 23, 24). Without evidence of person-to-person transmission, it is proposed that humans are infected from environmental sources. There have been previous studies examining drinking water supplies in the United States for NTM. Generally, these studies have focused on the occurrence of MAC organisms (*M. avium* and *M. intracellulare*) only and examined samples from a limited geographical area, i.e., Boston (6), Los Angeles (9), and the northeastern United States (24). The present study examined samples from widely dispersed drinking water utilities and other environmental samples to determine not only the occurrence of MAC organisms but also of other NTM which may be clinically significant.

(This study was presented in part at the 97th General Meeting of the American Society for Microbiology, Miami, Fla., May 4 to 8, 1997 [4a].)

MATERIALS AND METHODS

A total of 139 samples were collected from five different types of sources. Sources included public drinking water utilities, cisterns, bottled waters, drinking water treatment samples, and ice samples from commercially available sources and ice machines on hospital patient floors. Drinking water samples were collected from geographically dispersed sites (21 states) (Fig. 1), including drinking water supply systems that use a variety of treatments (filtered, nonfiltered, disinfected, and not disinfected) and source water (surface and ground). From each site one to four samples was collected according to *Standard Methods* (1) in 2-liter sterile sample bottles containing sodium thiosulfate (0.1 mg/liter). Samples were collected from cold taps, warm taps, or showers. The taps or showers were run for 1 to 2 min to clear the service lines prior to sample collection. The temperatures of the samples were recorded, and total and free chlorine analyses were performed by the *N*,*N*-diethyl-*p*-phenylenediamine colorimetric method (1). The age of the sample site (residence or hospital), the type of drinking water treatment, and the source water information were provided with the samples. Noncarbonated bottled water samples were collected from multiple bottlers. Ice samples were collected in 4-liter sterile wide-mouth sample bottles and allowed to melt at room temperature before the analyses. Drinking water treatment samples included a reservoir sample, a membrane filter effluent, and water samples from ice machine treatment cartridges. Samples were transported to the laboratory and were kept at 1 to 4°C until analysis. All samples were analyzed within 48 h of collection.

Samples were analyzed for NTM by the method reported by Glover et al. (9), except that a 30-min exposure to 0.04% cetylpyridinium chloride (Sigma Chemical Co., St. Louis, Mo.) was used to reduce background organism levels. Heterotrophic plate counts (HPC) were performed in duplicate on drinking water and bottled water samples by the R2A membrane filter method (1). Three 500-ml sample volumes were filtered through 0.45-μm-pore-size, black-grid, HABG 47-mm membrane filters (Millipore Corp., Bedford, Mass.) by vacuum filtration after exposure to cetylpyridinium chloride (CPC). After filtration the membrane filters were rinsed with buffer (1) and aseptically transferred to Middlebrook 7H10 agar (Difco Laboratories, Detroit, Mich.) plates containing 500 mg of cycloheximide (Sigma Chemical Co.) per liter. The plates were sealed in gas-permeable bags (Fisher Scientific, Pittsburgh, Pa.) and incubated at 37°C with 10% CO_2. The filters were examined at weekly intervals for 8 weeks with a stereoscopic microscope. NTM colony types were acid-fast stained, enumerated, and transferred to Middlebrook 7H10 agar slants and Middlebrook 7H9 broth (Difco Laboratories) for further identification. The selection of NTM colonies was based on the description of colonial morphologies by Glover et al. (9).

We used a PCR-mediated partial sequence analysis of the gene encoding the 16S rRNA to identify the isolates (20). DNA was extracted from the isolates grown either on Middlebrook slants or broth by the rapid freeze-thaw lysis

* Corresponding author. Mailing address: U.S. Environmental Protection Agency, National Exposure Research Laboratory, 26 W. Martin Luther King Dr., Cincinnati, OH 45268. Phone: (513) 569-7318. Fax: (513) 569-7117. E-mail: Covert.Terry@epamail.epa.gov.

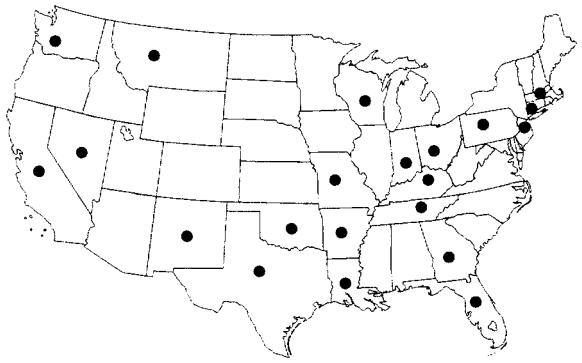

FIG. 1. States where public drinking water supply systems were sampled.

technique reported by Reischl et al. (18). After cell lysis the suspension was centrifuged at 14,000 rpm for 3 min (Eppendorf microcentrifuge) to remove the disrupted cell walls, and the lysate containing the genomic DNA was transferred to a new microcentrifuge tube for subsequent amplification. The PCR was performed in a 50-μl reaction mixture (Perkin-Elmer, Foster City, Calif.) as described by Springer et al. (20). A single 1,037-bp fragment was detected in all reactions. Three separate amplification reactions were run for each isolate, and the PCR products were pooled before sequencing. PCR products were purified for sequencing by using Microcon 50 microconcentrators (Amicon, Beverly, Mass.). DNA template was prepared for fluorescence-based cycle sequencing by using an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Sequencing primers 244 and 259 (3.2 pmol each) were used for sequencing of hypervariable regions A (corresponding to *E. coli* positions 129 to 267) and B (corresponding to *E. coli* positions 430 to 500), respectively. Extension products were purified by using Centri-Sep spin columns (Princeton Separations, Adelphia, N.J.). Identification of the 16S rRNA sequence was performed with an ABI 373A Sequencer (Perkin-Elmer) with a 6% polyacrylamide-urea gel. Sequence data of hypervariable region A were generated for all isolates, and species identification was based on these data. Hypervariable region B sequence data were generated for selected isolates. Identification was based on an exact match with the sequence data reported by Springer et al. (20) or with published sequences in GenBank by using DNASTAR software (DNASTAR, Inc., Madison, Wis.).

RESULTS

Occurrence of NTM. NTM were isolated from 46 of the 139 samples analyzed (33%) and from three of the five sources examined (Table 1). The occurrence of NTM in drinking water samples from distribution systems that use groundwater was similar to distribution systems that use surface water: 31 and 36%, respectively. NTM were not isolated from cisterns, noncarbonated bottled waters, or reservoir samples. All of the ice samples from ice machines located on hospital patient floors were NTM positive, but NTM were not isolated from commercially bagged ice. Two of the ice machine treatment cartridges from hospitals were contaminated with NTM.

TABLE 1. Occurrence of mycobacteria

Sample source (no. of samples analyzed)	No. (%) of samples with mycobacteria detected
Bottled water (11)	0
Drinking water, ground[a] (16)	5 (31)
Drinking water, surface[b] (89)	32 (36)
Cistern (6)	0
Ice	
Commercially bagged (5)	0
Hospital ice machines (6)	6 (100)
Drinking water treatment	
Reservoir (1)	0
Membrane filter effluent (1)	1 (100)
Ice machine treatment cartridges[c] (4)	2 (50)
Total (139)	46 (33)

[a] Drinking water samples from utilities that use groundwater as source water.
[b] Drinking water samples from utilities that use surface water as source water.
[c] Water samples from ice machine treatment cartridges.

TABLE 2. Characteristics of drinking water samples

Characteristics	Types of drinking water samples		
	Ground source water	Surface source water	Cistern
HPC (CFU/ml)			
No. of samples	16	89	6
Mean ± SE	5,700 ± 2,426	6,000 ± 2,091	39,000 ± 22,700
Median (range)	65.0 (5–30,000)	90.0 (1–120,000)	7,100 (1–120,000)
Free chlorine (mg/liter)			
Mean ± SE	0.40 ± 0.10	0.61 ± 0.06	NC[a]
Median (range)	0.20 (<0.1–1.0)	0.40 (<0.1–2.5)	
Total chlorine (mg/liter)			
Mean ± SE	0.53 ± 0.10	0.90 ± 0.10	NC
Median (range)	0.50 (<0.1–1.0)	0.70 (<0.1–2.8)	
Age of sample site[b] (yrs)			
No. of sites	7	43	3
Mean ± SE	42.0 ± 15.5	48.0 ± 4.3	25.3 ± 1.2
Median (range)	20.0 (10–120)	40.0 (2–125)	26.0 (22–28)
Cold temp (°C)[c]			
No. of sites	7	50	3
Mean ± SE	20.3 ± 0.5	19.0 ± 0.8	22.3 ± 0.8
Median (range)	20.0 (18.8–25.0)	20.0 (10.8–27.0)	23.0 (20–24)
Warm temp (°C)[d]			
No. of sites	4	30	3
Mean ± SE	36.7 ± 1.6	41.4 ± 1.1	46.7 ± 3.3
Median (range)	36.0 (34.0–41.0)	40.0 (30.6–59.6)	48.0 (38.0–54.0)

[a] NC, not chlorinated.
[b] Age of building or residence where samples were collected.
[c] Temperature of cold tap water.
[d] Temperature of warm tap water or shower sample.

Sample characteristics. The highest mean HPC levels were from cistern samples (Table 2). The mean and median values for HPC CFU per milliliter of drinking water samples from groundwater and surface water sources were similar. Of the 37 NTM-positive samples from drinking water supply systems, 18 had an HPC of ≤500 CFU/ml. Of the NTM-positive samples, 19 had an HPC of >500 CFU/ml. The mean free and total chlorine levels were above 0.2 mg/liter, as specified by U.S. Environmental Protection Agency (USEPA) drinking water regulations (8). NTM were recovered from drinking water samples with free chlorine levels of up to 2.5 mg/liter and total chlorine levels of 2.8 mg/liter. Of the NTM-positive drinking water samples, 81% demonstrated detectable chlorine levels at the time of sampling; the mean free chlorine level of the NTM-positive samples was 0.7 mg/liter, and the mean total chlorine level was 1.0 mg/liter. NTM were isolated from sample sites ranging in age from 2 to 125 years. Of the drinking water samples from cold taps, 24 (65%) were NTM positive, and 13 samples (35%) from warm taps or showers were NTM positive. The highest water temperature in which NTM were isolated (*M. intracellulare*) was from a 50.5°C shower sample. Data submitted from sample collectors regarding the type of treatment the drinking water utilities used were in some cases incomplete; however, the majority of drinking water utilities

TABLE 3. Recovery of NTM categorized by sample source

Sample source	NTM species	
	No. (%) of NTM-positive samples	NTM species recovered
Drinking water		
Ground	5 (100)	*M. gordonae*
Surface	13 (41)	*M. mucogenicum*
	5 (16)	*M. intracellulare*
	3 (9)	*M. gordonae*
	3 (9)	*M. gastri/kansasii*
	1 (3)	MCRO 45[a]
	1 (3)	MCRO 19[a]
	1 (3)	X88911[b]
	1 (3)	*M. fortuitum*
	1 (3)	*M. peregrinum*
	1 (3)	*M. scrofulaceum*
	1 (3)	*M. avium*
	3 (9)	Unknown[c]
Ice (hospital patient floors)	5 (83)	*M. fortuitum*
	1 (17)	*M. peregrinum*
Drinking water treatment		
Membrane filter effluent	1 (100)	*M. chelonae*
Ice machine treatment cartridge	1 (50)	*M. fortuitum*
	1 (50)	*M. gordonae*

[a] Related to *M. simiae* (20).
[b] Related to *M. scrofulaceum* (27).
[c] The hypervariable region A and B sequences did not match or no match could be found for the hypervariable region B sequence.

used conventional treatment (coagulation, sedimentation, filtration, and disinfection).

Levels of NTM. The counts of NTM in water samples ranged from 1 CFU/500 ml to too numerous to count. The majority of samples (>80%) showed colony counts of 1 to 20 NTM colonies/500 ml. Despite measures to control contamination with CPC and cycloheximide, *Bacillus* spp. and fungal overgrowth was a major problem with some samples. This was particularly evident with reservoir and cistern samples.

Identification of isolates. Table 3 lists the identification of the organisms isolated in this study. Based on the sequencing of the hypervariable region A of the 16S ribosomal gene, a total of 113 isolates were placed in 13 distinguishable groups. The sequence of the hypervariable region B was generated for many of the isolates and used to confirm the initial identification. Nine of the thirteen groups could be assigned to a known species. Both rapid- and slow-growing mycobacteria were isolated, including species which are considered human opportunistic pathogens. Of the remaining four groups, two (MCRO 19 and MCRO 45) have sequences identical to those of several unidentified clinical mycobacterial isolates reported by Springer et al. (20) to be related to *M. simiae*. The sequence of one isolate (X88911) is identical to a published sequence from a slow-growing mycobacterium related to *M. scrofulaceum* (27). The last group of isolates are listed as unidentified (Table 3) either because their hypervariable region A and B sequences did not match or because no match could be found for the hypervariable region B sequence.

Recovery of NTM species by sample source. *M. gordonae* was the only NTM species recovered from drinking water samples from distribution systems that use groundwater as the source water (Table 3). *M. mucogenicum* was the most frequently isolated organism (40 of 113 isolates [35%]) and was the most frequently isolated NTM from drinking water samples from distribution systems that use surface water as the source water. *M. mucogenicum*, *M. intracellulare*, *M. gastri/kansasii*, and *M. gordonae* represented 75% of the isolates from drinking water. *M. fortuitum* was the isolate obtained most frequently from ice samples from hospital patient floors and was recovered from treatment cartridges from these ice machines. MAC organisms were isolated from 19% of the NTM-positive samples.

DISCUSSION

Differentiation of mycobacteria to the species level by evaluation of phenotypic and biochemical tests is time-consuming because of the slow growth rate of mycobacteria. Phenotypic and biochemical test results may vary depending on the growth conditions, sometimes leading to inaccurate results. In response to these limitations, other identification methods have been developed such as lipid analysis, restriction fragment length polymorphism (RFLP) analysis of the heat shock gene, and DNA sequence analysis of the rRNA genes. Identification by nucleic acid probes (Gen-Probe, Inc., San Diego, Calif.) is a rapid method, but it requires several probes and covers a limited range of mycobacterial species. We attempted to use the RFLP method of Telenti et al. (22) to identify the isolates. The RFLP patterns we observed were similar to those reported by Telenti et al.; however, the band sizes varied by 10 to 12 bp. Similar experiences were reported by Steingrube et al. (21) when using this method. The difference may be attributed to our band size measurements provided by a fluorescence analyzer, the Fluorimage SI (Molecular Dynamics, Inc., Sunnyvale, Calif.), with ImageQuant computer software (version 4.2; Molecular Dynamics, Inc.) versus the practice reported by Telenti et al. of measuring the running distance visually and rounding the size to the nearest 5 bp. Since we were unable to confidently identify our isolates by this method, the isolates were identified by the 16S ribosomal gene sequencing method. Of the 113 isolates, 97 were identified by this method. We were unable to identify a group of slow-growing mycobacteria isolates from a site in Florida. Although the region A sequences from these isolates matched exactly with previously published sequences, a majority of the isolates had a unique region B sequence for which a match could not be found in the GenBank and EMBL databases. Springer et al. (20) noted that one advantage of using ribosomal sequences for genotypic identification of bacteria is the possibility of recognizing previously undescribed species. More sequence data are needed for these isolates in order to determine whether they represent previously uncharacterized species of mycobacteria. One disadvantage of using this method is the inability to distinguish *M. gastri* from *M. kansasii*.

Because NTM disease in immunocompromised hosts is primarily disseminated to many organs, questions have been raised concerning the portal of entry of these mycobacteria. Before the discovery of AIDS and in the absence of evidence of person-to-person spread of NTM, pulmonary infection was thought to be due to aerosol inhalation (28). However, in AIDS patients and other immunocompromised hosts, infection can occur via the gastrointestinal tract, lungs, or both (13). The environment is considered a likely source (15). Thus, a wide range of sources, exposures, and modes of transmission need to be investigated. Having data available showing the occurrence of these important emerging opportunistic pathogens greatly aids the assessment of risks to susceptible hosts from exposure to drinking water and other environmental samples that may have these organisms.

NTM were isolated from 33% of all samples. Drinking water samples were collected from 42 drinking water supply systems from 21 states. NTM were isolated from 16 (38%) of 42 drinking water supply systems. Only one NTM species (*M. gordonae*) was isolated from groundwater supplies; however, there were

eight NTM species recovered from surface water drinking water supplies. This may be attributed to higher levels of organic matter, feces, and soil in surface water contributing to the mycobacterial flora (4, 7). The most frequently occurring isolate was *M. mucogenicum*, followed by *M. intracellulare*, *M. gordonae*, and *M. gastri/kansasii*. MAC organisms were isolated from 6 (19%) of 32 NTM-positive samples from four drinking water systems supplied by surface water, and *M. avium* was isolated from 1 (3%) of 32 NTM-positive samples from one of these systems. Overall, MAC organisms were found in 9% of the drinking water supplies examined. *M. fortuitum* and *M. pregrinum* were isolated from ice samples from hospital ice machines, and *M. fortuitum* and *M. gordonae* were isolated from ice machine treatment cartridges, indicating NTM-contaminated ice may pose a risk to immunocompromised patients in hospitals. Other studies have shown that contaminated ice machines were associated with nosocomial infections (14, 16). No NTM were observed in the bottled waters tested, a finding similar to the observations of Holtzman et al. (12). No NTM were isolated from cistern or reservoir samples due to the high heterotrophic bacteria levels, which possibly inhibited development of mycobacterial colonies on the membrane filters.

NTM or MAC organisms were not found predominantly in hot water as du Moulin et al. had reported (5). Heterotrophic bacterial levels, chlorine concentration, or the age of the sample site were not found to correlate with the occurrence of NTM. Glover et al. (9), in their examination of Los Angeles drinking water, also reported no correlation between heterotrophic bacteria or chlorine levels and the occurrence of NTM. The resistance of NTM to disinfection (3, 7, 17, 23, 24) contributes to the ability of these organisms to persist in drinking water distribution systems. Haas et al. (10) concluded that conventional chlorination even at low pH does little to reduce the numbers of mycobacteria in water, and Caroli et al. (2) observed that levels of mycobacteria in samples they examined were not related to the degree of chlorination. The occurrence of NTM was related to the "complexity" of the plumbing of the sample site. The greatest occurrence of NTM was observed in samples from hospitals (61%), followed by public buildings such as state laboratories, hotels, motels, and office buildings (43%) and samples from private residences (22%). Large buildings and hospitals would be more likely to have plumbing dead ends containing biofilm which may include NTM. The higher incidence of NTM in hospitals may also be attributed to the type of plumbing. Many hospitals use galvanized (zinc) plumbing for their water supply system. There is some evidence that zinc may contribute to the persistence of MAC organisms in these distribution systems (7).

MAC and other NTM have been isolated from numerous environmental sources, including water, aerosols, soil, and plants. In this study MAC organisms were isolated from 19% of the NTM-positive samples. In a 1986 study of mycobacterial contamination of the Boston, Massachusetts, water supply system, du Moulin and Stottmeier (6) isolated MAC organisms from the public water distribution system and from various hospital sites such as hot and cold water taps, ice machines, heated nebulizers, reservoirs, bedside carafes, and sprays from shower heads. Serological tests confirmed that *M. avium* isolates from patient specimens and water sources were identical. Glover et al. (9) investigated the Los Angeles water supply as a possible source of MAC complex organisms infecting AIDS patients. NTM was recovered from 92% of reservoirs, 95% of the homes, and 100% of the 10 hospitals sampled. Of the NTM isolates, 34% were found to be positive by using DNA probes specific for MAC organisms. Serotyping and multilocus enzyme electrophoresis results showed a genetic relatedness between some *M. avium* water and clinical isolates. As a part of an epidemiological study of MAC infections in San Francisco by Yajko et al. (29), water, food, and, soil samples were collected from the home environment of 290 persons with HIV infection and cultured for mycobacteria. Although mycobacteria were recovered from numerous environmental samples, isolates reactive with MAC-specific probes were recovered from only 4 of 528 water samples and only 1 of 397 food samples. In contrast, MAC organisms were recovered from 55% of the soil samples from the patients' homes. In a study examining the incidence of MAC organisms in the United States, Finland, Zaire, and Kenya by von Reyn et al. (24), MAC organisms were isolated from all geographic areas and from 24% of the samples. MAC organisms were isolated from 20% of the drinking water supply samples; however, in the United States and Finland 32% of the drinking water samples were MAC probe positive. The occurrence of MAC organisms reported in this study is lower than the occurrences found in other studies. This result may be attributed to the limited number of samples from each site, to regional differences in the prevalence of MAC organisms in the United States, or to a greater proportion of samples from hospitals, which frequently have a higher occurrence of NTM. Although the methods for recovering MAC organisms and other NTM in the environment need improvement, the present study suggests that MAC organisms may not be ubiquitous in drinking water in the United States.

A number of outbreaks of nosocomial disease caused by rapidly growing NTM have been reported (7, 14, 16, 19). In this study *M. mucogenicum* (formerly known as *M. chelonae*-like organism) was isolated from 41% of the NTM-positive samples. *M. mucogenicum* has been associated with outbreaks of peritonitis associated with automated peritoneal dialysis machines (25). This organism was recovered from patient peritoneal fluid, the automated dialysis machines, and tap water used to supply the machines. The most frequent diseases associated with *M. mucogenicum* are catheter sepsis and posttraumatic skin infections. The ecological niche of *M. mucogenicum* is unknown, but drinking water has been suggested as a possible source of the organism (25). *M. mucogenicum* was the most frequently occurring NTM in this study and was isolated from all geographic areas. This indicates that the organism may be ubiquitous in drinking water and may pose a potential health risk.

In summary, NTM were isolated from 38% of the drinking water supplies examined from a wide geographic area and 33% of all samples. Not only were MAC organisms isolated from 19% of the NTM-positive samples but other clinically significant mycobacterial opportunist pathogens such as *M. kansasii*, *M. mucogenicum*, and *M. peregrenium* were also present. These organisms were isolated from well-operated, well-maintained drinking water utilities with HPC levels of $\leq$500 CFU/ml and chlorine residuals of up to 2.8 mg/liter. This study, along with other studies, indicates that exposure to drinking water containing NTM could pose a health risk to immunocompromised hosts and, to a lesser degree, to immunocompetent individuals.

There is a pressing need for improvement in methods to recover NTM from environmental samples. Studies are needed to describe the contributing factors for persistence of NTM in distribution systems. Although there is evidence that the environment is the source of NTM that infect patients, additional studies are needed to correlate the relatedness of patient isolates to environmental isolates.

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