Rapid Detection and Determination of the Aerodynamic Size Range of Airborne Mycobacteria Associated with Whirlpools

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Novel environmental air and water mycobacteria sampling and analytical methods are needed to circumvent difficulties associated with the use of culture-based methodologies. To implement this objective, a commercial, clinical, genus DNA amplification method utilizing the polymerase chain reaction (PCR) was interfaced with novel air sampling strategies in the laboratory. Two types of air samplers, a three-piece plastic, disposable filter cassette and an eight-stage micro-orifice uniform deposit impactor (MOUDI), were used in these studies. In both samplers, 37-mm polytetrafluoroethylene (PTFE) filters were used. Use of the MOUDI sampler permitted the capture of airborne mycobacteria in discrete size ranges, an important parameter for relating the airborne mycobacteria cells to potential respirable particles (aerodynamic diameter <10 μm) capable of causing health effects. Analysis of the samples was rapid, requiring only 1–1.5 days, as no microbial culturing or DNA purification was required.

This approach was then used to detect suspected mycobacteria contamination associated with pools at a large public facility. PCR was also used to analyze various water samples from these pools. Again, no culturing or sample purification was required. Water samples taken from all ultraviolet light/hydrogen peroxide–treated whirlpools tested positive for the presence of mycobacteria. No mycobacteria were detected in the chlorine-treated pools and the water main supply facility. All air samples collected in the proximity of the indoor whirlpools and the associated changing rooms were strongly positive for airborne mycobacteria. The airborne mycobacteria particles were predominantly collected on MOUDI stages 1–6 representing an aerodynamic size range of 0.5 to 9.9 μm. In conclusion, using this approach permits the rapid detection of mycobacteria contamination as well as the routine monitoring of suspected pools. The approach circumvents problems associated with culture-based methods such as fungal overgrowth on agar plates, and the presence of nonculturable or difficult to culture mycobacteria strains.

Keywords Mycobacteria, Polymerase Chain Reaction (PCR), Whirlpools, Hot Tubs, Bioaerosol

Public pools contaminated with microorganisms constitute a potential threat to public health. In addition, there are concerns that workers involved in the cleaning and maintenance of contaminated pools may be at risk for acquiring occupational microbial-induced illnesses. For example, studies have clearly associated various species of nontuberculous mycobacteria (NTM) with pools and natural waters.[1–6] Nosocomial outbreaks have been linked to the presence of NTM from various water sources.[7]

Recently, reports linked exposure to NTM-contaminated whirlpools to the development of various respiratory illnesses. In 1997, a healthy young adult exhibited clinical symptoms suggesting alveolitis after using a whirlpool referred to as a hot tub. The NTM was identified as *M. avium*.[8] A separate cluster of five cases of respiratory illness characterized by bronchitis, fever, and "flu-like" symptoms suggestive of hypersensitivity pneumonitis (HP) was reported in healthy individuals after using whirlpools contaminated with NTM. Cultures of all sputum samples, lung biopsy specimens, lung lavage, and water samples were positive for the NTM species identified as *M. avium* complex.[9]

Until recently, there was limited documentation relating microbial induced allergic lung diseases in healthy workers to microbial contaminated reservoirs in their work environment. In 1998, a published report described "Lifeguard Lung" or granulomatous lung disease resulting from an indoor microbial contaminated swimming pool with water spray features. Thirty-three lifeguards were affected.[10] In machine workers exposed to metalworking fluids contaminated with NTM, symptoms of HP and allergic alveolitis have been found.[11,12] Anecdotal

reports of occupational lung disease resulting from exposure to microbial agents continue.

Problems associated with the current environmental microbial sampling and analytical techniques have hampered the ability to undertake more extensive studies to address contamination issues and how to eradicate the contamination. Culturability among the NTM species is variable. Some environmental species of NTM appear to be viable but are not culturable. An outbreak of 29 cases caused by *Mycobacterium ulcerans* was linked to an irrigation system within the outbreak area using PCR. All cultures from the area were negative.[13] Furthermore, aerosolization and sampling stress can result in viable but nonculturable microbial states even for normally culturable microbes.[14−16]

In one study it was demonstrated that air samples subjected to culture methods resulted in a 2–3 log underestimation of the total bacterial burden in the air. These observations led the researchers to conclude that procedures other than culture methods are needed to evaluate bacteria in aerosol samples, especially if the quality of public health in indoor air environments is to be determined.[14] An underestimated 2–3 log exposure factor could explain allergic responses in individuals, simply from exposure to large amounts of microbial proteins.[14]

To circumvent the culturing difficulties, a sensitive commercial PCR method, originally designed for detecting *M. tuberculosis* in clinical samples, was adapted for environmental detection of mycobacteria at the genus level. This method targeted a highly conserved mycobacteria 16S RNA genetic region for amplification by PCR.[17] Previously, laboratory studies interfaced this method with two air samplers using *M. tuberculosis* surrogates, *M. bovis* BCG, and *M. tuberculosis* H37Ra.[18,19]

In 1997, the National Institute for Occupational Safety and Health (NIOSH) received a request from a state health department for technical assistance in evaluating a possible occupational HP case potentially linked to microbial contaminated whirlpools located in a large public facility. Previous cultural analysis of various water samples suggested possible NTM proliferation in the whirlpools. The NTM species were identified by an environmental laboratory as *M. avium*, *M. smegmatis*, *M. fortuitum*, and *M. gordonae*. NIOSH conducted two site visits, in March and September 1998, collecting air and water samples for both culture and non-culture analyses. Fungal contamination limited the analysis by culturing methods, as small amounts of fungi quickly overgrew many of the cultures submitted for mycobacteria analysis, resulting in the loss of the air samples as well as many of the water samples. (For additional details pertaining to the NIOSH response, see HETA 98-0051.)

The objectives of this study were: 1) To evaluate the utility of adapting the laboratory air sampling methods for environmental studies of mycobacteria at the genus level; 2) To evaluate the potential use of these approaches as general, rapid, sensitive screening methods for the detection of indoor NTM-contaminated water reservoirs; and 3) To determine the aerodynamic size distribution of aerosolized NTM associated with NTM-contaminated whirlpools without culturing the samples.

Therefore, a sampler such as the MOUDI, capable of particle size fractionation using filter collection, was tested.

MATERIALS AND METHODS

Culture Preparations for Laboratory Studies

In the laboratory, an avirulent mycobacterial model using *M. tuberculosis* H37Ra (ATCC 25177) was selected as a substitute for virulent *M. tuberculosis* to test the sampling and analytical methods. Stocks of mycobacteria obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, were cultured on Löwenstein-Jensen Gruft slants (Difco Laboratories; Detroit, MI) at 37°C in 7.5% CO_2 and were then stored at 4°C. Broth cultures of mycobacteria were grown in Bacto Dubos broth (5% glycerol; 0.25% Tween 80) at 35°C–37°C, and vortexed daily to minimize clumping. The initial number of rod-shaped mycobacteria particles per mL in early or mid-log liquid culture was determined using a Petroff Hausser Counting Chamber (Hausser Scientific Partnership, Horsham, PA). Approximately 50–80 percent of the cells in the culture appeared as single or double rods. The remaining percentage of cells, in general, contained 3–5 cells. No large clumps, containing 10 or more mycobacteria, were present.

Bioaerosol Chamber and Air Sampling in the Laboratory

The horizontal bioaerosol chamber (Torrington Research Company, Torrington, CT) used in this study is shown in Figure 1. The stainless steel chamber, 30 inches × 30 inches × 19 feet, contained baffles downstream of the bioaerosol inlet in order to generate a uniform bioaerosol concentration within the chamber. The supply and exhaust air were purified by passing the air through prefilters and high-efficiency particulate air (HEPA) filters. The chamber air flow rate was set at 750 cubic feet per min using calibrated air flow nozzles and was maintained at this level by measuring differential pressure with an oil manometer. Vertical and horizontal velocity traverses were performed using a hot-wire anemometer. The velocity throughout the cross-sectional area of the sampler location was uniform and determined to be approximately 100 feet per min.

The air flow rates of two Andersen six-stage samplers were adjusted to 28.3 L/min using a Gilian AirCon2 Constant Flow Sampler (Sensidyne, Clearwater, FL). The air flow rates through two MOUDI eight-stage samplers (Model 110, MSP Corporation, Minneapolis, MN) were adjusted to 30 L/min using a Dwyer air flow meter (Michigan City, IN). The air flow rates through three-piece filter cassettes (Gelman Sciences, Ann Arbor, MI) connected to personal air sampler pumps (SKC Airchek Sampler, Model 224-PC X R7, SKC, Inc., Eighty Four, PA) were calibrated to 4 L/min using a Mini Buck Calibrator (A.P. Buck, Inc., Orlando, FL).

Two calibrated Andersen six-stage samplers loaded with Bacto Dubos Oleic Agar plates (Difco), two filter cassette samplers with 37 mm (1.0 μm pore size) PTFE filters (Costar No. 130810), and two non-rotating MOUDI samplers with PTFE
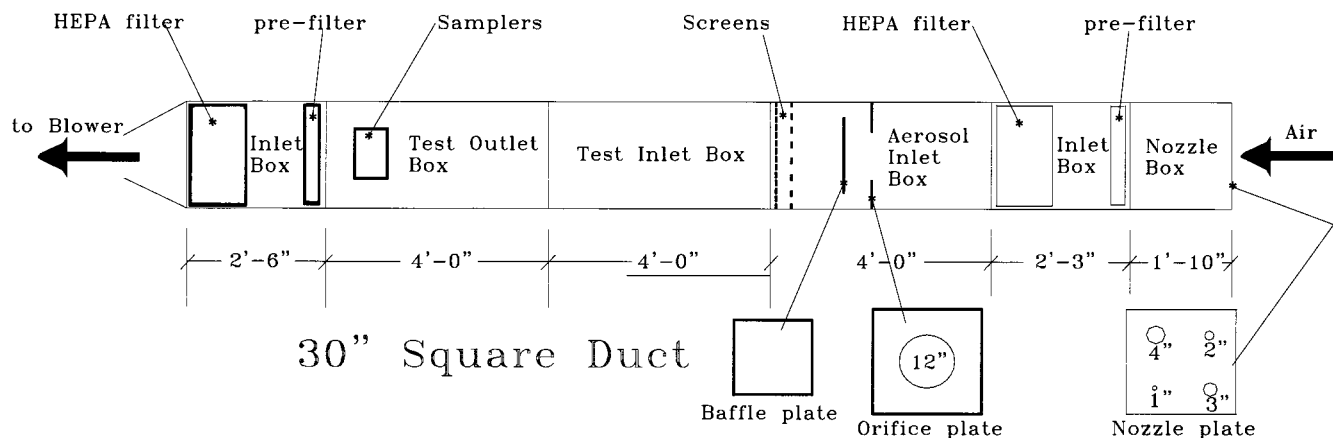
FIGURE 1
Schematic of the bioaerosol chamber.

filters were placed in the chamber through one of the side hatches. For each experiment, the filter cassette samplers were attached to a ring stand, one above the other, with these samplers positioned horizontally toward the air flow in the chamber. The Andersen six-stage samplers and the MOUDI samplers were placed side by side inside the chamber. The air flow rate through the bioaerosol chamber was adjusted to a velocity of approximately 100 feet per min prior to engaging the Collison nebulizer containing the microbial suspensions. Negative controls for each type of air sampler were obtained by operating the chamber under conditions similar to the microbial aerosolization experiments but omitting the microbial suspension.

Laboratory Microbial Aerosol Generation and Collection

Fresh cultures of *M. tuberculosis* H37Ra cells were enumerated using light microscopy and a Petroff Hausser Counting Chamber. Aliquots of the *M. tuberculosis* H37Ra cultures were then diluted into sterile deionized water to final concentrations of 1×10^5 cells/mL to 1×10^7 cells/mL. One hundred mL of microbial suspension was placed in a six-jet modified MRE-type Collison Nebulizer (BGI Inc., Waltham, MA). Filtered compressed air (138 kPa) was used to generate the bioaerosols. After a 10 min period of bioaerosol generation, the Andersen agar plates were removed and incubated at 37°C in 7.5% CO_2 for 2–3 weeks. The filter cassettes were placed in individual plastic bags and stored at −20°C prior to DNA analysis. The filters from each of the MOUDI stages were removed with tweezers and placed in individual 50 mm sterile plastic Petri dishes (Gelman Sciences or Falcon Number 1006). These were stored at −20°C until analysis.

Enumeration of Cultured Mycobacteria

The agar plates were examined daily for the presence of mycobacteria colonies. Visible colonies were manually counted at least twice on a Model C-110W colony counter (New Brunswick Scientific Co., Edison, NJ). A positive hole statistical adjustment was not applied as most of the plates contained low numbers of colonies, and the colonies generated by "multiple hits" were easily detected due partially to the small size of most of the colonies.

Preparation of Airborne Samples from Filter Collection Substrates for PCR

All filters were processed as previously described.[19]

Roche Amplicor *M. tuberculosis* Test

PCR samples were prepared according to the manufacturer's protocol (Roche Diagnostic Systems) as previously described.[19] Laboratory samples containing *M. tuberculosis* H37Ra amplicons were then divided in half. One half of the samples were immediately frozen for gel electrophoresis and the other half were analyzed using the Roche Amplicor *M. tuberculosis* Detection Kit as previously described.[19] This latter detection method is a qualitative method, and positive samples are detected by measuring the amount of yellow color (optical density or absorbance at 450 nm or A_{450}) in the sample. Environmental samples were removed from the thermal cycler and immediately frozen at −20°C. The samples were stored at this temperature until analysis was undertaken using agarose gel electrophoresis.

Agarose Gel Electrophoresis

PCR-amplified DNA, representing a highly conserved mycobacteria 16S rRNA genetic region common to species of mycobacteria in the genus, resulted in a 0.58 kb product, which was detected using 2% NuSieve 3:1 (FMC BioProducts, Rockland, ME) agarose in $1 \times$ TBE containing 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3 (Sigma). The gels, 16×15 cm, were cast in a submarine gel chamber (DNA SubCell, Bio-Rad). After loading samples containing 10 μL sample mixed with 2 μL of Gel Loading Solution (Sigma) and submerging the gel in $1 \times$ TBE, electrophoresis was carried out at 200 volts. The gels were stained post-electrophoresis with SYBR Green I nucleic acid gel stain (FMC BioProducts), visualized using a UV

illuminator (Photodyne, New Berlin, WI) and photographed with Polaroid Type 57 film.

Environmental Sample Collection and Analyses

Water samples of 100 mL were collected in sterile plastic containers and stored at 4°C. Two-mL aliquots (in duplicate) were transferred to 2.0 mL sterile microcentrifuge tubes and centrifuged at 12,000 × g for 10 minutes at room temperature. The supernatant was decanted, and the invisible pellets resuspended in 1.0 mL of Sputum Wash Solution or 10 mM Tris, pH 8.0, containing 1% Triton X-100. The suspensions were re-centrifuged at 12,000 × g for 10 minutes at room temperature, supernatants decanted, and the pellets stored at −20°C prior to analysis.

To detect airborne mycobacteria, aerosols were collected using the three-piece filter cassettes with calibrated flow rates of 3 L/min and a non-rotating MOUDI eight-stage sampler, 30 L/min. Since it was not known how long air sampling should be conducted before sufficient aerosolized mycobacteria would be detected for the determination of the aerodynamic diameter size range, various sampling periods were used. Air was sampled for 7 hours using the plastic filter cassettes, and either 1 or 3 hours using the MOUDI. To prevent cross-contamination, each plastic filter cassette was individually sealed in the laboratory in plastic storage bags prior to use in the field. After sampling in the field, the plastic filter cassettes were placed back into the respective storage bags for shipment to the laboratory. For the MOUDI sampler, extra sets of filter-containing impaction plates were prepared in the laboratory. These plates were changed in the field for each sampling site. All filters were removed from the impaction plates after the sets were returned to the laboratory for analysis. All filters were stored at −20°C. All samples were analyzed as described above. Air samples were collected at eight interior building locations, which included some rooms that did not contain any type of pool. Outdoor air samples were also collected and at least one area (patio terrace) was not in the vicinity of any type of pool.

RESULTS

Comparison of Amplicon Detection Methods

The commercial mycobacteria 16S RNA PCR method was interfaced with a sensitive, *M. tuberculosis*–specific detection system utilizing a microtiter plate containing a bound DNA probe.[17] Previous studies using this detection method had demonstrated that a single genomic copy could be detected.[19] To detect amplicons representing NTM, a different detection method would be needed in order to detect amplicons from members of the genus. Thus, agarose gel analysis was used in place of the commercial species-specific detection system. To determine if the use of agarose gel analysis would permit a comparable sensitivity for the detection of amplicons from just a few mycobacteria cells, serial dilutions of small quantities of cultured *M. tuberculosis* H37Ra were placed in sample tubes and processed as described in Material and Methods.

Aliquots of the amplicons were simultaneously used in both detection methods, the detection of yellow color in the microtiter plate reading at A_{450} and the detection of DNA bands (amplicons) in the agarose gels after staining with SYBR Green I. Some commercial siliconized sample tubes were also included to determine whether siliconization of polypropylene microcentrifuge tubes enhanced the detection of amplicons from small quantities of the mycobacteria. In general, all gel lanes containing detectable 0.58 kb amplicons corresponded with the positive (+) or negative (−) microtiter plate readings at A_{450} (Figure 2A). In
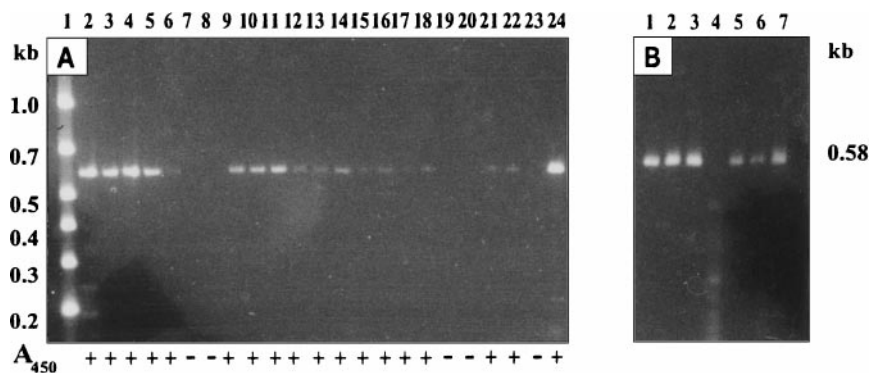

FIGURE 2

(A) Detection of amplicons by agarose gel analysis and A_{450} from small quantities of mycobacteria processed in both silicon-coated, lanes 3–8, and non-silicon-coated, lanes 9–23, polypropylene tubes. Lane 1, DNA size markers; lane 2, positive PCR control; lanes 3–5, 125 cells; lanes 6–8, 12 cells; lanes 9–11, 125 cells; lanes 12–14, 12 cells; lanes 15–17, 1 cell; lanes 18–23, <1 cell; lane 24, positive PCR control. Qualitative A_{450} results, + or −, are shown below the gel. (B) Efficiency of mycobacteria recovery after subjecting two sets of sample filters to different orientations, PTFE side up and PTFE side down, during the mycobacteria removal step. Lanes 1–3, amplicons from recovered mycobacteria after stripping the filters with PTFE side up; lane 4, negative PCR control; lanes 5–7, amplicons from recovered mycobacteria after stripping the filters with PTFE side down.

summary, the limit of detection of both methods is comparable, and both methods can result in the detection of amplicons from samples containing fewer than 10 mycobacteria cells.

The use of siliconized versus non-siliconized microcentrifuge tubes was less clear. Stronger visible DNA (amplicons) gel bands were detected from the siliconized tubes compared to the non-siliconized when approximately 125 mycobacteria cells were used (Figure 2A lanes 3–5 vs. lanes 9–11). However, at lower concentrations, the use of non-siliconized tubes resulted in the detection of more positive samples (Figure 2A, lanes 6–8 vs. lanes 12–14). No detectable amplicons resulted when less than 12 mycobacteria cells were processed in siliconized tubes (results not shown). However, amplicons were detected at these lower concentrations when the non-siliconized tubes were used to process the samples (Figure 2A, lanes 12–23). Therefore, use of siliconized tubes is not necessary for processing the samples when the samples contains small numbers of mycobacteria cells.

Filter Stripping Efficiency

The filter used in the air sampling experiments is a polytetrafluoroethylene (PTFE) filter laminated to a polypropylene backing for support. Both surfaces are hydrophobic and not readily distinguishable in appearance. The membranes are inserted into the air samplers PTFE side up. However, it was unclear whether the PTFE side had to remain up during the stripping process after the filters are transferred to the Petri dishes and covered with the microbial stripping solution. As demonstrated in Figure 2B, lanes 1–3, higher recoveries of the mycobacteria cells, as reflected in the intensity of the amplicon gel bands, are achieved if the PTFE side of the filter remains up throughout the stripping process. Stripping filters with the polypropylene backing side up consistently resulted in the detection of fewer amplicons reflecting the less efficient recovery of the mycobacteria cells (Figure 2B, lanes 5–7).

Determination of Aerodynamic Size in the Laboratory

Previous studies had validated the plastic disposable filter cassette as an efficient sampler for capturing airborne mycobacteria.[18,19] Therefore, the cassette was used as a positive control sampler for the detection of airborne mycobacteria and placed side-by-side with experimental samplers in the bioaerosol chamber. Aerosolized mycobacteria studies were undertaken in the laboratory prior to field activities in order to determine the aerodynamic size range of the well-characterized rod-shaped cells. Such data would also permit a direct comparison to field results.

To characterize the complete aerodynamic size range of aerosolized mycobacteria coupled to PCR, the MOUDI with cut sizes ranging from 0.18–18 μm was used. The same PTFE filter was employed as a collection substrate on each of the stages. The placement of the filter cassettes and the MOUDI impactor in the bioaerosol chamber is shown in Figure 3. To determine which MOUDI stages consistently captured the most airborne
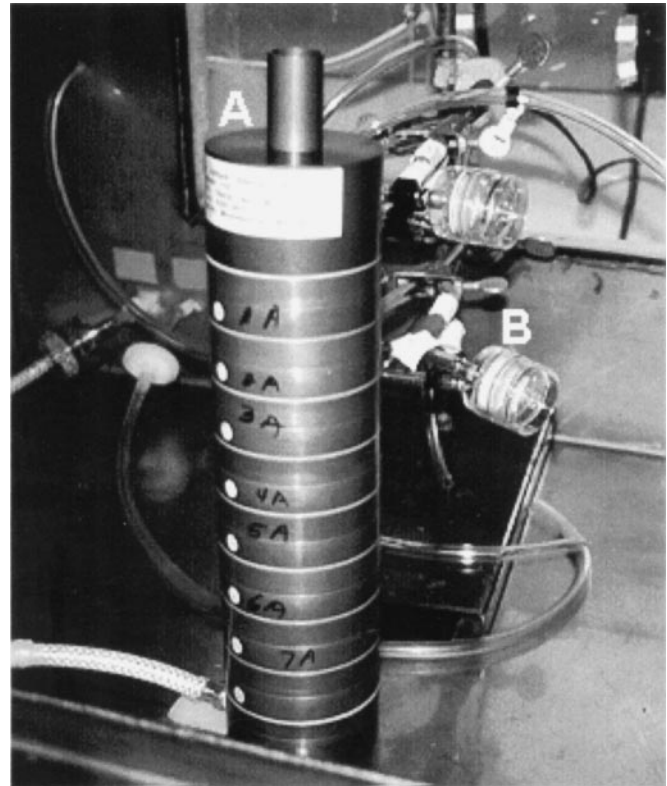

FIGURE 3
Depicts how the samplers are positioned inside the bioaerosol chamber. (A) MOUDI sampler and (B) 3-piece plastic cassette samplers.

mycobacteria cells, repetitive experiments were undertaken and the collected mycobacteria cells from the various stages were subjected to PCR. Use of cultured *M. tuberculosis* H37Ra for this laboratory effort permitted the detection of PCR products by both detection methods, agarose gel analysis and the detection of yellow color by measuring the A_{450}. The PCR-generated DNA products resulted in the expected single 0.58 kb DNA band on the gels with most of the mycobacteria amplicons associated with the samples from MOUDI stages 4 and 5 (Figure 4, lanes 8–9 and lanes 17–18), representing aerodynamic sizes 1.8 μm and 1.0 μm, respectively. Because a small quantity of amplicons were detected in the sample from stage 7, some airborne mycobacteria cells were also captured on this stage, which has a 0.3 μm aerodynamic cutpoint. In general, airborne mycobacteria larger than that size would be collected on stage(s) above stage 7—that is, each stage collects aerosolized particles equal or greater in size than the cutpoint.

The detection of H37Ra PCR amplicons from the MOUDI filters analyzed using the qualitative *M. tuberculosis* commercial Detection Kit is presented in Table I. Positive samples are identified by readings at A_{450}. All filter samples from the various stages that resulted in a 0.58 kb amplicon band on the gel also tested positive when processed with the *M. tuberculosis*
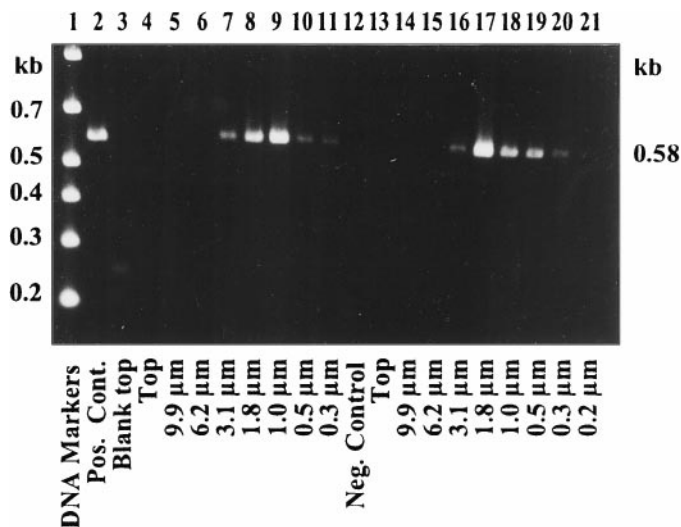
FIGURE 4

Determination of the aerodynamic size range of mycobacteria aerosolized in the bioaerosol chamber using a Collison nebulizer. Lane 1, DNA size markers; lane 2, positive PCR control; lane 3, negative PCR control; lanes 4–11, shows the MOUDI sampler 1, 18 μm (Top) to 0.3 μm aerodynamic size range results; lane 12, negative PCR control; lanes 13–21, depicts the MOUDI sampler 2, 18 μm (Top) to 0.2 μm aerodynamic size range results.

Detection Kit. For this side-by-side comparison, the gel results from Figure 4 are presented as positive ($+$) or negative ($-$) in Table I. The commercial detection kit is primarily a very sensitive qualitative test and little information can be obtained in

TABLE I

Aerodynamic size range of captured aerosolized *M. tuberculosis* H37Ra generated in the bioaerosol chamber using the MOUDI sampler (samples analyzed by two analytical methods: gel analysis and A_{450})

Stage	Aerodynamic size (μm)	MOUDI #1		MOUDI #2	
		Gel analysis	A_{450}	Gel analysis	A_{450}
Top	18	$-$	$-$	$-$	$-$
1	9.9	$-$	$-$	$-$	$-$
2	6.2	$-$	$-$	$-$	$-$
3	3.1	$+$	$+$	$+$	$+$
4	1.8	$+$	$+$	$+$	$+$
5	1.0	$+$	$+$	$+$	$+$
6	0.5	$+$	$+$	$+$	$+$
7	0.3	$+$	$+$	$+$	$+$
8	0.2	a	$+$	$-$	$-$

Gel analysis = detection of stained PCR products in agarose gels.
A_{450} = detection of yellow color, as an indicator of the presence of mycobacteria PCR products.
a, Sample lost; $+$, Positive; $-$, Negative.

regard to quantitative levels of the captured mycobacteria cells. However, gel analysis does indicate which filters captured the most mycobacteria cells. As more copies of the mycobacteria genome are added to the PCR tubes, the larger or more intensive is the appearance of the 0.58 kb gel bands (Figure 4). However, a precise quantization is not possible under these experimental conditions.

Detection of NTM Associated with Whirlpools

Water and air samples were collected from various types of pools at the large public facility in March 1998 and again in September 1998. All whirlpools at the facility were treated with ultraviolet light/hydrogen peroxide to control microbial contamination. All other pools were treated with chlorine. Two different air samplers, the plastic filter cassette, operated at 3 L/min for 7 hours, and the MOUDI, operated at 30 L/min for either 1 or 3 hours, were simultaneously used at the facility.

Air sampling was conducted at the perimeter of the pools approximately 2 feet above the pools and in several rooms that did not contain any type of pool. The March 1998 air sampling results using the filter cassettes are presented in Figure 5. Air samples from both indoor whirlpools, 1 and 2, were strongly positive (Figure 5A, lanes 9 and 11). Furthermore, the air samples from the whirlpool change rooms adjoining the indoor whirlpools were also positive, although the concentration levels of the airborne NTM, as reflected by the lower intensities of the 0.58 kb gel bands of the PCR amplicons, were somewhat less (Figure 5A, lanes 10 and 12).

Results obtained from the second sampling period, September 1998, are presented in Figure 5B. Duplicate PCR analysis of each sample was conducted. Air samples taken at the perimeter approximately 2 feet above the outdoor whirlpool, referred to as whirlpool 3, did not result in the detection of any airborne NTM (Figure 5B, lanes 8 and 9). Ambiguous results were obtained for room 1, which did not contain any type of pool, as one of two air samples was weakly positive and, as shown in the original gel photo, the second sample may be a false positive (Figure 5B, lanes 6 and 7). As before, air samples from the whirlpool 1 change room (lanes 10 and 11), and whirlpool 1 (lanes 19 and 20), were all strongly positive. Lower concentration of PCR products representing the whirlpool 2 change room can be seen in lanes 14 and 15. Whirlpool 2, however, yielded strongly positive air samples, shown in lanes 16 and 17. Both air samples from room 2 were weakly positive (lanes 12 and 13) although the room did not contain any pools. There was no evaluation of air flow patterns to determine if air intake valves could have captured the aerosolized mycobacteria particles from the whirlpools. Water samples from the various pools were also analyzed. In summary, the water samples from all whirlpools tested strongly positive for NTM contamination. All other pools tested negative (unpublished data).

To determine the aerodynamic size range of the airborne NTM as well as which MOUDI stages captured the highest number of NTM cells, aliquots of the PCR products from each stage
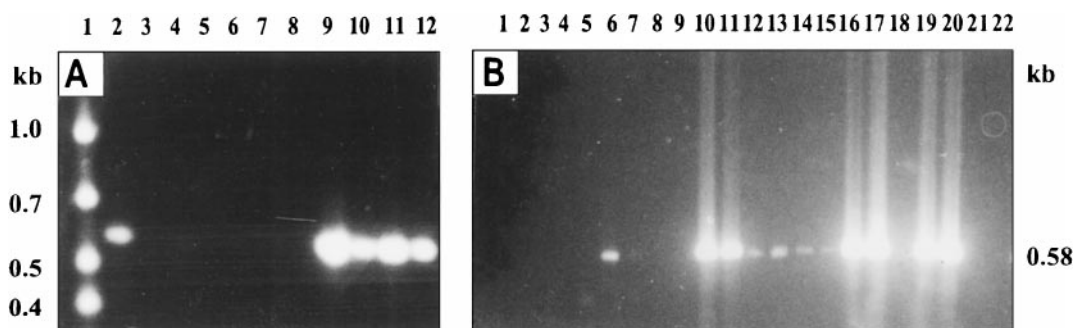
FIGURE 5

Detection of aerosolized NTM at the public facility using the plastic three-piece filter cassette sampler. (A) March 1998 results. Lane 1, DNA size markers; lane 2, positive PCR control; lane 3, outdoor control; lane 4, room 1; lane 5, outdoor control; lane 6, room 3; lane 7, room 4; lane 8, room 2; lane 9, whirlpool 1 (WP1); lane 10, whirlpool 1 change room (WP1-WRm); lane 11, whirlpool 2 (WP2); lane 12, whirlpool 2 change room (WP2-WRm). (B) September 1998 results. Lanes 1 and 2, room 3; lanes 3 and 4, outdoor control; lane 5, negative PCR control; lanes 6 and 7, room 1; lanes 8 and 9, outdoor whirlpool (WP3); lanes 10 and 11, room WP1-WRm; lanes 12 and 13, room 2; lanes 14 and 15, room WP2-WRm; lanes 16 and 17, WP2; lane 18, negative PCR control; lanes 19 and 20, WP1; lanes 21 and 22, room 4.

were analyzed. The PCR-generated DNA products resulted in a single 0.58 kb DNA band and most of the NTM cells were captured on stages 1–6 representing an airborne aerodynamic size range of 1.0 to 9.9 μm (Figures 6A and 6B). The environmental airborne NTM-containing droplets exhibited a wider aerodynamic size range compared to the laboratory results. The laboratory results showed that the mycobacteria cells were predominantly captured on the lower stages of the MOUDI representing aerodynamic sizes of 1 to 1.8 μm. During both sampling periods, more airborne NTM cells, as reflected in the intensities of the DNA gel bands representing the PCR amplicons, were captured when air sampling at the perimeter of whirlpool 1 compared to air sampling at the perimeter of whirlpool 2. Because substantial quantities of NTM amplicons were detected during the March 1998 site visit, 1-hour as well as a 3-hour MOUDI sampling periods were used during the September 1998 site visit. Substantial quantities of NTM PCR amplicons were detected for the 1-hour sampling period (Figure 6B). A 3-hour sampling period conducted the same day yielded higher levels of amplicons as expected (data not shown). A qualitative summary of the results from both sampling periods are present in Table II. The NTM species was determined by a commercial environmental laboratory to be predominantly *M. avium* based on traditional cultural analysis of the water samples from the pools.[20]

DISCUSSION

Generation of airborne small droplets from bubbles bursting at the water surface of whirlpools has been well documented. In two studies, respirable airborne water droplet size distributions from 1–8 μm were determined for several whirlpools.[21,22] However, the presence or absence of microbial agents in the water droplets was not documented in that study. Because aerodynamic size determines the site and extent of deposition in

the respiratory tract, such data could facilitate the linking of a respiratory illness to a contaminated whirlpool. In another study by other investigators using electrostatic methods and *S. marcescens*, it was demonstrated that not only is the microbial-contaminated water aerosolized, but the concentration of the aerosolized bacteria can be increased 10- to 20-fold in the bubbles bursting at the surface. In addition, approximately one half of these aerosolized *S. marcescens*–containing droplets exhibited aerodynamic sizes <10 μm.[23] In indoor environments, the concentration of airborne microbial-containing droplets <10 μm can increase over time if they are not removed by ventilation or other engineering controls and may pose a health hazard to

TABLE II

Aerodynamic size range of environmental airborne NTM associated with indoor whirlpools using the MOUDI sampler

| | | Gel analysis | | | |
| | | March 1998 | | September 1998 | |
Stage	Aerodynamic size (μm)	WP1 (3 h)	WP2 (3 h)	WP1 (1 h)	WP2 (1 h)
Top	18	+	+	+	+
1	9.9	−	+	+	+
2	6.2	+	+	+	+
3	3.1	+	+	+	+
4	1.8	+	+	+	+
5	1.0	+	+	+	+
6	0.5	+	+	+	+
7	0.3	+	+	+	+
8	0.2	+	−	+	+

Gel analysis = detection of stained PCR products in agarose gels.
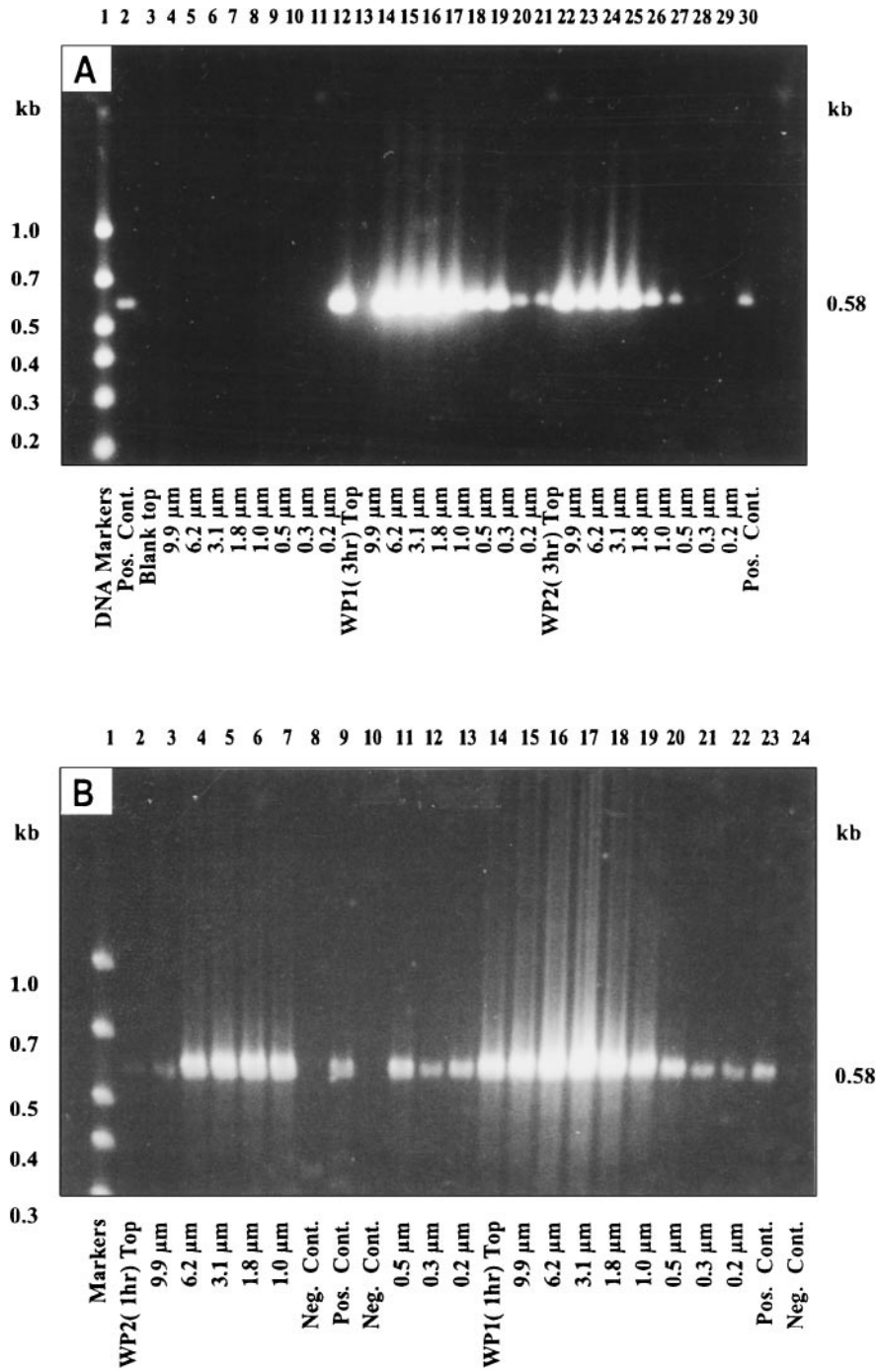+, Amplicons; −, No amplicons; WP, Whirlpool.

FIGURE 6

Use of the MOUDI sampler to determine the aerodynamic size range of environmental airborne NTM generated by the indoor whirlpools at the public facility. (A) March 1998. Lane 1, DNA size markers; lane 2, positive control; lanes 3–11, negative air sample control; lanes 12–20, WP1; lanes 21–29, WP2; lane 30, positive PCR control. (B) September 1998. Lane 1, DNA size markers; lanes 2–7 and 11–13, WP2; lanes 8 and 10, negative PCR controls; lane 9, positive PCR control; lanes 14–22, WP1; lane 23, positive PCR control; lane 24, negative PCR control.

susceptible individuals inhaling the contaminated air.[24] In fact, NTM-induced pulmonary disease is increasingly recognized in previously healthy persons.[25] Therefore, it is increasingly important to detect NTM bioaerosols.

Some of the environmental NTM clearly are not readily culturable. Several studies attempting to link *M. ulcerans*, for example, to an natural reservoir, route of infection and transmission mechanism failed using traditional culturing methods. However, use of an *M. ulcerans* PCR method readily detected the presence of *M. ulcerans* from water samples taken from the site of several *M. ulcerans* outbreaks.[26] To circumvent difficulties associated with culturing methods and to develop a rapid, sensitive method that would permit routine screening of indoor air for NTM contamination, a mycobacteria genus PCR method was successfully interfaced with air sampling methods. Both the filter cassette and the MOUDI samplers permitted the coupling of PCR to the air sampling methods. The samplers yield different information about the airborne NTM. Use of the filter cassette permits the capture of the total amount of airborne NTM cells while the use of the MOUDI sampler permits the determination of the aerodynamic size range of the airborne NTM. The aerodynamic size range, 1.0 to 9.9 μm, of the NTM obtained using the MOUDI sampler indicates that most of the airborne NTM could penetrate the lower respiratory tract or the alveoli regions and potentially cause such diseases as hypersensitivity pneumonitis (HP) or other allergic reactions in addition to colonization in susceptible individuals. The aerodynamic size range of the whirlpool samples is broader than the aerodynamic size range of the mycobacteria-containing aerosols captured in our bioaerosol chamber. It is possible that the whirlpool aerosolized mycobacteria represent mostly small aggregates, instead of predominantly single particles. Alternately, the whirlpool aerosolized mycobacteria may simply be part of tiny aerosolized water droplets. The mycobacteria aerosolized in the bioaerosol chamber contain little, if any, water residue and are predominantly single particles.

The PCR method can also be used to evaluate water samples without major modifications and, therefore, only one PCR method is needed for detecting mycobacteria at the genus level in both air and water samples. Use of this method for water samples clearly associated the captured airborne NTM to the contaminated whirlpools. The detection of significant levels of airborne NTM only occurred in the indoor environment. Although the outdoor whirlpool was contaminated with NTM, no airborne NTM was detected when sampling was conducted at the perimeter of this pool. Thus, the water and air data together indicate that transmission in indoor environments of the NTM from the water to the air and then to the human respiratory system is possible as the generation of airborne NTM-containing droplets are small enough to enter and deposit in the lungs.

Using a PCR method to detect mycobacteria to the genus level permits the rapid detection of many different species of mycobacteria. Positive samples can then be subjected to additional analyses if needed. For many environmental situations, the effort could simply focus on eliminating the contamination from the water and the air without NTM species identification. However, if species identification were necessary, immediate identification of the NTM could be undertaken using species-specific probes.[27]

The whirlpool results clearly indicate a need to evaluate the disinfection methods used for these pools. All whirlpools at the facility were treated with ultraviolet light/hydrogen peroxide to control microbial contamination. All other pools were treated with chlorine. It is possible that the NTM may have formed biofilms somewhere in the whirlpools or the plumbing system(s) associated with the whirlpools, resulting in the constant shedding of NTM into the water. Biofilms are usually more resistant to biocides than microbial cells in aqueous suspensions so treating the pools would not necessarily eliminate the contamination.

In conclusion, two air sampling methods coupled to a genus PCR method permitted the rapid detection of airborne NTM as well as the aerodynamic size range of the aerosolized NTM-containing droplets. The PCR method also permitted the detection of NTM in water samples and demonstrated that the whirlpools were the source of the airborne NTM. This approach was also successfully used to test additional pools in another state (unpublished data). It is proposed that this approach be used as a general screening method for the detection of environmental NTM, and its routine use could prevent both public and occupational exposures to NTM from contaminated water reservoirs. The above approaches would also permit the evaluation of engineering controls for the containment or removal of the NTM from the contaminated air and water as well as the testing of various disinfection methods.

DISCLAIMER

Mention of commercial names or products does not constitute endorsement by the Centers for Disease Control and Prevention.

ACKNOWLEDGMENTS

REFERENCES

1. American Thoracic Society: Diagnosis and Treatment of Disease Caused by Nontuberculous Mycobacteria. Am J Respir Crit Care Med 156:S1–S25 (1997).

2. Covert, T.C.; Rodgers, M.R.; Reyes, A.L.; et al.: Occurrence of Nontuberculous Mycobacteria in Environmental Samples. Appl Environ Microbiol 65:2492–2496 (1999).

3. Leoni, E.; Legnani, P.; Mucci, M.T.; et al.: Prevalence of Mycobacteria in a Swimming Pool Environment. J Appl Microbiol 87:683–688 (1999).

4. Parker, B.C.; Ford, M.A.; Gruft, H.; et al.: Epidemiology of Infection by Nontuberculous Mycobacteria. Am Rev Respir Dis 128:652–656 (1983).

5. Stinear, T.; Davies, J.K.; Jenkin, G.A.; et al.: Identification of *Mycobacterium ulcerans* in the Environment from Regions in Southeast Australia in Which It Is Endemic with Sequence Capture-PCR. Appl Environ Microbiol 66:3206–3213 (2000).

6. Wendt, S.L.; George, K.L.; Parker, B.C.; et al.: Epidemiology of Infection by Nontuberculous Mycobacteria. Am Rev Respir Dis 122:259–263 (1980).

7. Wallace, Jr., R.J.; Brown, B.A.; Griffith, D.E.: Nosocomial Outbreaks/Pseudo-Outbreaks Caused by Nontuberculous Mycobacteria. Ann Rev Microbiol 52:453–490 (1998).

8. Kahana, L.M.; Kay, J.M.; Yakrus, M.A.; et al.: *Mycobacterium avium* Complex Infection in an Immunocompetent Young Adult Related to Hot Tub Exposure. Chest 111:242–245 (1997).

9. Embil, J.; Warren, P.; Yakrus, M.; et al.: Pulmonary Illness Associated with Exposure to *Mycobacterium avium* Complex in Hot Tub Water. Chest 111:813–816 (1997).

10. Rose, C.S.; Martyny, J.W.; Newman, L.S.; et al.: "Lifeguard Lung": Endemic Granulomatous Pneumonitis in an Indoor Swimming Pool. Am J Pub Health 88(12):1795–1800 (1998).

11. Kreiss, K.; Cox-Ganser, J.: Metalworking Fluid-Associated Hypersensitivity Pneumonitis: A Workshop Summary. Am J Indus Med 32:423–432 (1997).

12. Shelton, B. G.; Flanders, W.D.; Morris, G.K.: *Mycobacterium* sp. as a Possible Cause of Hypersensitivity Pneumonitis in Machine Workers. EID 5:1–4 (1999).

13. Ross, B.C.; Johnson, P.D.R.; Oppedisano, F.; et al.: Detection of *Mycobacterium ulcerans* in Environmental Samples During an Outbreak of Ulcerative Disease. Appl Environ Microbiol 63:4135–4138 (1997).

14. Heidelberg, J.F.; Shahamat, M.; Levin, M.; et al.: Effect of Aerosolization on Culturability and Viability of Gram-Negative Bacteria. Appl Environ Microbiol 63:3585–3588 (1997).

15. Marthi, B.; Fieland, V.P.; Walter, M.V.; et al.: Survival of Bacteria During Aerosolization. Appl Environ Microbiol 56:3463–3467 (1990).

16. Stewart, S. L.; Grinshpun, S.A.; Willeke, K.; et al.: Effect of Impact Stress on Microbial Recovery on an Agar Surface. Appl Environ Microbiol 61:1232–1239 (1995).

17. Tevere, V.J.; Hewitt, P.L.; Dare, A.; et al.: Detection of *Mycobacterium tuberculosis* by PCR Amplification with Pan-*Mycobacterium* Primers and Hybridization to an *M. tuberculosis*-Specific Probe. 34:918–923 (1996).

18. Schafer, M.P.; Fernbach, J.E.; Jensen, P.A.: Sampling and Analytical Method Development for Qualitative Assessment of Airborne Mycobacterial Species of the *Mycobacterium tuberculosis* Complex. Am Indus Hyg Assoc J 59:540–546 (1998).

19. Schafer, M.P.; Fernback, J.E.; Ernst, M.K.: Detection and Characterization of Airborne *Mycobacterium tuberculosis* H37Ra Particles, a Surrogate for Airborne Pathogenic *M. tuberculosis.* Aerosol Sci Tech 30:161–173 (1999).

20. Martinez, K.F.: Close out letter of June 3, 1999, from K.F. Martinez, Division of Surveillance, Hazard Evaluations, and Field Studies, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Public Health Service, U.S. Department of Health and Human Services. HETA 98-0051 (1999).

21. Baron, P.A.; Willeke, K.: Respirable Droplets from Whirlpools: Measurements of Size Distribution and Estimation of Disease Potential. Environ Res 39:8–18 (1986).

22. Mangione, E.J.; Remis, R.S.; Tait, K.A.; et al.: An Outbreak of Pontiac Fever Related to Whirlpool Use, Michigan 1982. JAMA 253:535–539 (1985).

23. Blanchard, D.C.; Syzdek, L.D.: Water-to-Air Transfer and Enrichment of Bacteria in Drops from Bursting Bubbles. Appl Environ Microbiol 43:1003–1005 (1982).

24. Wells, W.F.: Sanitary Ventilation. In: Airborne Contagion and Air Hygiene, W.F. Wells, Ed., pp. 155–173. Harvard University Press, Cambridge, MA (1995).

25. Mangione, E.J.; Huitt, G.; Lenaway, D.; et al.: Nontuberculous Mycobacterial Disease Following Hot Tub Exposure. EID 7(6):1–9 (2001).

26. Roberts, B.; Hirst, R.: Immunomagnetic Separation and PCR for Detection of *Mycobacterium ulcerans.* J Clin Microbiol 35:2709–2711 (1997).

27. Stauffer, F.; Haber, H.; Rieger, A.; et al.: Genus Level Identification of Mycobacteria from Clinical Specimens by Using an Easy-to-Handle *Mycobacterium*-Specific PCR Assay. J Clin Microbiol 36:614–617 (1998).