ORIGINAL RESEARCH

Association between *Mycobacterium avium* Complex Pulmonary Disease and Mycobacteria in Home Water and Soil

A Case–Control Study

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Abstract

Rationale: Nontuberculous mycobacteria (NTM), including *Mycobacterium avium* complex (MAC), are emerging pathogens that can opportunistically cause debilitating pulmonary disease in susceptible human hosts. Potential sources of exposure in homes include point-of-use water sources, such as taps and showerheads, as well as gardening soils. The relative human health impacts of NTM in these home environments remain poorly understood.

Objectives: This study tested associations between MAC pulmonary disease and NTM colonization of five potential point-of-use sources of pathogen exposure in homes.

Methods: A case–control study was conducted of Washington and Oregon residents who had been diagnosed with MAC pulmonary disease, and population controls were matched by age, sex, and geography. Samples were collected from bathroom faucets, kitchen faucets, shower aerosols, indoor soil, and outdoor soil. Mycobacteria in environmental samples were identified in a blinded fashion by using bacteriological culture combined with polymerase chain reaction. The isolation of NTM from case homes ($n = 56$) versus control homes ($n = 51$) was quantitatively compared using conditional logistic regression models with adjustment for potential confounding variables.

Results: NTM were isolated from shower aerosols collected in case homes more often than in control homes. An adjusted conditional logistic regression analysis showed that NTM isolation from shower aerosols had a high odds ratio associated with disease (odds ratio, 4.0; 95% confidence interval, 1.2–13). Other home environmental samples (tap water, soils) did not exhibit this association.

Conclusions: The results implicate shower aerosols as uniquely significant sources of NTM exposure in homes.

Keywords: NTM; built environments; aerosol; *Mycobacterium avium*; exposure

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Certain nontuberculous mycobacteria (NTM) species are opportunistic and environmentally acquired bacterial pathogens. Infection of susceptible hosts by NTM can result in chronically debilitating pulmonary disease. NTM pulmonary disease (NTM-PD) has known host risk factors that include advanced age, thoracic structural anomalies, preexisting pulmonary disease, and autoimmune disease (1–8). Increasing annual prevalence of NTM-PD has been reported (9). Within NTM, one of the most

common causes of pulmonary disease in the United States is *Mycobacterium avium* complex (MAC).

Among patients of integrated healthcare delivery systems in the United States, the reported period prevalence of NTM-PD ranged from 1.4 to 6.6 per 100,000 people (10, 11). Hospital setting studies are relevant, given the concentration of susceptible hosts in such environments (11). However, they do not address residential environments, where many people may acquire the disease.

Pathogenic NTM grow and persist in premise plumbing, defined as the water distribution system and pipes downstream of water meters in residential buildings and workplaces. NTM resist biocide treatments and ultraviolet irradiation (12, 13) and are commonly isolated from point-of-use water sources despite ozonation and filtration (14). They form biofilms with other microorganisms, allowing them to persist in diverse environments after leaving water treatment facilities (12, 15–18). Studies of the homes of patients with MAC pulmonary disease (MAC-PD) have found MAC isolates in potting soil (19); showerheads (15, 20); shower water, bathtub water, and drain outlets (12, 21, 22); residential hot water sources (23); and shower aerosols (24). These studies identified potential sources of infection in homes, and several used genotypic analyses to identify isolates from homes that matched isolates from corresponding cases.

Specific household water fixtures have been hypothesized to play particularly important roles in NTM exposure. Aerosol-generating fixtures such as showerheads are frequently found to be colonized with NTM. In some cases, isolates from shower heads and shower aerosols have been matched to isolates from cases of human disease using molecular typing methods (15, 17, 24, 25). A study by Angenent and colleagues (25) found that *Mycobacterium* species preferentially partition into aerosols, suggesting a pathway by which colonized fixtures could effectively seed the airways of exposed humans. However, past studies of NTMs in showerheads have rarely used comparative analytical designs to detect associations between human disease and the presence of NTM in aerosol-generating fixtures. Two exceptions include a recent study by Gebert and colleagues (26) and another by Lande and colleagues (20). Gerbert and colleagues used culture-independent metagenomic methods to quantify the presence of *Mycobacterium* and other bacterial genera in showerhead biofilms collected by citizen-scientists from 656 households in the United States and Europe (26). Strikingly, "hot spots" in the United States with high abundance of certain lineages of *Mycobacterium* overlapped significantly with geographical regions where NTM lung disease is most prevalent (26). This finding associated NTM colonization of showerheads with a relevant human health outcome on the ecological level. On the individual level, Lande and colleagues isolated mycobacteria from biofilm swabs collected from a variety of point-of-use sources in the homes of MAC-PD cases and control subjects. Isolates were seen slightly more often in case homes than in control homes, but the study included too few individuals to have the statistical power to detect associations between residential point-of-use site colonization and disease status (20). It therefore remained uncertain whether showers or other household sites played a significant role in NTM exposure leading to disease.

The current study sought to rigorously test the connection between residential exposure to NTM and human health suggested by the studies above. It isolated mycobacteria from home environments of patients with clinically diagnosed MAC-PD in Washington and Oregon and from the homes of age-, sex-, and geography-matched population control subjects. The objective was to determine whether mycobacterial colonization of specific home environments is more common in the homes of MAC-PD cases than in matched control homes. The residential point-of-use sources and sampling techniques—shower aerosols,bulk water from bathroom and kitchen faucets, and aerosols generated from outdoor and indoor soil samples—were selected on the hypothesis that aerosol generation during routine hygiene, cooking, and gardening is an important step in disease acquisition. Because MAC isolation from environmental sources is uncommon in comparison with the broader NTM category, the latter was used in the analysis as an indicator of environments that are conducive to colonization by diverse mycobacteria, including MAC.

Methods

Study Design and Participants

This analysis continued a population-based case–control study of MAC-PD conducted in Washington and Oregon. Details of recruitment, inclusion and exclusion criteria, participant demographics, and the results of the interview-based portion of the study were previously published (3). In brief, cases met 2007 American Thoracic Society diagnostic criteria and were recruited between January 2009 and January 2011 (3). Control subjects matched by age and sex were identified and recruited between May 2010 and July 2011 via random-digit dialing (3). Telephone numbers used to screen for eligible control subjects were formed using the first 7 digits of each case's 10-digit primary telephone number and completed with 3 random digits. All participants received a home visit that included informed consent and an interview; if a case still lived in the same home at the time of the visit as on their date of diagnosis, environmental samples were collected from five point-of-use sites in their home and the home of their matched controls (3). The environmental sampling portion of the study included 56 cases and 51 control subjects (3). Institutional review boards at University of Washington and Oregon Health and Science University approved the study.

In-Home Environmental Sampling

Bulk water samples were collected from bathroom and kitchen taps. Both hot and cold water taps were opened until the stream reached the participants' usual hand-washing temperature, and then samples were collected. This was done to ensure that water from the cold and hot water supplies were both sampled and to mimic routine behavior. For both kitchen and bathroom taps, 1-L samples were collected using a sterile 1-L Nalgene bottle (Thermo Fisher Scientific). Samples of outside soil and (if available) inside soil were collected by using an autoclaved hand trowel to transfer material into pre–ultraviolet-irradiated plastic bags. If a subject's indoor soil came from multiple pots, a composite sample was collected (3).

Shower aerosols were collected from the bathroom most often used by the subject for showering or bathing. If

participants preferred showers to baths, they were asked to adjust the shower to their usual showering temperature and usual pressure. Then, a BioStage single-stage cascade impactor powered by an attached Quick-Take 30 High Flow Pump (SKC Inc.) was placed in the shower chamber outside of the direct path of the showerheads' spray for 10 minutes (flow rate of 30 L/min). Two 10-minute samples were collected, one with the impactor loaded with a Petri dish with Middlebrook 7H10 agar with Oleic Albumin Dextrose Catalase (OADC) enrichment, and the other with the impactor loaded with a Petri dish with Middlebrook 7H10 agar enriched with 0.001% malachite green. If participants preferred baths to showers, participants were asked to fill their bathtub until it reached the participant's usual temperature and level. The impactor and pump were held near the running faucet for 2 minutes while the bath was filling and then held over the surface of the water in the tub for 8 minutes. If the participants' bath had jets, the procedure was slightly modified by holding the impactor and pump for 2 minutes near the running faucet with jets on and for 6 minutes over the surface of the water in the tub (3).

Sampling methods are described in greater detail in the online supplement.

Laboratory Processing

Laboratory processes, including NTM isolation and identification, are presented in detail in the online supplement and outlined in Figure E1 (*see* online supplement). After collection, environmental samples were transported to the University of Washington Environmental and Occupational Health Microbiology Laboratory in a cooler with ice packs. Microbiologists and polymerase chain reaction (PCR) technicians in the lab were blinded to the case versus control status of samples after the creation of primary culture plates.

Primary plating of bulk water samples was accomplished by vacuum filtration. A total of 250 ml of the water samples were poured into a sterile polyphenylsulfone magnetic filter funnel (Pall Life Sciences) containing 0.45-μm, 47-mm filters (EZ-Pak; MilliporeSigma). Filters were removed and placed on the primary culture plates with Middlebrook 7H10 agar with OADC enrichment (various manufacturers). A second 250 ml of the water samples was treated with 1.25 ml of 1% cetylpyridinium chloride to decontaminate the sample for 30 minutes, vacuum filtered, and also plated on Middlebrook 7H10 agar with OADC enrichment. The remainder 500 ml of the sample was filtered and the filter archived at −80°C.

The Petri dish in the BioStage cascade impactor constituted the primary culture plate for shower aerosol samples. For primary plating of soil samples, an autoclavable soil aerosolization chamber was designed specifically for this study (Figure E2). The chamber was custom fabricated from a 5-gallon milk pail and modified to include a soil delivery cylinder on top and sampling port on the side. A BioStage single-stage cascade impactor was attached to the side sampling port of the soil aerosolization chamber using a stainless steel elbow, gasket, and hose clamp. Soil samples from a subject's home were divided into two portions that were separately dropped in through the delivery cylinder. For the first portion, the impactor was loaded with a Petri plate of Middlebrook 7H10 agar with OADC enrichment and for the second portion with Middlebrook 7H10 agar with OADC enrichment and 0.001% malachite green (wt/vol), modeled after De Groote and colleagues (19). The sampling pump was run for 10 minutes after the soil was released at 30 L/min.

Steps after the creation of primary culture plates were conducted in blinded fashion. Primary plates were incubated in sealed bags at 37°C and observed for 8 weeks or until the culture became desiccated or molded. Each week, new colonies and their morphotypes were recorded. New colonies were selected in proportion to their morphotype (up to a maximum of 10 colonies/wk) and transferred by sterile loop to new plates by streaking for isolation. All subculture plates were similarly incubated and observed for 8 weeks or until the culture became desiccated or molded. This process was repeated until subculture plates only had a single morphotype.

Because of mold or desiccation, some samples, isolates, and subcultures were lost. Table E1 shows the distribution of primary plate loss between cases and controls. Blinding of the process after primary plating helped assure that losses in subsequent subculture steps were not biased by differential effort or care by laboratory personnel.

DNA was extracted from purified colonies by a boil-prep, heat-lysis method. Each tube was boiled in a thermocycler at 96°C for 10 minutes, cooled to 4°C, and then centrifuged for 2 minutes. Aliquots of each supernatant (5 μL) were then subjected to a multiplex PCR described by Wilton and Cousins (27). PCR primers from Wilton and Cousins (27) were used as outlined in Table E2. PCR conditions were as follows: 95°C for 30 seconds followed by 35 cycles of 98°C for 10 seconds, 62°C for 15 seconds, and 72°C for 10 seconds. The resulting PCR products were electrophoresed in 2% agarose gel with 90 volts for 90 minutes. Detected band sizes in the gel were correlated with genus and species identifications as outlined in Table E3.

Statistics

Statistical analyses were performed using STATA/SE v. 14.2. Our analysis asked whether there were associations between NTM colonization of the residential environment and MAC-PD disease status. These associations were measured with odds ratios obtained from conditional logistic regression models, to account for case–control matching and enable consideration of potential confounding variables. The exposure variable was binary: any NTM isolated versus none.

Potential confounders were selected *a priori*. Advanced age and female sex had known associations with disease status (1, 3, 8, 9), and socioeconomic status and geography were considered likely to be associated with access to diagnosis and case ascertainment. These four factors were also considered possibly associated with colonization with NTM via age of home and the availability of time, skills, and funds for gardening and home maintenance.

We considered it unlikely that bias due to age would be consistent in magnitude and direction throughout the range of participant ages. Thus, for each point-of-use site sampled in subject homes, we performed three conditional logistic regressions with increasing adjustment for potential confounding: an (unadjusted) crude model, a model adjusted for extreme age, and a fully adjusted model. The crude model compared each case to his/her respective age-, sex-, and geography-matched control using only the variables of interest, namely disease status and the isolation of NTM. The second model included an additional variable for whether

subjects were >80 years old. The fully adjusted model contained the previously mentioned variables (disease status, isolation of NTM, age > 80 yr) in addition to race and education level as proxies for socioeconomic status (which was not explicitly asked). These analyses were repeated with age as a continuous variable in lieu of the dichotomous variable for age > 80 years. They were also repeated with MAC isolation in particular, rather than any NTM, as the exposure of interest. Separate models were completed for each different point-of-use source.

Although sample collection was noted previously (3), results of analysis of environmental samples have not previously been reported.

Results

NTM and MAC Isolated from Case and Control Homes

The number of case and control homes that had NTM and MAC isolated from each point-of-use source are shown in Tables 1 and 2, respectively. The broader NTM category was used in the main analysis because diverse environmental *Mycobacterium* species were likely to thrive in similar environments to MAC, whereas the relative large numbers of NTM were expected to facilitate statistical analysis.

Association between NTM Isolation from Shower Aerosols and MAC-PD

The results of our conditional logistic regressions for each point-of-use site hypothesized to be involved in exposure to NTM are shown in Table 1. In our crude, unadjusted models, the association between disease and NTM isolation from shower aerosols was positive, with an odds ratio of 3.2 (95% confidence interval [CI], 1.1–8.9). No significant associations were found for the other four sites, namely kitchen taps, bathroom taps, indoor soil, or outdoor soil. In our models adjusted for age > 80 years, again, only shower aerosols had a positive association between NTM isolation and disease, with an odds ratio of 3.8 (95% CI, 1.2–12). Our fully adjusted models, which considered age > 80 years, race, and education, also showed NTM isolation from shower aerosols had a positive association with MAC-PD disease, with an odds ratio of 4.0 (95% CI, 1.2–13).

Shower aerosols were significantly associated with disease whether or not hypothesized confounding variables were included in the regression models. Adjustment for any of the potential confounders did not considerably affect the magnitude of the OR estimates. When analyses were repeated with age as a continuous variable rather than as a dichotomous variable, the results were unchanged (not shown). When looking more specifically at MAC colonization at point-of-use sources (as opposed to NTM in general), no statistically significant associations were detected, possibly because of lower frequency of detection overall (Table 2).

Discussion

Our study compared the relative importance to human disease of disparate potential sources of NTM exposure. We found more NTM colonization in shower aerosols collected from case homes than in samples from homes of age-, sex-, and geography-matched control subjects. Other point-of-use sites did not exhibit this association within the number of pairs assessed here.

Our first report on this case–control study found evidence that MAC-PD is strongly associated with host risk factors and did not find evidence of association between MAC-PD and behaviors believed to increase aerosolization of water and soil in the residential environment (3). In that report, we concluded there was more evidence that MAC-PD is a disease that affects "susceptible persons" than evidence that it results from an "unusual dose." This report refines that understanding. Although our previous study (3) did not observe an association between MAC-PD and increased aerosol-generating behavior, the current study observed an association between MAC-PD and the microbiological content of aerosols. To our knowledge, this is the first case–control study of associations between human health and exposure to NTM in aerosols generated in the residential environment.

NTM have previously been isolated from showerheads and shower aerosols, potentially resulting in airborne exposure (20–25). An additional study identified shower aerosols as a reservoir in the home for diverse opportunistic infectious agents, including NTM (15). Showerheads may offer a dark, damp, nutrient-rich environment that supports opportunistic microbes. Angenent and colleagues (25) found that *Mycobacterium* species preferentially partitioned into aerosols compared with other bacteria species.

Table 1. Association of residential nontuberculous mycobacteria and *M. avium* complex pulmonary disease by point-of-use source

Household site	*N* (Positive)*		Unadjusted Analysis		Age-adjusted Analysis†		Fully Adjusted Analysis‡	
	Cases	Control Subjects	Odds Ratio	95% CI	Odds Ratio	95% CI	Odds Ratio	95% CI
Bathroom faucet	40 (23)	48 (20)	1.7	0.8–4.0	1.8	0.7–4.3	2.1	0.8–5.5
Kitchen faucet	40 (23)	48 (22)	1.6	0.7–4.0	1.4	0.6–3.5	1.6	0.6–4.2
Shower aerosols	39 (18)	46 (10)	3.2	1.1–8.9	3.8	1.2–11.7	4.0	1.2–13.4
Indoor soil	30 (17)	38 (13)	2.0	0.7–5.4	1.6	0.6–4.6	1.4	0.5–4.4
Outdoor soil	39 (10)	46 (9)	1.2	0.4–3.4	1.1	0.4–3.2	1.2	0.4–3.4

Definition of abbreviations: CI = confidence interval; *M. avium* = *Mycobacterium avium*.
*Some case–control pairs had more than one control. Positives were samples with at least nontuberculous mycobacteria isolate.
†Adjusted for age > 80 years.
‡Adjusted for age, race, and education level.

Table 2. Association of residential *M. avium* complex and *M. avium* complex pulmonary disease by point-of-use source

Household site	*N* (Positive)*		Unadjusted Analysis		Age-adjusted Analysis†		Fully Adjusted Analysis‡	
	Cases	Control Subjects	Odds Ratio	95% CI	Odds Ratio	95% CI	Odds Ratio	95% CI
Bathroom faucet	40 (15)	48 (11)	1.8	0.7–4.5	1.7	0.6–4.5	1.8	0.6–4.9
Kitchen faucet	40 (15)	48 (14)	1.2	0.5–2.6	1.1	0.5–2.5	1.2	0.5–2.8
Shower aerosols	39 (10)	46 (6)	2.6	0.7–10.4	2.9	0.7–12.5	2.9	0.7–12.4
Indoor soil	30 (7)	38 (4)	1.9	0.5–6.4	1.3	0.3–5.0	1.1	0.3–4.5
Outdoor soil	39 (8)	46 (8)	1.0	0.3–3.1	0.9	0.3–2.8	0.9	0.3–2.8

Definition of abbreviations: CI = confidence interval; *M. avium* = *Mycobacterium avium*.
*Some case–control pairs had more than one control. Positives were samples with at least nontuberculous mycobacteria isolate.
†Adjusted for age > 80 years.
‡Adjusted for age, race, and education level.

Conceivably, mycobacteria in premise plumbing can detach from biofilms and aerosolize at showerheads, effectively seeding the airways of users.

This model was supported by the recent findings of Gebert and colleagues (26) and Lande and colleagues (20). Gebert and colleagues identified "hot spots" in the United States with high abundance of certain lineages of *Mycobacterium* in showerhead samples and showed at the ecological level that these overlapped regions of the country where NTM lung disease is most prevalent (26). The study of Gebert and colleagues had unusual geographical breadth, but it did not measure associations at the individual level and it did not compare showers to other water-associated sources of household exposure to NTM (26). The recent study by Lande and colleagues did measure exposure to *Mycobacteria* in multiple point-of-use sources at the individual level, but the number of individuals studied was too small to estimate associations (20). Therefore, our results both confirm and extend the notion that shower aerosols are significant sources of NTM exposure leading to human disease

As with Gebert and colleagues (26), we did not attempt to generate evidence that the species and strains of environmental mycobacteria isolated in our study were involved in initial MAC-PD infections. No attempt was made to genotypically match environmental MAC isolates with clinical isolates (the latter were not available to the study), and most of the environmental NTM isolates observed here were likely to be species other than MAC. Moreover, the MAC-PD cases included in this study were diagnosed with their diseases as early as 1997, and initial infections may have occurred earlier than that, whereas sampling took place from 2009 through 2010. Despite this, shower aerosols had significantly more NTM in case homes than in control homes. Case homes may have had favorable environments for colonization by diverse *Mycobacterium* species, a condition that could increase the probability of exposure to disease-causing species such as MAC. Alternatively, chronic exposure to NTM (including but not limited to MAC) could exacerbate an existing MAC-PD infection, either through reinfection or by stimulating immune responses leading to symptoms. Chronic NTM exposure and a corresponding escalating immune response could even prime the host for primary MAC infection (e.g., through macrophage recruitment). This would increase the likelihood of diagnosis and inclusion in our case group. Longitudinal studies with larger numbers are needed to distinguish these possibilities.

Our study had additional limitations. Our microbiological and molecular methods may not have detected all NTM present in the samples, and some plates were lost to mold overgrowth and other problems. However, the blinding of laboratory personnel to case and control status helped prevent bias in handling and technique. Limitations in our microbiologic techniques would have applied to both case and control samples and therefore were unlikely to explain the association between disease status and NTM in shower aerosol. However, they might have limited our ability to identify similar associations in the other sample types. In addition, we were unable to address potential bias due to batch effect and collection times of case and control environmental samples. Although all samples were processed in the same way, proficiency could conceivably have improved over the course the study. Because case homes were sampled earlier in the study, on the average, than control homes (3), such effects would have been expected to bias toward the null. Moreover, such effects would have been unlikely to differentially affect point-of-use sources (e.g., shower aerosols vs. soils). Additional limitations were that the study was not powered to divide results into high, low, and no colonization or to see subtle associations that may exist with low colonization. A larger study may have detected weaker associations between NTM and disease in point-of-use sources other than shower aerosols.

In summary, we observed that shower aerosols collected in the homes of patients with MAC-PD more often have environmental mycobacteria than those of age-, sex,- and geography-matched healthy control subjects. Other sites in the homes (water taps, soil) did not exhibit this association. These results confirm and extend past suggestions that shower aerosols are potentially significant sources of NTM exposure in homes. Future epidemiological investigations should look more at residential exposures, including but not limited to shower aerosols, in the homes of individuals with compromised immune or pulmonary systems. Such studies will help to discern additional potential risk factors in residential environments. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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