Association between Mycobacterium avium Complex Pulmonary Disease and Mycobacteria in Home Water and Soil: A Case-Control Study

Connie L. Tzou, M. Ashworth Dirac, Annie L. Becker, Nicola K. Beck, Kris M. Weigel, John S. Meschke, Gerard A. Cangelosi

ONLINE DATA SUPPLEMENT

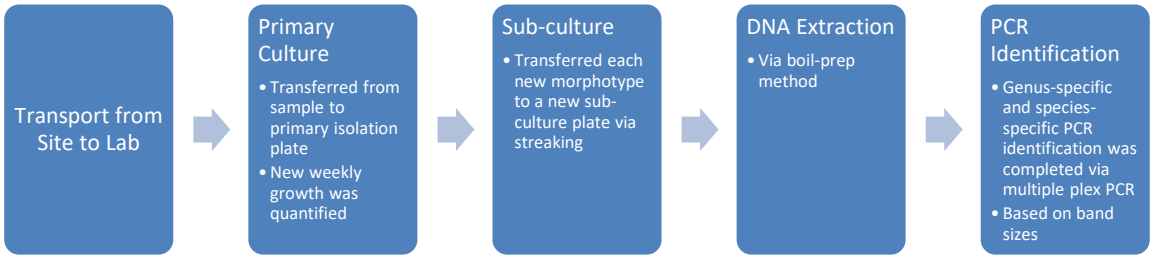

Figure E1. Flow diagram of laboratory processes.

Figure E2. Custom-built Soil Aerosolization Chamber (SAC) for generation and collection of soil aerosols prior to primary plating.

Table E1. Frequency of sample loss due to mold overgrowth or dessication.

	Percent of samples lost to mold overgrowth or dessication	
	Cases	**Controls**
Bathroom faucet	8.7%	9.4%
Kitchen faucet	7.8%	8.2%
Shower aerosols	17.6%	19.2%
Indoor soil	17.9%	21.4%
Outdoor soil	22.7%	21.2%

Table E2. Expected product size of primer pairs used in the multiplex PCR

Primer pairs	Primer concentrations, respectively (nM)	Product Size (bp)
Mycgen-F and Mycgen-R	250 + 250	1030
Mycgen-F and Mycav-R	250+70	180
Mycint-F and Mycgen-R	350 + 250	850
TB1-F and TB1-R	200 + 200	372

Abbreviations: nM, nanomolar; bp, base pair

Table E3. PCR product sizes and species interpretation.

Species	Detected Product Size (bp)
M. avium	180
M. avium	1030+180
M. avium	180+EB[1]
M. avium	1030+180+EB
M. avium or M. intracellulare	1030+850+180
M. avium or M. intracellulare	850+180+EB
M. avium or M. intracellulare	1030+850+180+EB
M. avium or M. intracellulare	850+180
M. intracellulare	850
M. intracellulare	1030+850
M. intracellulare	850+EB
M. intracellulare	1030+850+EB
Mycobacterium	1030
Mycobacterium	1030+EB
Negative/No species detected	Only EB
Negative/No species detected	No reaction

Abbreviations: bp, base pair; EB, extra bands

MICROBIOLOGICAL PROTOCOLS

Environmental sample collection. The following samples were collected: 1) one liter of water from the kitchen tap, 2) one liter of water from the bathroom tap, 3) a composite of arbitrarily sized samples of soil from an arbitrary number of the subject's indoor potted plants, 4) a composite of arbitrarily sized samples of soil from an arbitrary number of the subject's outdoor potted plants and garden plots, 5) aerosols collected while running the shower.

Bulk water samples were collected by turning on both hot and cold water, and letting the mixture run until the stream came to what was subjectively considered "hand-washing" temperature, to ensure that water from the cold and hot supply were both sampled. Then, an autoclaved, 1-liter Nalgene bottle was filled.

Soil samples were collected by scooping soil into a plastic bag pretreated with ultraviolet (UV) light using either an autoclaved hand trowel brought by study staff, a spoon or trowel belonging to the subject, or by hand, inverting the bag to serve as a plastic glove. Soil samples could be collected in 2 Mil Plain Premium Red Line Reclosable Zipper Bags (Minigrip, Kennesaw, GA), Ziploc Brand Freezer Bags with the Smart Zip Seal or Storage Bags with the Smart Zip Seal (SC Johnson, Racine, WI), or Whirl-Pak Bags (Nasco, Salida, CA).

Shower aerosols were collected using a BioStage Single-stage Viable Cascade Impactor (SKC Inc, Eighty Four, PA) attached to a Quick-Take 30 High Flow Pump (SKC Inc, Eighty Four, PA) and loaded with a Petri dish containing Middlebrook 7H10 agar with OADC enrichment (various manufacturers). Air-flow through the set up was measured using a 30L/min rotameter. The subject was then asked to turn on the shower to the temperature and pressure they normally use, and to otherwise recreate the typical scenario for their shower (for example, to turn on a fan or heat lamp, open a window, etc.) The pump was then run for ten minutes, with the sampler in the shower chamber, but not directly in the spray. The shower was then turned off and the Petri dish was exchanged for one containing Middlebrook 7H10 agar (various manufacturers) enriched with both OADC and malachite green 0.001% (wt/vol), modeled after De Groote *et al* 2006. The shower was turned on to the same temperature and pressure and the pump was run for an additional ten minutes.

If the subject reported that they exclusively took tub baths prior to index date, they were asked to start filling the bathtub in their usual fashion, and aerosols were collected by holding the sampler near the stream from the running faucet for 2 minutes, then turning off the water and holding the sampler above the still surface of the collected water for 8 minutes. If the tub had jets, the sampler was run 2 minutes near the faucet stream, 2 minutes above the collected water with jets on, and 6 minutes above the still collected water. The Petri dishes were stored in clean, UV-treated plastic bags during transit. The bags specified for transport and incubation of Petri dishes throughout the study were 2 Mil Plain Premium Red Line Reclosable Zipper Bags (Minigrip, Kennesaw, GA)

Several steps were included in the protocol to help prevent cross-contamination between subjects, excessive growth of organisms that compete with NTM, or degradation of NTM. These were 1) to wear gloves when handling Petri dishes and filters. 2) To prepare all autoclavable equipment for home-visits by autoclaving, washing, wrapping in paper, and autoclaving a second time. (When multiple visits were made on a single trip, the protocol was to take unique autoclavable equipment for each visit.) 3) To prepare non-autoclavable equipment by washing with vesphene and treating with UV light for 10 minutes or longer. (When multiple visits were made during a single trip, the protocol was to treat non-autoclavable equipment by washing with vesphene and 70% ethanol between visits.) 4) To transport samples from the subject's home to the UW Environmental & Occupational Health Microbiology Lab (EOHML) in a cooler with artificial ice packs. 5) To process water and soil samples immediately or store them in the refrigerator until processing. 6) To transfer plates with aerosol samples from the plastic bag used in transit to a new, UV-treated plastic bag, before placing them upside-down in an incubator at 37 degrees Celsius. 7) To freeze and store filters with cultured aerosol samples at -20°C.

Initial sample processing. Water samples were processed by vacuum filtration in three portions. EZ-Pak 0.45µm, 47mm, white gridded filters (Millipore, Billerica, MA) were placed on 300-mL or 500-mL polyphenylsulfone magnetic filter funnels (Pall Life Sciences, Ann Arbor, MI) and placed in a three-station stainless steel vacuum manifold. Two-hundred and fifty milliliters of sample were poured into the filter holder with stoppers closed, stoppers were opened, and when the portion finished filtering, stoppers were closed and the filter was removed and placed on Middlebrook 7H10 agar with OADC enrichment (various manufacturers). Another filter was placed on the filter holder and 500mL of sample were filtered in like fashion. The filter was removed and placed in a 50mL plastic conical tube. Then, 1.25mL of 1% cetylpyridinium chloride (CPC) was added to the portion of the sample remaining in the Nalgene bottle, mixed by inverting the closed bottle 3-4 times, and allowed to decontaminate for 30

minutes. NTM are relatively resistant to CPC, so CPC was used to kill organisms other than NTM that might be in the sample and out-compete NTM when the concentrated sample was incubated. The decontaminated sample was then filtered, and the filter was placed on Middlebrook 7H10 agar with OADC enrichment (various manufacturers).

Several steps were included in the water sample processing protocol to help prevent cross-contamination between subjects, excessive growth of organisms that compete with NTM, or degradation of NTM. These were 1) to wear gloves were and change them frequently. 2) To handle filters with tweezers treated with 70% ethanol and flame (before and after the first and third portions were filtered) and with Eliminase decontaminant (Decon Labs Inc., King of Prussia, PA), followed by 70% ethanol and flame before and after the second portion was filtered. 3) To process only samples from the same subject side-by-side. 4) to treat autoclavable equipment between samples from different subjects by autoclaving, washing, wrapping in paper and autoclaving again. 5) To treat non-autoclavable equipment between samples from different subjects by cleaning with vesphene and treating with UV for 10 minutes. 6) To treat surfaces between samples from different subjects with vesphene or 10% bleach followed by 70% ethanol. 7) To put plates in new, clean plastic bags before placing them upside down in an incubator set to 37°C. 8) To freeze conical tubes with filters at -20°C.

Soil samples were divided in three portions for processing: one portion weighing approximately 0.5g and two other approximately equal portions made with the remainder of the sample. The 0.5-gram portion was placed in a drying oven at 60C during processing and weighed afterwards to calculate the wet-to-dry weight ratio for the sample. The other two portions were aerosolized in the containment unit designed for this project. A Petri dish containing Middlebrook 7H10 agar with OADC enrichment (various manufacturers) was placed in a BioStage Single-stage Viable Cascade Impactor (SKC Inc, Eighty Four, PA), the impactor was attached to a Quick-Take 30 High Flow Pump (SKC Inc, Eighty Four, PA), and air-flow through the set-up was measured using a 30L/min rotameter. The inlet of the impactor was then attached to the outlet line of the containment unit using a stainless steel elbow, gasket, and hose clamp. One portion of soil was placed in the closed chute on top of the unit. The soil was dropped into the unit and the pump was turned on for 10 minutes. The Petri dish was then removed from the impactor and replaced by a Petri dish containing Middlebrook 7H10 agar with OADC enrichment (various manufacturers) enriched to 0.001% (wt/vol) malachite green, modeled after De Groote *et al* (2006). The second soil sample was placed in the chute. The soil was released and the pump was run for ten minutes.

Several steps were included in the soil processing protocol to help prevent cross-contamination between subjects, excessive growth of organisms that compete with MAC, or degradation of MAC. These were 1) To process only samples from the same subject side-by-side. 2) To empty the containment unit, wipe it with a paper towel, and treat it with UV for ten minutes between indoor and outdoor samples from the same subject. 3) To handle filters with tweezers treated with Eliminase decontaminant (Decon Labs Inc., King of Prussia, PA) followed by 70% ethanol and flame. 4) To place Petri dishes in new UV-treated plastic bags before placing them upside down in a 37°C incubator. 5) To place filters in 50mL conical tubes and freeze them at -20°C.

Observation and processing of primary isolation plates. Primary isolation plates from processing bulk water, soil, and shower aerosols were incubated at 37°C and observed approximately weekly for 8 weeks or until overgrown with mold or another organism that was obviously not NTM. At each observation every plate in the incubator was classified as "Overgrown", showing "No Growth", showing only the growth that was already marked on a previous day ("Marked Growth Only"), or showing "Some Unmarked" (presumably new) growth. Plates overgrown with mold were always classified as "Overgrown" and were thrown away. Plates overgrown with bacterial growth unlikely to be MAC or with yeast were sometimes classified as "Overgrown" and thrown away, and sometimes classified as "Some Unmarked" and treated as described below. Plates classified as "No Growth" or "Marked Growth Only" were returned to the incubator.

Plates with any unmarked growth were observed on a Quebec colony counter. Unmarked growth was marked with permanent marker and described in terms of the total number of distinct colony morphotypes that could conceivably be MAC, the number of distinct morphotypes that clearly were not MAC, and the number of colonies of each morphotype. Occasionally, primary isolation plates were also photographed, and if it was unclear whether unmarked growth was truly new, it was compared to previous weeks' photographs. Primary isolation plates were either bagged and returned to the incubator to await streaking colonies for isolation or immediately had colonies streaked for isolation. For a small number of observations early in the study, the primary isolation plates were left bagged on the lab bench to await streaking colonies for isolation, but this practice was abandoned because they sometimes sat at room temperature for several days, preventing them from continuing to grow at 37°C.

From each primary isolation plate classified as "Some Unmarked", 10 colonies were selected from among the colonies new that week. If fewer than 10 colonies grew since last observation, all colonies were selected. If a subset was selected, colonies were chosen to roughly represent the relative abundance of each morphotype, but when rounding to whole numbers, the morphotypes that most resembled clinical and laboratory MAC cultures were favored. Colonies were streaked on Middlebrook 7H10 agar with OADC enrichment, using disposable plastic inoculating loops. Subcultures and primary isolation cultures were then returned to the incubator.

Several protocol steps were included to help prevent cross-contamination of cultures derived from different samples or contamination by ambient organisms. These were 1) to clean the colony counter and lab bench by soaking with a layer of vesphene or 10% bleach for 10 minutes, then spraying with 70% ethanol, prior to observing primary isolation plates. 2) To streak colonies for isolation in a biological safety cabinet that was wiped with vesphene or sprayed with 70% ethanol and then treated with UV for 10 minutes. 3) To wear gloves were during plate observations and subculture preparation. 4) To change gloves after handling plates overgrown with mould or other contaminants. 5) To place primary isolation plates and subcultures in new plastic bags before being placed in the incubator.

Data collected when observing primary isolation plates and streaking colonies for isolation were originally recorded on paper forms, and later recorded on blank paper. The data were entered into the study database after completion of fieldwork.

Observation and processing of isolates. Subcultures were classified as either moldy ("Moldy"), having insufficient growth to process ("Insufficient Growth"), having one morphotype ("Monomorphic"), or having multiple morphotypes ("Polymorphic"). "Moldy" subcultures were thrown away. Subcultures with "Insufficient Growth" were bagged and returned to the 37°C incubator, if less than 56 days old, and thrown away if they were 56 days old or older. The subcultures with sufficient growth to process were bagged and left on the bench until processed.

First-generation subcultures with one morphotype were colony-purified by streaking a single colony on Middlebrook 7H10 agar with OADC enrichment (various manufacturers). Second-generation (or third, fourth, etc.) subcultures with one morphotype were scraped into 2-mL, screw-top, free-standing, freezer-safe microcentrifuge tubes (various manufacturers) with 0.5-1mL 15% glycerol and stored at -80°C.

For subcultures of any generation with more than one morphotype, records were searched to find a description of the colony originally selected from the primary isolation plate to make the subculture, and one of the colonies of the type most resembling that description was streaked for isolation on Middlebrook 7H10 agar with OADC enrichment. If the description was unavailable, or multiple types resembled the original description equally well, either multiple colonies were streaked for isolation on separate plates, or the colony most resembling clinical and laboratory MAC cultures was streaked for isolation. Colony-purification of polymorphic subcultures continued over multiple rounds of processing until a grossly uniform culture was achieved. The subcultures so processed were bagged and stored at room temperature. The newly colony-purified subcultures were bagged and placed upside down in the 37°C incubator.

Subcultures were retained at room temperature until archives were made. Retained subcultures were occasionally reviewed to assess whether they were still needed. Those whose descendants were already archived were thrown away. Those whose descendants were thrown away for being moldy or showing no growth at 56 days were colony-purified again.

To prevent cross-contamination of cultures derived from different samples or contamination with ambient organisms, the lab bench was cleaned with 10% bleach or vesphene for 10 minutes and then sprayed with 70% ethanol prior to observation of subcultures. Colony-purification and archiving was done using disposable plastic loops and took place either on the bench or in a biological safety cabinet cleaned with vesphene or 70% ethanol and treated with UV for ten minutes.

Determination of genus, species, and subspecies. Archived environmental isolates were divided into groups containing all environmental isolates for 3-9 subjects. Matched case-control pairs were generally placed in the same group, although a small number of pairs were split between groups to keep group-size manageable. Isolates obtained from the homes of cases for whom no matched control was found were excluded from this analysis. Laboratory workers were blind to the identity and case-control status of the subjects. Laboratory workers further divided the groups of environmental isolates into batches to be grown in a common bag and then photographed, processed for DNA extraction, and analyzed by PCR at the same time. Each batch consisted of approximately equal numbers of isolates from each subject in the larger group.

After batching, laboratory workers streak archived isolates onto Middlebrook 7H10 agar with OADC enrichment (various manufacturers), and incubate them upside-down in plastic bags in a 37°C incubator. When the cultures grow they photograph them. Then, from each culture, they suspend a 1uL loop of cells in 100uL of 1x tris-ethylenediaminetetraacetic acid (TE) in a PCR tube. The tube is boiled in a PTC-200 thermo cycler (MJ Research Inc., Waltham, MA) at 96°C for 10 minutes and cooled to 4°C. The contents of the tube, termed "boil-prep", are then spun down in a microcentrifuge (Fisher Scientific International, Hampton, NJ) for 2 minutes and 5uL of supernatant is used as template.

Boil-preps from all isolates were subjected to a multiplex PCR designed to identify members of genus *Mycobacterium* and species *M. avium* (MA) and *M. intracellulare* (MI), as described in the text. Master mixes for these reactions contained 17.5uL Phusion High-Fidelity Master Mix with HF Buffer (New England BioLabs Inc., Ipswich, MA), 0.6125uL of 25mM MgCl2, 16.69uL water, and primers: 50ng MYCOGEN-F, 50ng MYCOGEN-R, 50ng MYCAV-R and 75ng MYCINT-F. All reactions were 40uL in volume with 35uL master mix and 5uL template. The thermo cycler conditions were as follows: 95°C for 30sec followed by 98°C for 10 sec, 62°C for 15 sec, and 72°C for 10 sec. This was repeated 34 times followed by a final extension of 72°C for 7 minutes and holding at 4°C. 7uL of PCR product was run in a 2% agarose gel at 90V for 90 minutes. Band sizes were determined by running 1kb Plus DNA Ladder (Invitrogen, Life Technologies, Grand Isle, NY) alongside each batch of products. The band sizes produced were 1030bp to indicate *Mycobacterium* species, 1030bp + 180bp to indicate MA, or 1030bp + 850bp to indicate MI.

Boil-preps from isolates that are determined to be MA were then subjected to a multiplex PCR designed to identify *M. avium* subspecies *hominissuis* (MAH) and *M. avium* subspecies *avium/silvaticum* (MAA/MAS), using primers from Moravkova *et al*.[149] Master mixes for these reactions contained 17.5uL Phusion High-Fidelity Master Mix with HF Buffer (New England BioLabs Inc., Ipswich, MA), 1.75uL of 25mM MgCl2, 13.93uL Betaine, and primers: 10pmol IS901, 15pmol IS1245 and 40pmol dnaJ. The additional primers from Moravkova *et al* were not included in these reactions. All reactions were 40uL in volume with 35uL master mix and 5uL template. The thermo cycler conditions were as follows: 95°C for 30 sec followed by 98°C for 10 sec, 60°C for 20 sec, and 72°C for 15 sec. This was repeated 45 times followed by a final extension of 72°C for 5 minutes and holding at 4°C. 7uL of PCR product was run in a 2% agarose gel at 90V for 90 minutes and band sizes were determined by running 1kb Plus DNA Ladder (Invitrogen, Life Technologies, Grand Isle, NY) alongside it. The band sizes produced were 577bp + 385bp + 140bp to indicate MAA/MAS or 385bp + 140bp to indicate MAH.

Before beginning template preparation and PCR, the laboratory workers cleaned their workspace and all tools with a thick layer of vesphene for 10 minutes, followed by a 70% ethanol rinse and treatment with Eliminase decontaminant (Decon Labs Inc., King of Prussia, PA). To prevent cross-subject contamination, they wore gloves constantly and change gloves frequently. They included laboratory controls with each batch of isolates when boil-prepping and doing PCR. These controls were: H2O, *Staphylococcus aureus*, *Mycobacterium smegmatis*, and a MAH strain, 104.

A pre-printed batch-list was used to select archived isolates for re-growth, and accompanied plates through growth, template preparation, PCR set-up; images from visualizing PCR products were ultimately taped to the pre-printed batch-list. Gel-images were read by laboratory staff and entered directly into Excel spreadsheets (Excel 2007, Microsoft Corporation, Redmond, WA) for future importation into the study database.

Reference:

De Groote, M. A., N. R. Pace, K. Fulton, and J. O. Falkinham, III. 2006. Relationships between Mycobacterium isolates from patients with pulmonary mycobacterial infection and potting soils. *Appl Environ Microbiol* 72:7602-7606.