Cooccurrence of Free-Living Amoebae and Nontuberculous Mycobacteria in Hospital Water Networks, and Preferential Growth of *Mycobacterium avium* in *Acanthamoeba lenticulata*

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The incidence of lung and other diseases due to nontuberculous mycobacteria (NTM) is increasing. NTM sources include potable water, especially in households where NTM populate pipes, taps, and showerheads. NTM share habitats with free-living amoebae (FLA) and can grow in FLA as parasites or as endosymbionts. FLA containing NTM may form cysts that protect mycobacteria from disinfectants and antibiotics. We first assessed the presence of FLA and NTM in water and biofilm samples collected from a hospital, confirming the high prevalence of NTM and FLA in potable water systems, particularly in biofilms. *Acanthamoeba* spp. (genotype T4) were mainly recovered (8/17), followed by *Hartmannella vermiformis* (7/17) as well as one isolate closely related to the genus *Flamella* and one isolate only distantly related to previously described species. Concerning mycobacteria, *Mycobacterium gordonae* was the most frequently found isolate (9/17), followed by *Mycobacterium peregrinum* (4/17), *Mycobacterium chelonae* (2/17), *Mycobacterium mucogenicum* (1/17), and *Mycobacterium avium* (1/17). The propensity of *Mycobacterium avium* hospital isolate H87 and *M. avium* collection strain 104 to survive and replicate within various FLA was also evaluated, demonstrating survival of both strains in all amoebal species tested but high replication rates only in *Acanthamoeba lenticulata*. As *A. lenticulata* was frequently recovered from environmental samples, including drinking water samples, these results could have important consequences for the ecology of *M. avium* in drinking water networks and the epidemiology of disease due to this species.

Lung disease caused by nontuberculous mycobacteria (NTM) is an increasingly important global health problem, with an estimated prevalence in the United States of 30 to 60 cases per 100,000 individuals (1). *Mycobacterium avium* complex (MAC) organisms, including *Mycobacterium intracellulare*, are responsible for ~80% of NTM lung disease cases, characterized by bronchiectasis, nodules, and cavities (2). Chronic lung disease due to NTM is often recalcitrant to available therapy (3).

Free-living amoebae (FLA) are unicellular protozoa that are widely distributed in natural and human-made environments (4–6). FLA are ubiquitous phagocytes that can survive harsh conditions, partially due to the ability of many of them to switch from motile trophozoites to immobile cysts during starvation, desiccation, hypoxic conditions, and extreme temperature changes (7). During the trophozoite phase, FLA actively feed on bacteria, fungi, or algae. Although the general population is frequently exposed to FLA with no major health consequences (8), FLA can cause keratitis and devastating central nervous system disease (9).

FLA share many of the same aquatic niches as NTM and are abundant in soil, water, and biofilms (10–12). A study examining a water treatment plant in France discovered that while purification reduced the number and types of FLA, these organisms survived all phases of the water sanitation process, including ozonation and chlorination (11). In a study comprised of 2,454 water and biofilm samples taken from 467 homes, 79% of the households contained FLA, with showerheads and kitchen sprayers being most likely to harbor FLA, mainly *Acanthamoeba* and *Hartmannella* species (13). Since cultivation on nonnutritive agar (NNA) plates covered with *Escherichia coli* was used in those stud-

ies, it is likely that many FLA species could not be recovered under these limited growth conditions.

NTM have also been frequently detected in water treatment lines and in water distribution systems (14). High concentrations of NTM have been found in the surrounding air and water of hot tubs and therapy pools, with one study from a U.S. hospital yielding an NTM prevalence of 72% (15). Numerous experimental studies have substantiated the ability of different NTM species to survive and grow in FLA, and in some cases, NTM are considered to be “endosymbionts” of FLA (10, 16–19). *Acanthamoeba polyphaga* cysts have been shown to harbor MAC organisms, and the 11 MAC strains examined were culturable following excystment (20). Furthermore, *Acanthamoeba* cysts have been shown to resist inactivation by chlorine and other treatments used to sanitize drinking water (21) as well as to resist inactivation by glutaraldehyde and other disinfectants used in hospitals (22). Those studies suggested that encysted FLA may protect NTM from being killed during various disinfection processes (23). In addition, it was reported that *Acanthamoeba castellanii* can protect *Mycobac-*

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terium avium from the effect of antibiotics, thus raising concern about the possible transmission of *M. avium* through this vector (24).

While the relationship between NTM and FLA has been described in the literature, and it is speculated that FLA contribute to the transmission and pathogenesis of diseases caused by NTM (17, 25), it remains unclear if NTM, particularly MAC organisms, can grow in any FLA frequently recovered from water networks, i.e., *Acanthamoeba* species that belong to the T4 and T5 genotypes based on 18S rRNA gene sequences and *Hartmannella vermiformis*. Here, we report the recovery, identification, and coincidence of FLA and NTM from biofilm and water samples collected at a U.S. medical center. We also examined differential growth patterns of *M. avium* in various environmental and laboratory strains of amoebae, including representatives of the genera *Acanthamoeba*, *Hartmannella*, and *Naegleria*.

MATERIALS AND METHODS

Hospital. Multiple areas of a U.S. medical center were sampled. The hospital water system was recirculating. At the heat source, hot water was measured at 50°C. The temperature of hot water in a building far from the heat source was measured at 42°C.

Samples. In order to focus on clinically relevant areas, we sampled showerheads and faucets in patient rooms, drinking fountains, the hospital therapy pool, and disinfection units used to sterilize bronchoscopes and endoscopes.

A total of 88 samples were collected, including 23 water samples and 65 biofilm samples. Four patient rooms were examined. For each one, we sampled the shower and sink by swabbing the interior surface of the showerhead and faucet as well as each drain. Additionally, we collected two 500-ml water samples in succession from each water source sampled. For the hospital therapy pool, we sampled five locations with water stagnation, including the tile floor surrounding the pool, two water filters, the pool water, and a water dispenser used to feed water into the pool. We also collected biofilm samples from two disinfection unit stations. We also sampled eight sinks used to rinse the medical equipment. In addition, we swabbed three containers holding an aldehyde-based disinfectant or enzymatic cleaner. We also swabbed the two tubings that deliver the formulations to the sink used for washing scopes. For each sampling location involving disinfection, 2 to 3 swabs were taken.

Sample collection. Biofilms were sampled by swabbing the inside of the pipe with a sterile cotton-tipped applicator (CardinalHealth, McGaw Park, IL). After sampling, the entire applicator was placed into 5 ml of sterile phosphate-buffered saline (PBS), kept at 4°C, and processed within 48 h for isolation of FLA and NTM.

For liquid samples, 1 liter of water was sampled from each faucet or shower that was swabbed. Water was also collected from drinking fountains, standing water in sinks, and a hospital therapy pool. Both hot and cold taps were turned on, and water was collected into two 500-ml sterile bottles. The temperature of one of the water samples was recorded immediately after sampling. After collection, all water samples were filtered through a 0.2- μ m cellulose nitrate membrane (Sartorius AG, Germany). After filtration, the water was discarded, the filter was cut into pieces using an aseptic technique, and the pieces were suspended in 5 ml sterile PBS. Samples were processed within 48 h for isolation of FLA and NTM.

Recovery of cultivable FLA and cultivable NTM. The swab and water suspensions were centrifuged at $800 \times g$ for 10 min. Each pellet was resuspended in 1 ml of Page amoeba saline (PAS), and three 250- μ l aliquots were spread onto three nonnutritive agar (NNA) plates plated with a lawn of *Escherichia coli* ATCC 25922 for cultivation of indigenous amoebae. Supernatants were transferred into new sterile tubes and centrifuged at $2,800 \times g$ for 30 min. New pellets were resuspended in 1 ml PAS before being processed to recover cultivable NTM (see below).

Identification of indigenous amoebae. NNA plates were incubated at 28°C in a humidified atmosphere and examined daily for 12 days for the presence of amoebae. When positive, amoebae were subcultured on new NNA plates with *E. coli* to obtain clonal populations. After 3 subcultures, amoebae were harvested by scraping and resuspended in 1 ml of PAS. Ziehl-Nielsen staining was used to detect mycobacteria in indigenous FLA. To identify amoebae, DNA was extracted with the AquaPure Genomic DNA extraction kit and proteinase K (Bio-Rad), and partial 18S rRNA PCR and sequencing were performed with combinations of primers Ami6F1 and Ami9R or JDP1 and JDP2, as previously described (26, 27).

Coculture of environmental samples with an axenic FLA to recover NTM. Axenic *Acanthamoeba castellanii* ATCC 30010 was inoculated with water and biofilm samples as described previously (11). On day 6, the amoebal cocultures (generation F0) were subcultured on fresh amoebae, and subcultures (generation F1) were incubated for 14 days at 32°C. Ziehl-Neelsen staining was systematically performed by taking 50 μ l of cocultures F0 and F1 after 6 and 14 days of incubation, respectively. When acid-fast bacilli were detected, two 100- μ l aliquots were seeded onto 7H10 agar and incubated for 2 months at 32°C or 37°C.

Identification of NTM to the species level. Different strategies were used to identify mycobacterial isolates depending on their phenotypic characteristics after growth on 7H10 plates. All yellow isolates that developed rapidly on 7H10 plates (likely to be *M. goodii* according to our experience [26]) were identified by using partial 16S rRNA gene amplification and sequencing with primers FD1 and Rp2 as well as internal primers (28, 29). All white-ivory isolates (more likely to be pathogenic isolates for which the 16S rRNA gene sequence sometimes fails to discriminate species, as described for *Mycobacterium kansasii* and *Mycobacterium gastri*) were identified by partial sequencing of the *rpoB*, *secA*, and *hsp65* genes with primers based on published sequences (30, 31). Sequences were analyzed by comparison to known sequences in the nonredundant database at the NCBI website by using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and creating CLUSTAL alignments (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The results of these two tests determined the identification of NTM to the species level.

Intra-amoebal growth of *M. avium*. We examined the ability of clinically relevant *M. avium* strain 104 and *M. avium* hospital isolate H87 to infect, survive, and proliferate within seven distinct amoebal isolates. We used four *Acanthamoeba* strains that belong to the T4 genotype: *A. castellanii* ATCC 30010, *A. castellanii* ATCC 30234, *A. polyphaga* CCAP 1501/18 (identical to Linc-Ap1), and *Acanthamoeba* field isolate "H16," which was isolated from a sink drain in a patient room during this study and was successfully cultivated in peptone-yeast extract-glucose (PYG) broth without bacteria. We also included *A. lenticulata* ATCC 30841 (genotype T5), *H. vermiformis* ATCC 50237, and *Naegleria clarki* CCAP 1518/15. Briefly, *Acanthamoeba* spp. were cultivated in PYG broth, whereas *H. vermiformis* and *N. clarki* were cultivated in ATCC 1034 culture medium and in modified Chang's medium, respectively (32). For infection experiments, amoebae were cultivated in sterile broth until they reached confluence. Subsequently, FLA were suspended in PAS buffer and adjusted to 7.5×10^5 amoebae/ml. Ten milliliters of this suspension plus 10 ml of sterile PAS buffer were distributed in a 150-cm² cell culture flask and incubated at room temperature for 1 h for amoebae to adhere to the bottom of the flask.

To create fluorescently labeled mycobacteria, *M. avium* 104 and *M. avium* H87 were transformed with the red fluorescent protein variant mCherry by electroporation with the plasmid pCHERRY3 (33). This transformation was previously shown to have no effect on growth characteristics and virulence of mycobacteria (33). The two *M. avium* strains transformed with mCherry were cultivated for 10 days at 37°C on 7H10 plates with kanamycin (200 μ g/ml). Several colonies were then collected in 10 ml PAS, sonicated for 3 min, and filtered with a 5- μ m filter to remove aggregates. Optical densities were then adjusted to reach 3×10^8 bacteria/ml, and 250 μ l was distributed into every flask to reach a multi-

TABLE 1 List of samples from which amoebae and/or mycobacteria were isolated^a

Sample location	Sample type ^b	Amoeba(c)	Mycobacterium species
Patient room 1, shower drain	B	<i>Acanthamoeba</i> sp. genotype T4	
Patient room 1, sink drain	B	<i>H. vermiformis</i>	<i>M. chelonae</i>
Patient room 2, showerhead swab	B	<i>H. vermiformis</i>	<i>M. gordonae</i>
Patient room 2, sink drain	B	<i>H. vermiformis</i> and <i>Acanthamoeba</i> sp. genotype T4 ^c	<i>M. chelonae</i>
Patient room 2, shower drain	B	<i>H. vermiformis</i> and <i>Acanthamoeba</i> sp. genotype T4	<i>M. peregrinum</i>
Patient room 3, sink drain	B		<i>M. peregrinum</i>
Patient room 3, sink water	W	<i>H. vermiformis</i>	<i>M. gordonae</i>
Patient room 3, shower drain	B	2 different <i>Acanthamoeba</i> species, genotypes T3 and T4	Ziehl +
Patient room 4, sink faucet head	B		<i>M. gordonae</i>
Patient room 4, sink drain	B	<i>H. vermiformis</i> and <i>Flamella</i> sp.	Ziehl +
Patient room 4, sink water	W		<i>M. gordonae</i>
Patient room 4, showerhead swab	B	<i>H. vermiformis</i>	
Patient room 4, shower drain	B	<i>Acanthamoeba</i> sp. genotype T3	Ziehl +
Patient unit, water fountain faucet head	B	<i>Acanthamoeba</i> sp. genotype T4	<i>M. gordonae</i>
Patient unit, kitchen ice/water machine drain	B	Distantly related to <i>Allovalhikampfia</i> sp.	<i>M. peregrinum</i>
Hospital therapy pool tile floor	B		<i>M. gordonae</i>
Pool filter 1 (end of pool)	B		<i>M. mucogenicum</i>
Pool water dispenser	B	<i>Acanthamoeba</i> sp. genotype T4	
Research building, water fountain dispenser	B		<i>M. peregrinum</i> and <i>M. gordonae</i>
Enzymatic cleaner container	B		<i>M. gordonae</i>
Diagnostic center sink, running water	W		<i>M. avium</i> and <i>M. gordonae</i>

^a We mention "Ziehl +" only when acid-fast bacteria were observed after amoebal coculture but could not be recovered on 7H10 plates.

^b B, biofilm; W, water.

^c This isolate was successfully grown in PYG broth without bacteria and is termed "*Acanthamoeba* H16" in the text.

plicity of infection (MOI) of 10 bacteria per amoeba. Flasks were incubated overnight (16 h) at 28°C. The supernatant was then gently removed and replaced with PAS with 200 µg/ml amikacin. After incubation for 2 h at 28°C, the supernatant was gently removed and replaced with 12 ml sterile PAS, and amoebae were detached by tapping the flasks. The recovered suspension was centrifuged at 500 × *g* for 10 min, and the pellet was resuspended in PAS to reach approximately 7.5 × 10⁵ amoebae/ml. One milliliter of this suspension was distributed into 5 wells of a six-well plate and completed with 1 ml of a 1/5 dilution of PYG with 40 µg/ml amikacin, resulting in a final concentration of 1/10 PYG plus 20 µg/ml amikacin (verified to be bacteriostatic). At days 1, 2, 4, and 7 after infection, infected amoebae were collected by scraping the content of one well and then centrifuged at 500 × *g* for 10 min and adjusted to 7.5 × 10⁵ amoebae/ml in PAS. One hundred microliters (corresponding to 7.5 × 10⁴ cells) was deposited into 4 wells of a sterile 96-well µ-Clean microplate and allowed to attach to the bottom of the wells for 1 h in the dark. mCherry fluorescence was then measured in each well by using a FLUOstar Omega microplate reader (BMG Labtech) (excitation, 584 nm; emission, 620 nm; 10 by 10 scanning mode; gain value, 4,000). After fluorescence reading, pictures were taken by using an IX71 inverted microscope equipped with fluorescence (Olympus), and microplates were stored at −80°C before CFU were counted.

For counting of CFU, contents of the 4 identical wells were thawed, pooled, and pelleted by centrifugation at 21,000 × *g* for 10 min. The pellet was suspended in 0.1% Triton X-100 in PBS for 2 h and vortexed 4 times for 30 s during incubation to release intra-amoebal bacteria. The suspension was then serially diluted on 7H10 plates that were incubated for 8 days at 37°C.

In a second series of experiments, we used fluorescence readings to measure the ability of *M. avium* H87 to survive and grow within the same amoebal species described above. We then tested survival and growth of *M. avium* 104 and *M. avium* H87 in 8 additional *Acanthamoeba* strains that all belonged to the T4 genotype (*A. castellanii* CCAP 1501/10 and 7 field isolates described previously [11, 26], all tested to be free of endosymbionts), 2 additional *Naegleria* strains (*N. gruberi* CCAP 1518/17 and an *N. clarki* field isolate), and *H. vermiformis* field isolate 172 (26).

Statistical analysis. The mean numbers of mycobacterial strains isolated from amoeba-colonized samples were compared to the mean numbers of strains isolated from other samples by using the chi-square test (STATA software, version 7.0; Stata Corporation, College Station, TX). All infection experiments were repeated 3 times, and statistical analysis was performed by using one-way analysis of variance (ANOVA).

Nucleotide sequence accession numbers. Amoebal sequences have been deposited in GenBank under accession numbers [KC669503](#) to [KC669519](#). NTM sequences have been deposited in GenBank under accession numbers [KC669520](#) to [KC669536](#) and [KC669491](#) to [KC669502](#).

RESULTS

Recovery of cultivable FLA from the hospital water system. Of the 88 samples collected, 65 (73.9%) were swabbed (biofilm) samples and 23 (26.1%) were water samples. FLA were recovered from 13 of 88 (14.8%) hospital samples, mostly from biofilms. From the 13 samples containing cultivable amoebae, 17 distinct isolates were recovered. The majority of amoebae isolated were identified as *Hartmannella* sp. (*n* = 7/17; 41.2%) or *Acanthamoeba* sp. (*n* = 8/17; 47.1%) (Table 1). All *Hartmannella* isolates were assigned to the species *H. vermiformis* since 450- to 650-bp portions of their 18S rRNA gene sequences presented ≥98% identity with sequences of collection strains. *Acanthamoeba* isolates belonged mostly to genotype T4 (6/8 isolates); only two belonged to genotype T3. One of the genotype T4 isolates could then successfully be grown in PYG sterile broth without bacteria and was used in infection experiments. Another cultivable FLA recovered was closely related to the genus *Flamella* (100% identity with the 18S rRNA gene sequence of *Flamella balnearia* over 624 bp). The last isolate was distantly related to the genus *Allovalhikampfia* (92% identity with the 18S rRNA gene sequence of *Allovalhikampfia spelaea*). We did not observe bacteria that were positive by Ziehl-

FIG 1 Number of *M. avium* 104 bacteria per amoeba recovered after lysis of infected amoebae and seeding of released bacteria on 7H10 plates. The following amoebal strains were infected: *Naegleria clarki* CCAP 1518/15 (◇), *Hartmannella vermiformis* ATCC 50237 (□), *Acanthamoeba castellanii* ATCC 30234 (●), *A. polyphaga* CCAP 1501/18 (▲), *A. castellanii* ATCC 30010 (△), *Acanthamoeba* field isolate H16 (◆), and *A. lenticulata* ATCC 30841 (■). Data are shown as mean values of three independent experiments; error bars represent upper and lower limits for 95% confidence intervals for the means.

Neelsen staining in the indigenous FLA recovered from the hospital water system.

Cultivable NTM were more likely to be recovered from samples containing cultivable FLA. Overall, 26 of the 88 environmental samples cultured with axenic *A. castellanii* stained positive for acid-fast bacteria. Of the positive samples, 15 (12 biofilm and 3 water samples) were culture positive for 17 identifiable NTM isolates (Table 1). Nine isolates were identified as *M. gordonae*, four were identified as *M. peregrinum*, two were identified as *M. chelonae*, one was identified as *M. mucogenicum*, and one was identified as *M. avium*. Of the 13 environmental samples from which cultivable FLA were recovered, NTM were isolated from 7 (53.8%), whereas of the 75 samples in which no FLA were found, NTM was isolated from 8 (10.7%). There was a statistically significant relation between the presence of cultivable mycobacteria and the presence of cultivable FLA ($P < 0.001$). NTM could not be isolated from the 11 remaining coculture samples positive for acid-fast bacilli. Of these, three were also positive for indigenous FLA. Acid-fast-positive but culture-negative samples included samples taken from patient rooms (sink water, sink, and shower drains), from the water fountain dispenser and drain, and from one sterilizer unit (including sink, enzymatic cleaner container, and disinfectant container).

Differential growth of *M. avium* in different isolates of FLA. First, we compared two techniques for monitoring intracellular growth of *M. avium* 104 labeled with mCherry: measurement of fluorescence intensity and counting of CFU on 7H10 agar. There was marked variation in the number of mycobacteria recovered from *A. lenticulata* infected with *M. avium* 104 after 1 to 7 days compared to the number of mycobacteria recovered from different FLA isolates (Fig. 1). There was a good correlation ($R^2 = 0.74$; $P < 0.05$) between the fluorescence intensity measurement and CFU counts (Fig. 2 and 3A). *A. lenticulata* ATCC 30841 was more efficiently infected by *M. avium* 104 than the 6 other amoebal strains tested, resulting in approximately 25 intra-amoebal mycobacteria after 7 days of infection, whereas fewer than 2 mycobac-

teria/amoeba were counted for all other amoebal strains ($P < 0.05$) (Fig. 1). This was confirmed by microscopic observation of infected amoebae. Microscopy revealed large clumps of fluorescent bacteria within *A. lenticulata*, whereas smaller clumps were seen within other *Acanthamoeba* spp. and *H. vermiformis* (Fig. 4). For *N. clarki*, only single, primarily extracellular bacilli were observed (Fig. 4). Of note, *H. vermiformis* tended to form cysts faster than *Acanthamoeba* and *Naegleria* spp. (Fig. 4E and F), which could have a negative impact on intracellular replication of mycobacteria.

Similar results were obtained when measuring the fluorescence intensity of amoebae infected with *M. avium* field strain H87 (Fig. 3B). Additional infection experiments were performed to evaluate the capacity of both *M. avium* strains to infect other *Acanthamoeba* isolates, an *N. clarki* environmental isolate, *N. gruberi* CCAP 1518/17, and an *H. vermiformis* environmental isolate. None of these additional FLA strains yielded levels of mycobacterial replication similar to that observed for *A. lenticulata*, with all

FIG 2 Correlation curve between fluorescence intensity data and CFU (R^2 [logarithmic] = 0.74; $P < 0.05$).

FIG 3 Fluorescence of amoebal cells infected with *M. avium* 104 mCherry (A) or *M. avium* NJH 87 mCherry (B). The following amoebal strains were infected (from left to right): *Naegleria clarki* CCAP 1518/15 (□), *Hartmannella vermiformis* ATCC 50237 (▤), *Acanthamoeba castellanii* ATCC 30234 (■), *A. polyphaga* CCAP 1501/18 (■), *A. castellanii* ATCC 30010 (▨), *Acanthamoeba* field isolate H16 (▩), and *A. lenticulata* ATCC 30841 (▧). Day 0 corresponds to fluorescence of noninfected amoebae. Data are shown as mean values of three independent experiments; error bars represent upper and lower limits for 95% confidence intervals for the means.

fluorescence measurement values staying at the same level during the course of infection, suggesting intracellular survival with low or no replication (data not shown).

DISCUSSION

In previous studies, Falkinham et al. examined the presence of MAC organisms and other NTM in biofilms and found that 69% of bacterial isolates found in the biofilms of drinking water distribution systems were acid fast (34). Of 450 acid-fast organisms isolated, 267 were species of *Mycobacterium*. The average concentration of mycobacteria within biofilms was 600 CFU/ml. How-

ever, levels as high as 2,850 CFU/ml were recovered (34). In a previous study of biofilm samples taken from showerheads across U.S. cities, rRNA gene sequencing revealed ~6,000 unique rRNA sequences, of which *Mycobacterium* was the most common genus, accounting for approximately one-third of all clones isolated (12). FLA also reside in household water systems and are known to be enriched in the presence of biofilms (35). The present study confirms that FLA and NTM are readily recovered from potable water sources, including hospital water systems (26). Overall, we recovered 17 cultivable FLA isolates and 17 cultivable NTM isolates from nearly all hospital areas sampled. The showers and sinks in

FIG 4 Amoebae infected with mCherry-transfected *M. avium*. *A. lenticulata* ATCC 30841 (A and B), *Acanthamoeba* H16S1 (C and D), *H. vermiformis* ATCC 50237 (E and F), and *Naegleria clarki* CCAP 1518/15 (G and H) were infected with *M. avium* H87 (left column) or *M. avium* 104 (right column) according to the described protocol and observed 4 days after infection.

patient rooms yielded the most FLA, NTM, or both, including the clinically relevant *M. chelonae*. Furthermore, samples containing cultivable FLA were significantly more likely to yield cultivable NTM. This supports the notion that FLA and NTM cohabitate in biofilms within plumbing systems that distribute potable water (6, 26). However, we did not detect NTM directly in FLA isolates cultivated from hospital water and biofilm samples, suggesting that colonization of cultivable FLA by NTM in these ecosystems is not particularly frequent. Instead, the presence of *Acanthamoeba* within the water system could potentiate saprozoic growth of NTM by releasing extracellular metabolites that can be used as

nutrients by NTM (25). Alternatively, the method used to isolate FLA can also have a negative impact on recovery of intra-amoebal NTM since FLA develop relatively quickly on NNA plates with *E. coli*, and only small portions of the migration fronts are subcultivated for purification and identification of FLA. Infected amoebae initially present in the original sample might be lost during this process. In addition, it cannot be excluded that FLA that cannot be grown on NNA plates with *E. coli* were present in the samples that we analyzed and can support intracellular growth of cultivable (or noncultivable) NTM; these limitations have to be kept in mind when interpreting the results presented here.

While no FLA were cultured from samples taken within a facility used to disinfect diagnostic medical equipment, 7 of these samples were positive for acid-fast organisms, likely indicating the presence of noncultivable NTM. This is troubling considering that bronchoscopes and endoscopes are cleaned within this facility and that tap water is used to rinse equipment following chemical disinfection. *M. gordonae* was cultured from a container used to store an enzymatic cleaner and the running water used to wash scopes. However, this isolate was fully inactivated ($>5\text{-log}_{10}$ reduction) when exposed to diluted enzymatic cleaner for 24 h at room temperature (data not shown). Therefore, it is likely that the container's surface was contaminated by tap water containing *M. gordonae* and that the pure enzymatic cleaner stored in the container did not contain NTM. *M. avium*, which is one of the most common causes of NTM lung disease in the United States (3), was also isolated from the faucet in the cleaning facility. *M. avium* has been isolated from other hospital water networks as well as from household plumbing (36, 37).

There have been few studies that have investigated the relationship between *M. avium* and amoebae. Recently, Berry and colleagues (38) reported that amoebal strains (mostly of the T4 genotype) freshly isolated from the environment yielded fewer bacteria than laboratory strains when experimentally infected with *M. avium* 104. However, reported differences were low, with 6 to 10 bacteria/amoeba observed in the field isolate and 3 to 5 bacteria/amoeba in the laboratory isolate during the course of infection. We investigated the capacity of *M. avium* collection strain 104 and *M. avium* H87 recovered from the hospital water system to proliferate within a large number of amoebal strains, including *H. vermiformis*, which is frequently isolated from water networks but has never been tested for its ability to harbor NTM. Our results demonstrate that both *M. avium* isolates survive but do not proliferate in *H. vermiformis* and *Naegleria*. Interestingly, similar results were observed for the proliferation of *M. avium* isolates in other *Acanthamoeba* strains tested. The only exception was *A. lenticulata*, for which significant intracellular proliferation was observed. In addition, *M. avium* field strain H87 had a better capacity to invade *Acanthamoeba* spp. than the collection strain *M. avium* 104 ($P < 0.05$). This might be due to attenuated virulence of the laboratory strain since virulence correlates with the ability of *M. avium* strains to invade FLA (39). We believe that proliferation of *M. avium* in *A. lenticulata* may significantly impact the epidemiology of this pathogenic NTM in drinking water networks. Indeed, *A. lenticulata*, the only representative species of genotype T5, is frequently isolated from environmental and clinical samples. In a large study by Fuerst et al., *A. lenticulata* was the second most frequent species recovered from environmental samples after species of the T4 genotype (40). Half of the *Acanthamoeba* isolates recovered from rivers, canals, and lakes in Arizona were *A. lenticulata* (41). *A. lenticulata* has also been isolated from biofilm samples collected from hospitals and swimming pools in Brazil (42, 43) as well as soils and tap water samples in Florida (44). However, to our knowledge, the present study is the first examination of mycobacterial growth in *A. lenticulata*, with all other previous studies using species that belong to genotype T4: *A. castellanii* strains ATCC 30010 and ATCC 30234 or *A. polyphaga* LincAp-1 (10). Our results suggest that *M. avium* survives but does not replicate efficiently in these strains. Importantly, Carroll et al. demonstrated that fluorescence emitted by mycobacteria transformed with the plasmid pCHERRY3 is related directly to

viability, thus ensuring that the measured fluorescence corresponds to living bacteria (33).

Acanthamoeba lenticulata can have different susceptibilities to infection by various microorganisms compared to *Acanthamoeba* spp. that belong to genotype T4. For example, we recently demonstrated that Lausannevirus, a "giant" virus, infected and rapidly killed all *Acanthamoeba* isolates that belonged to the T4 genotype but not *A. lenticulata* (45). The same was demonstrated for *Parachlamydia acanthamoeba* strain Bn9, which could not infect two different *A. lenticulata* strains, whereas it efficiently infected nine *Acanthamoeba* strains that belonged to genotype T4 (46). These host selectivity aspects could dramatically impact the distribution of acanthamoebal genotypes in water networks and subsequently affect the proliferation of select mycobacterial species (45). We believe that these observations urge the need for additional experiments to understand the distribution of *A. lenticulata* in water networks. In addition, further studies should more extensively test the ability of *A. lenticulata* strains to harbor intracellular replication of *M. avium* and other pathogenic NTM. Mycobacterial strains modified with the plasmid pCHERRY3 will be very useful to reach these goals.

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