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Short Communication

Methylobacterium spp. as an indicator for the presence or absence of *Mycobacterium* spp.

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ABSTRACT

Objective/Background: A published survey of bacteria in showerhead biofilm samples revealed that *Methylobacterium* spp. and *Mycobacterium* spp. seldom coexisted in biofilms.
Methods: To confirm that information, biofilm samples were collected from household plumbing of *Mycobacterium avium* patients and *Methylobacterium* spp. and *M. avium* numbers were measured by direct colony counts.
Results: The results demonstrated that if *Methylobacterium* spp. were present, *Mycobacterium* spp. were absent, and the opposite.
Conclusion: The data demonstrate that microbial populations in biofilms can influence the presence or absence of opportunistic premise plumbing pathogens and, thereby, increase the range of strategies to reduce exposure to waterborne pathogens. Finally, by assessing for the visual presence of methylobacteria as pink pigmentation on showers and shower curtains, homeowners and managers of hospitals and other buildings can quickly determine whether a premise plumbing biofilm sample has mycobacteria with a high degree of assurance.

Introduction

Nontuberculous mycobacteria (NTM) are opportunistic human pathogens whose source of infection is the environment [1]. *Mycobacterium* species are found in drinking water distribution systems [2], hospital plumbing [3], and household plumbing [4] and cause chronic and life-threatening pulmonary infections that are difficult to treat [5,6].

The incidence of NTM disease in the USA and Canada is rising [7,8]. In Toronto, Canada NTM disease incidence has risen from 1.5 per 100,000 to 9.0 per 100,000 over the period 1997–2003 [7]. Similarly, NTM disease is increasing in the USA based on reports of NTM lung disease in hospitalized persons [8]. A major fraction of these cases are found in older, slender women, lacking any of the classic risk factors for NTM disease, yet have a greater tendency than the general population to develop NTM pulmonary disease [9–11]. It follows that as the population of the USA continues to age—25% of the US population will be over 60 years by 2025 [12]—the incidence of NTM pulmonary disease will continue to increase. Further, as

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NTM-infected patients are subject to the reemergence of infection or reinfection by other environmental NTM [13], it is of value to identify means to reduce NTM exposure.

A recent study demonstrated the widespread presence and high numbers of *Mycobacterium* spp. and *Mycobacterium avium* in showerhead biofilms across the USA [14]. Although not highlighted by the authors, examination of the data indicate a potentially important pattern; namely, the presence of clones of pink-pigmented *Methylobacterium* spp. were associated with the absence of *Mycobacterium* spp. and the presence of *Mycobacterium* spp. with the absence of *Methylobacterium* spp. [14]. Specifically, of 10 clone libraries with >37.5% *Methylobacterium* spp. sequences, nine had <6.4% *Mycobacterium* spp. sequences [14]. Correspondingly, of eight clone libraries with >50% *Mycobacterium* spp. sequences all had >7.5% *Methylobacterium* spp. sequences [14]. In only one showerhead sample was there both substantial *Mycobacterium* spp. (46.7%) and *Methylobacterium* spp. (38.9%) sequences [14].

Like *M. avium* and other NTM, *Methylobacterium* spp. are normal inhabitants of drinking water distribution systems and buildings, including hospitals [15–21]. Further, a substantial proportion of *Methylobacterium* spp. isolates are chlorine-resistant [22], form biofilms [23,24], and belong to the group of amoeba-resisting bacteria of drinking water [25]. Household environments influenced by municipal water are evidently also habitats, as *Methylobacterium* spp. have been shown to be abundant among DNA clones recovered from shower curtains [26].

For this study, it was hypothesized that the presence of the pink-pigmented *Methylobacterium* spp. would be associated with the absence of *Mycobacterium* spp. and that the presence of *Mycobacterium* spp. would be associated with the absence of *Methylobacterium* spp. to test the hypothesis, household water systems were sampled to directly measure numbers of *Methylobacterium* spp. and *Mycobacterium* spp.

Materials and methods

Households

The Falkinham laboratory is currently participating in a study of households of patients from the Philadelphia, Pennsylvania area with *M. avium* pulmonary disease, with the objective of determining whether their household plumbing (including showerheads) could be the source of their infection. Approval for that study was granted by the Main Line Health Hospitals Institutional Review Board.

Collection of household water and biofilm samples

Surface biofilms of water taps and showerheads from households of patients with NTM pulmonary disease and their neighbors were swabbed (5–10 cm^2) by study personnel and placed in 3 mL of tap water from the residence. Containers and swabs were sent to the Virginia Tech laboratory for isolation, enumeration, and identification of mycobacteria and methylobacteria. Patients did not collect samples, lest patient *M. avium* contamination occur.

Isolation of Mycobacterium spp. and Methylobacterium spp. from biofilm samples

Isolation, enumeration, and identification of *Mycobacterium* spp. was performed as described [27]. In addition, samples were also spread on R2A agar (BD, Sparks, MD, USA) and incubated at 30 °C for 3 days for isolation and enumeration of the pink-pigmented, presumptive *Methylobacterium* spp.

Identification of Methylobacterium spp. isolates

Pink-pigmented colonies on R2A agar were picked, purified using single colony isolation, and identified on the basis of cultural, biochemical, and enzyme tests as described [19].

Results

Isolation of Methylobacterium spp. and Mycobacterium spp. in household plumbing biofilm samples

To confirm that the presence of *Methylobacterium* spp. was associated with an absence of *Mycobacterium* spp. [14], 153 biofilm samples from the plumbing of 20 Philadelphia NTM patient households and neighboring control households received by the Virginia Tech laboratory were processed to isolate, enumerate, and identify pink-pigmented colonies as well as *Mycobacterium* spp. Pink-pigmented colonies were picked and purified. Seven percent of pink colonies proved to be yeast by microscopic morphology, 9% were cocci (presumably *Deinococcus* spp.), and the remaining 84% were gram-negative rods. The rod-shaped isolates were identified as *Methylobacterium* spp. on the basis of the presence of catalase activity, absence of urease activity, their failure to grow at 37 °C or 42 °C, absence of growth on MacConkey agar, inability to hydrolyze Tween 80, or change the pH of triple sugar iron agar [19]. A majority grew on glycerol or xylose as sole carbon sources, but failed to grow on glucose, lactose, or mannitol [19]. As noted by others [19], a high percentage (92%) of the *Methylobacterium* spp. isolates recovered here spontaneously aggregated in Tryptic Soy broth cultures.

All the acid-fast isolates from the biofilm samples collected from the plumbing of the patients' and neighbors' households sampled for this study proved to be *M. avium*.

Coexistence of Methylobacterium spp. and Mycobacterium spp. in biofilms

Based only on the number of *Methylobacterium* spp. isolates, the results (Table 1) show that biofilm samples with *Methylobacterium* spp. seldom yielded *Mycobacterium* spp. and that samples lacking *Methylobacterium* spp. were more likely to yield *Mycobacterium* spp. The criterion for the presence of *Methylobacterium* spp. colonies in samples required $\geqslant$10 or more colonies (i.e., 300 CFU/cm^2) on RA2 agar. Any sample yielding an *M. avium* isolate was considered positive. Based on the assumption that the distribution of *Mycobacterium* spp. and *Methylobacterium* spp. should be equal and random, the variation in the four groups was significantly different than expected (p = .0015, Fisher's exact test). Thus, the results