Title: Environmental Risk of Nontuberculous Mycobacterial Infection: Strategies for Advancing Methodology

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Abstract:

The National Institute of Allergy and Infectious Diseases organized a symposium in June 2022, to facilitate discussion of the environmental risks for nontuberculous mycobacteria exposure and disease. The expert researchers presented recent studies and identified numerous research gaps. This report summarizes the discussion and identifies six major areas of future research related to culture-based and culture independent laboratory methods, alternate culture media and culturing conditions, frameworks for standardized laboratory methods, improved environmental sampling strategies, validation of exposure measures, and availability of high-quality spatiotemporal data.

I. Introduction

Nontuberculous mycobacteria (NTM) are ubiquitous environmental pathogens, frequently causing disease in those with underlying health conditions, such as cystic fibrosis (CF) [1-3]. NTM pulmonary disease rates have increased in recent decades, in both these high-risk populations and the general population [4-8]. Preventing infection and disease from NTM, both among persons with CF and others, is a significant public health need.

Identifying NTM sources and routes of transmission and subsequent infection are central to prevention efforts. NTM have been isolated from a variety of environmental reservoirs, including soil, natural water bodies, and water and biofilms in the built environment and premise plumbing. However, not all have been definitively linked to human disease [9]. A better understanding of how environmental exposure contributes to disease is needed [10]. In 2017, the National Institute of Allergy and Infectious Diseases (NIAID) held a workshop attended by diverse experts and published a roadmap for future research [11]. The experts at these workshops identified numerous research gaps and suggested foci for the future.

In the intervening years between the 2017 workshop and this report, a significant amount has been done to fill the gaps identified. In June 2022, NIAID organized a symposium in Fort Collins, Colorado, in conjunction with the 2022 Colorado State Mycobacterial meeting, to summarize research related to the environmental risks of NTM disease and discuss critical research gaps. In this report, we summarize key developments and remaining research questions related to environmental risk factors for NTM disease (Table 1). We anticipate that this summary will guide future research efforts and policy decisions.

II. Recent advances in NTM environmental risk research

Environmental sampling and laboratory methods.

The geographic distribution of NTM is predicted by a number of environmental and climatic factors [12]; specific environmental conditions are likely optimal for different mycobacteria species/strains. Researchers at the University of Colorado, Boulder have been quantifying the pH and temperature growth optima of a variety of mycobacteria, investigating different optima for pathogenic versus non-pathogenic species/strains. Because pH and temperature influence NTM biogeography, these variables have been found to predict the presence or virulence of mycobacteria across different systems [13-15]. While the effects of other environmental factors, such as oxygen availability or soil features [16, 17], on NTM distribution are under study, the research will benefit from broader sampling strategies.

Sampling efforts have been varied across the landscape. In Hawai'i, an area with high disease incidence, community science efforts have promoted sampling in a variety of geographic niches including natural and indoor environments [18-21]. Community science is being used in other areas, and greater efforts are being made to sample widely. Efforts should also include sampling of various locations within the built environments where most humans spend the majority of their time [22, 23]. Studies of colonization in the built environment have historically been focused on premise plumbing but, as with outdoor environments, data from diverse sampling efforts, such as air and aerosol sampling, will further elucidate sources of NTM exposures [24, 25]. A valid method for measuring human exposure to NTM, rather than disease, will also propel the field forward. For *Mycobacterium tuberculosis* (TB), noninvasive oral swabs were used in a 2019 study as an alternative sample type to sputum to detect up to 90% of cases [26]. Sputum can be difficult and hazardous to collect and challenging to process in the laboratory. Using oral swabs to detect NTM exposures, rather than infection, may be feasible and could be further explored.

For any study to succeed, laboratory methods must have high sensitivity and specificity for the presence of mycobacteria in samples. These requirements are affected by contamination and overgrowth of bacteria and fungi in culture. In the last few years, new NTM isolation media that have entered the commercial market may improve NTM recovery [27].

Epidemiology and analytic methods

NTM incidence has been increasing worldwide. In Canada, increases of both culture positivity and disease are a growing concern. The cause of the 2014 surge in *Mycobacterium avium* isolation in the Toronto area is still unknown [28]. Drinking water source-type and treatment and quality variables among the 42 largest water treatment systems in the province of Ontario, Canada, were used in modeling regional rates of NTM disease. Although numerous trends were identified, the power to identify significant effects may have been limited by methodology and the small sample size. With the analysis at the level of the region, each water treatment region in the province contributed its own rate and models were constructed based on only 42 rates. With so few regions, the sample size may have been too small to see an effect [29].

In the US, research regarding water quality factors and NTM infections have identified an association between concentrations of the trace metals vanadium and molybdenum in the municipal water supply and NTM pulmonary infection risk [30-32]. Although the specific factors influencing this increased risk have not been elucidated, some evidence suggests that the presence of these metals is important for mycobacterial metabolism, thereby increasing NTM abundance, and subsequently increasing the risk of exposure and infection. This hypothesis is supported by prior studies showing a significant correlation nationally between state-specific disease prevalence and showerhead mycobacterial relative abundance [15]. Alternatively, or in addition, these metals may confer increased host susceptibility in humans [32, 33].

In Queensland, Australia, NTM infections have remained notifiable since the inception of statewide TB services. The increasing incidence of *M. avium* complex (MAC) cases spurred an evaluation of geospatial risk [8]. NTM clusters have been found, with the best models including a spatial component. Adjusted models revealed geographic and temporal trends, with cyclic incidence patterns associated with temperature and rainfall [34]. Conversely, in the US, state-level NTM reporting is not consistently required. Only twenty states have some form of mandatory reporting, with few specifying the *Mycobacterium* species (Figure 1). To identify burden, trends, and clusters in the US, researchers must combine data from multiple independent sources including federal and state sources, as well as patient registries and electronic health records.

Some variables have consistent effects across space. Disease is associated with population density [4, 35], possibly due to more susceptible individuals living in high-density

areas near specialized health care providers or clinics. Higher population density is also associated with more complicated water distribution systems and premise plumbing that may be associated with enhanced growth of NTM in pipe biofilm and dissemination of NTM to households [36]. With limited understanding of the incubation period for NTM in a host, i.e. the lag time between NTM exposure and disease onset, it is difficult to determine the true effect of events related to time, such as time spent in a specific geographic area [37] or temporal changes made to water treatment. As the environment changes and more extreme weather events are predicted, frequent screening in susceptible populations will provide invaluable temporal data [38].

To analyze geographic trends, methods are needed to systematically identify high-risk areas where the risk of disease incidence is significantly higher than what is expected under some hypothesis of constant risk across all geographic regions (potentially after adjusting for explanatory variables). For these approaches, precise location information for cases is necessary, and standardized data are ideal for consistency across studies. To date, more precise spatial data has not been readily available, but more recent studies have used large linked deidentified datasets with patient residence geocoded to latitude and longitude within 1 km [39].

III. Knowledge gaps and areas of future research

1. Culture-based and culture-independent laboratory methods complement each other and should be used in tandem.

The choice between culture-based and culture-independent methods for detection of NTM typically depends on the proposed research or clinical question. For environmental samples, the yield from culture-based methods is limited by the potential for bacterial and fungal overgrowth, requiring harsh decontamination steps and/or selective media suppressing the growth of non-acid-fast bacilli, that can further impede mycobacterial growth. These steps and the slow growing nature of NTM, makes culture-based methods time-consuming. Additionally, culture-based methods may miss clinically relevant NTM that are difficult to cultivate. Culture independent methods may provide a broader landscape of NTM from different samples. However, to ascertain if a species/strain of NTM from the environment is disease-causing, genomic similarity with strains causing human disease is required.

Direct detection using qPCR (quantitative polymerase chain reaction) has been explored as an alternative to culture for detection of NTM in environmental samples [40]. While culturing can allow for the detection of NTM, even if they are in low abundance, culture-independent methods can allow for more rapid detection of diverse NTM taxa in environmental samples. Additionally, culture-independent methods may aid detection of clinically relevant NTM that are difficult to cultivate.

PCR methods perform more efficiently as a monitoring tool, and PCR positivity may then prompt further investigation using a culture-based method better for more targeted or in-depth analyses. Culture-based approaches additionally allow for variation in experimental methods, such as the use of selective media or cultivation across ranges of pH, temperature, or other environmental factors of interest. As studies evolve to investigate strain-specific optima of environmental factors, culturing methods will remain indispensable.

2. Additional study is needed for novel media that may improve culture validity.

Improved sensitivity and specificity of culture media is needed to improve the recovery and identification of mycobacteria in environmental samples. Each combination of NTM species and sampling matrix, such as water, biofilm or soil, presents a unique culturing challenge. Novel selective media have been described [27, 41, 42] and additional study of the costs and benefits of their use is warranted. The Rapidly Growing Mycobacteria (RGM) media (NTM Elite agar, bioMerieux, Marcy-l'Etoile France), for example, has shown promise in early studies [27], but has not yet been tested extensively across laboratories or in the context of diverse sample types.

The move toward more selective media is driven, in part, by the high concentration of competing bacteria in environmental samples, and the labor and materials required by the use of traditional NTM media. Depending on sample type, a decontamination step may be required before culture with Middlebrook 7H10/7H11 or Lowenstein-Jensen, and a proportion of plates may yet be eliminated due to contamination or overgrowth. Concerns that decontamination steps may remove NTM from samples also encourage development of selective media. The purpose of the culture will ultimately determine the media used, specific to NTM species, sample matrix, and study goals. The role played by more selective media in the future will depend on the results of further cost-benefit analyses comparing these media to traditional options.

3. Standardized laboratory methods are recommended, but effort and cooperation is necessary to establish a framework.

All laboratory methods have relative advantages and disadvantages, and their utility depends upon the research goals of a specific study or surveillance effort. A classic approach to limit bias is to standardize methods across studies. Clinical studies of NTM have some level of standardization, but such standardization for environmental monitoring efforts do not currently exist.

Historically, establishing standards for other bacterial genera has been difficult. Variations within standards result in lengthier approval times. At the same time, care should also be taken to assure that standards do not inhibit the implementation of new, improved methods, but allow for deviations. Methodological improvement must be measured against some baseline, however, and standardized methods provide such a baseline. This baseline does not currently exist for NTM.

Specific standard protocols are likely necessary for individual mycobacterial species and for various sample types. Decontamination or extraction steps will vary between soil and water samples. Culturing pH and temperature optima will differ among *Mycobacterium* species/strains. With culture-independent methods, standard target genes should be determined for each species. These standards, however, may still select for specific species and will not describe the entire microbial diversity (microbiome) in a sample, even in a culture-independent framework. Typically single genes are used for identification. Without appropriate reference databases, some samples sent for analysis may remain unspeciated. For example, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) methods have limited specificity, as results are only as good as the reference database. Expanding the reference library of MALDI-TOF MS to include more environmentally relevant species will improve success for environmental monitoring and epidemiologic investigations.

Improving laboratory standards will require cooperation among all relevant institutions, including clinical and public health laboratories, industry partners, and regulatory and public health agencies such as the FDA and CDC, both in the initial standardization phase as well as in the maintenance of robust reference databases. Efforts in other fields, such as food safety laboratories use of standardized methods for food and environmental sample analyses or the CDC's protocols for *Legionella*, may provide useful models moving forward.

4. The utility of exposure data depends on environmental sampling strategy.

The statistical power and generalizability of environmental findings regarding environmental exposures, infections, or disease will depend upon the number of samples taken and the strategy used to choose sampling locations. Much work has focused on sampling within the homes of NTM pulmonary disease cases. Such studies are important because most people spend most of their time within built environments. Within homes, site and mode of sampling can be important considerations. For example, a disease association study found that NTM isolation from shower aerosols is highly associated with MAC pulmonary disease, while isolation from bulk tapwater and soils, environments found in or near homes, was not [43]. In contrast, a cross-sectional study in Florida found an association between duration of soil exposure, particularly soil-related occupations, and *Mycobacterium avium* exposure [44]. Isolation of NTM from soil has been linked to patients with NTM disease [45, 46]. A combination of indoor and outdoor samples provides a broader view of NTM biogeography. Large-scale studies are needed in diverse locations, with samples from various substrates. In homes, samples of household dust and air filters would complement those obtained from premise plumbing. Sampling efforts focused on quantifying NTM distributions in soils or waterbodies would benefit from collaborations with state or federal agencies that sample widely. This approach may lower the expense and increase the feasibility of obtaining fine-scale environmental data.

5. High-risk populations allow for efficient epidemiology, while a measure of exposure, not infection, will allow for more precise associations.

NTM have been identified in numerous sources to which human populations are exposed, including water, soil, and aerosols. Future studies will be needed to test the efficacy of additional behavior modifications, point-of-use interventions, or other prevention efforts. Such studies should be conducted in cohorts where the incidence rate is high enough to obtain statistical power needed to detect the effect of the intervention [47, 48]. For example, patients with CF or individuals previously infected who have experienced culture conversion undoubtedly comprise the highest risk, while people with non-CF bronchiectasis and chronic obstructive pulmonary disease may also comprise feasible study populations

Epidemiological studies in these populations will necessarily be observational; randomizing high-risk patients to avoid potentially protective tools or behaviors may not be ethical. A validated method to detect exposure, not infection, would facilitate broader epidemiological studies possible in the general population. In such individuals, who are not at high risk, randomized intervention studies would be possible.

6. Environmental epidemiology studies require high-quality spatiotemporal resolution

Statistical methods focused on geographic areas are particularly useful for determining the effect of environmental variables on disease risk. Results vary, however, depending on how patients are grouped. The goal of analyses should be flexibility, to show any possible clustering, while remaining computationally feasible.

Standardized data with well-defined geographic information are not readily available. The optimal data comprise geocoded patient addresses, with residence information geocoded to some radius to protect the participants' privacy. Data are more often aggregated by zip code or county, and the best statistical methods are chosen based on the data available. The quality of available data, in particular groupings at broader spatial levels, limits analytic flexibility and hampers discovery of meaningful associations.

Analysis of temporal associations could also yield important information on the epidemiology of NTM. However, because the incubation period for NTM disease in a host is unknown, and is likely influenced by the exposure dose and the virulence of the infecting strain/species, these analyses have been limited. Nonetheless, one analysis of a large linked dataset representing Kaiser beneficiaries in Hawai'i did find a positive association between time of residence and risk of NTM infection [37]. Analysts with access to time-series or other temporally-structured datasets may find associations between disease incidence and water treatment or other historical changes. Such analyses, in turn, may give researchers clues about the host incubation period for NTM, as well as factors influencing disease risk.

IV. Summary and conclusions

NTM are ubiquitous environmental organisms that increasingly pose risk across diverse populations. This report summarizes the input of expert researchers of NTM environmental predictors who identified six major areas of focus for future research:

1. Simultaneous use of both culture-based and culture-independent laboratory methods.
2. Increased use of alternate media and broader culturing conditions, in addition to traditional media, to select for mycobacteria in culture.
3. Establishing a framework for standardizing laboratory methods.
4. Improved environmental sampling strategies with population-based or other sampling frameworks, to define the geographic area and allow increased generalizability.
5. Validation of a measure of exposure to conduct epidemiological studies in low-disease-risk populations.
6. Availability of high-quality spatiotemporal data for models of host NTM incubation periods and for flexible, yet efficient, identification of disease clustering.

The authors provide these recommendations to help guide future research and fill the knowledge gaps necessary for prevention and control of NTM lung disease.

Acknowledgements:

This work was supported in part by the Intramural Research Program at the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

The authors thank the conference organizers, speakers, and attendees for their contributions to the symposium and manuscript.

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Table 1: Important foci of future environmental NTM research.

Research Focus	Needs
Culture-based and culture-independent laboratory methods	Increase complementary use of both culture-based and culture-independent methods in environmental studies.
Role of novel media	Additional studies to assess the sensitivity and specificity of novel media
Standardized laboratory methods	Greater cooperation and effort to establish a standard framework, such as specific protocols or genetic reference databases
Exposure data	Larger-scale sampling studies including locations both within the built environment and in external environments
Measures of exposure	Validation of a measure of human exposure, for example, identification of NTM from noninvasive oral swabs
High-quality spatiotemporal resolution	Inclusion of highly specific spatial and temporal components in environmental studies (as possible when protecting participant privacy)

Figure 1 legend:

Nontuberculous mycobacteria case reporting in the United States. Fourteen states have some form of NTM reporting (CO, CT, LA, MD, ME, MN, MO, MS, NE, OR, TN, UT, VA, WI), including two where pulmonary and extrapulmonary NTM infection is a reportable condition (MN, CO). In addition, four states have extrapulmonary NTM infection only (MD, NE, OR, TN), and eight have NTM infection type not specified, e.g., specimen type or NTM species may not be reported (CT, LA, ME, MO, MS, UT, VA, WI). In MN and CO, Pulmonary NTM infection is reportable only in some counties.