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Cystic Fibrosis Lung Microbiome: Opportunities to Reconsider Management of Airway Infection

Lindsay J. Caverly, MD, Jiangchao Zhao, PhD, and John J. LiPuma, MD

Summary. The importance of infection in the pathogenesis of cystic fibrosis (CF) lung disease has been long recognized, and the use of antibiotics targeting bacteria identified in cultures of respiratory specimens has played a critical role in improving outcomes for individuals with CF. Over the past ~15 years, the use of culture-independent methods to assess airway microbiology in CF has revealed complex and dynamic CF airway bacterial communities. Recent areas of investigation of the CF lung microbiome have included exploring how bacterial community structures change over time, particularly with respect to disease progression or pulmonary exacerbation, and in response to antibiotic therapies. This review will discuss what has been learned from these studies as well as how these findings offer opportunities to further refine management of CF airway infection. **Pediatr Pulmonol. 2015;50:S31–S38.**

Key words: cystic fibrosis; microbiome; microbiota; airway bacterial community.

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INTRODUCTION

Identification of the important role of infection in the pathogenesis of cystic fibrosis (CF) lung disease dates back to the observation of bronchopulmonary infection with *Staphylococcus aureus* in autopsies in Andersen's initial description of CF in 1938.[1] The negative impact that infection with certain bacterial pathogens has on CF lung function became well recognized thereafter, and obtaining bacterial cultures of CF respiratory samples has been in practice since the 1950s. The results of respiratory cultures have been used to guide many aspects of CF care, particularly the choice of antibiotics to treat pulmonary exacerbations. More recently, methods to detect the presence of bacteria without having to recover viable organisms in culture have been applied to studies of CF microbiology. The use of these "culture-independent" methods has provided greater insight into the composition of CF airway bacterial communities. Improved understanding of these communities, their relationship to clinical course, and response to antibiotic therapy offers a promising path toward continued improvements to our management of CF airway infection.

CULTURE-INDEPENDENT MOLECULAR METHODS IDENTIFY GREATER NUMBERS OF BACTERIAL SPECIES

Initial culture-independent studies of CF airway microbiology employed a variety of molecular genetic methods that proved useful in detecting unusual and otherwise unreported bacteria from CF specimens.[2–6] These studies showed that standard bacterial cultures significantly underrepresent the number of species in CF airway samples. In an early study, Rogers et al.[3] detected an average of 13 bacterial species per sputum sample from adults with CF. Since then, studies employing next generation (or deep) sequencing of the bacterial 16S ribosomal subunit (16S rRNA) gene have provided even greater discrimination of bacterial community structure through detection of species that are difficult to recover in culture and/or present in low abundances. Culture-independent methods also allow for the estimation of bacterial community diversity, defined as a measure of the number of species present (community richness) and their relative abundances within the community (community

Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, Ann Arbor, Michigan.

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*Correspondence to: John J. LiPuma, MD, Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, Ann Arbor, MI 48109. E-mail: jlipuma@umich.edu

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evenness). Analyses of bacterial community diversity have deepened our understanding of CF airway infection and its association with clinical course.

BACTERIAL COMMUNITY DIVERSITY DECREASES WITH AGE AND LUNG DISEASE PROGRESSION

The age specific prevalence of respiratory pathogens based on bacterial culture of CF specimens is well known.[7] Culture-independent analyses have complemented this by providing greater insight into how respiratory infection changes over time in CF. The airway bacterial communities of infants and young children with CF appear to be typically dominated by *Streptococcus* and anaerobic genera such as *Veillonella* and *Prevotella.*[8] Based on a small number of longitudinal studies, airway bacterial diversity appears to increase during the first decade of life.[9] Diversity then decreases throughout adolescence and adulthood in parallel with lung function decline.[9–12] Cross-sectional analyses have similarly shown a correlation between low community diversity and poor lung function.[9,13–15] Deep sequencing of bacteria in lung explants taken at the time of transplant demonstrates the marked constriction of community diversity in end-stage lung disease.[16,17] Conversely, greater community diversity in adulthood is associated with less severe and more slowly progressing lung disease.[10,18,19] While the association between decreased bacterial community diversity and lung disease progression is clear, the causal relationship between these phenomena has yet to be fully elucidated.[20] Additional longitudinal studies, beginning in infancy, of the patterns of bacterial community structure, together with careful analysis of the factors that may influence this structure, will provide opportunities to refine current antibiotic management strategies to prevent or slow lung function decline.

CHANGES IN BACTERIAL COMMUNITY STRUCTURE ASSOCIATED WITH PULMONARY EXACERBATION

Despite the importance of pulmonary exacerbation in CF, many questions remain unanswered regarding the pathophysiology of these events.[21–24] Consequently, understanding the changes in airway microbial community structure and activity that occur around the time of exacerbation onset is an area of great interest. Contrary to conventional wisdom, culture-independent analyses indicate that total airway bacterial density typically does not increase with the onset of pulmonary exacerbation.[10,14,25,26] Further, predictable changes in overall bacterial community composition at the onset of exacerbation have not been found.[10,13,14,27] Nevertheless, changes in community composition with exacerbation onset have been observed in some individuals.[14,26] In one study, communities dominated by *Pseudomonas* experienced a *decrease* in both the relative and absolute abundances of *Pseudomonas* with exacerbation.[14] This intriguing finding suggests a potential role for less abundant species in contributing to pulmonary exacerbations. Whether or not individuals would benefit from antibiotic therapy directed at these species during an exacerbation remains to be determined. Deep sequencing studies also have identified airway bacterial communities that do not include typical CF pathogens. The use of culture-independent methods has the potential to shed light on the pathophysiology of such "culture-negative" exacerbations.[28] These findings suggest the potential for identifying changes in the airway microbiota that herald exacerbation onset. Such biomarkers could, in turn, provide new opportunities for more timely and appropriate treatment of pulmonary exacerbation.

RESILIENT BACTERIAL COMMUNITIES EVENTUALLY BECOME RESISTANT TO CHANGE

Greater understanding of CF lung microbiota has the potential for influencing the choice of antibiotic agent, route, and treatment duration for pulmonary exacerbations. The degree to which airway bacterial communities change with perturbation—such as that caused by acute antibiotic treatment in the setting of exacerbation—is dependent on their level of diversity. Bacterial communities with relatively high diversity (e.g., those found in younger children and in milder lung disease) can exhibit significant change in community structure with antibiotic therapy.[10,14] These changes tend to be temporary, however, and communities quickly return to their baseline structures.[12,13,29–31] In ecological terms, such communities are described as being *resilient*. In contrast, and as might be expected, the structures of communities with relatively low diversity (e.g., dominated by a single species, as is often observed in late stage disease) experience little apparent change with antibiotic treatment.[13,14,32] They lack the dynamic range of more diverse communities and are described in ecological terms as being *resistant* to perturbation.

Although the drivers of decreasing diversity and increasing community resistance that occur over time are not well understood, it appears that repeated antibiotic administration plays an important role. In children, community diversity is inversely correlated with antibiotic exposure.[39] In a longitudinal study of adults with CF, antibiotic use was found to be the primary predictor of decreased diversity,[10] and long-term antibiotic use in adults has been associated with decreased richness of bacterial communities.[33] The route, specific type, and number of antibiotics administered are likely important variables in shaping community structure in the long term.[34] Again, further study is needed to better define the role repeated antibiotic use has in determining the

fate of airway communities in both the short and long terms, and how therapies can be optimized accordingly.

ANAEROBES ARE FREQUENTLY DETECTED IN AIRWAY COMMUNITIES

Although the identification of anaerobic bacteria and their consideration as potential pathogens in CF dates back to the early 1980s,[35,36] the prevalence of these species in CF airways and the role they may have in contributing to lung disease has remained poorly defined. Among the impediments to a better understanding of these questions is that anaerobic bacterial culture requires techniques and conditions that are not routinely included in standard culturing protocols for CF airway samples. Culture-independent investigations of the CF airway microbiota, however, have illuminated the presence and diversity of anaerobic species in the CF airways.[2,3,5,13,19,37–39] Anaerobes are highly prevalent in CF respiratory samples irrespective of patient age and sampling method, including oropharyngeal (OP) swabs, expectorated sputum, bronchoalveolar lavage (BAL), and lower airway plugs obtained from lung explants.[37,40,41] Importantly, anaerobic species are frequently detected in abundances equal to or greater than those observed for typical pathogens such as *P. aeruginosa.*[13,40] Among the most prevalent anaerobic species in CF airways are *Prevotella* and *Veillonella*,[30,37] although a wide range of species has been described.

The detection of obligate and facultative anaerobic species, which are inhabitants of the healthy upper airway, has been attributed to "contamination" of respiratory specimens by oral microbiota. However, a number of studies provide evidence supporting the presence of anaerobes in the lower airways. Harris et al.[5] analyzed BAL samples from 28 children with CF. These samples would be expected to be only minimally contaminated by bacteria in the oropharynx, which is largely bypassed in BAL sampling. Nevertheless, rich bacterial communities were identified, composed of, among other species, obligate and facultative anaerobic species, including high relative abundance of *Streptococcus*, *Prevotella*, and *Fusobacterium*. In a recent study, Brown et al.[42] performed deep sequencing on sections of lung resected from a young child with severe CF lung disease. A diverse microbiota was detected in the lung tissue, including several anaerobic species that are inhabitants of the healthy oral cavity. Because the lung tissue was surgically removed, sectioned and processed under sterile conditions, the anaerobic species detected therein could not have resulted from oropharyngeal contamination during sampling.

Recent metagenomic and metatranscriptomic analyses of CF respiratory samples provide compelling evidence of microbial anaerobic respiration in the CF lung, suggesting that anaerobes not only reside in the airways but play a role in CF lung disease.[43] Although CF patients with or without anaerobes identified by deep sequencing did not differ in overall lung function in cross sectional studies,[37,40] Zemanick et al.[44] found that among children with CF, the relative abundance of *Prevotella* was positively associated with lung function and negatively associated with markers of airway inflammation. This suggests that the presence of certain anaerobes may be a marker of "healthier," more diverse bacterial communities, such as are found in children with mild lung disease that have not yet been treated with intensive antibiotic therapy. In contrast, facultative anaerobic species, including *Gemella*,[14] *Rothia*,[45] and members of the "*Streptococcus milleri*" group[46] have been associated with pulmonary exacerbations in adults with CF, and recently, DNA sequencing based microbial profiling has shown that fermentative anaerobes become more abundant during exacerbation.[47] Observations such as these have prompted renewed interest in reconsidering of the use of antibiotics with greater activity against anaerobic species in CF.

HOW REPRESENTATIVE IS SPUTUM OF THE LOWER AIRWAYS MICROBIOTA?

In order for studies of the CF lung microbiome to continue to advance our knowledge of CF microbiology and lead to further improvements in the management of airway infection, several technical aspects of conducting these studies are worth considering. The first involves the type of sampling method used to represent the airway microbiome. The use of expectorated sputum to assess the airway microbiota in CF (as well as in COPD and asthma) has been criticized as being poorly representative of microbiota present in the lower airways. The detection of bacterial species that are typical inhabitants of the healthy human oropharynx, particularly anaerobes, has exacerbated these concerns, although several studies provide strong evidence that anaerobes do indeed reside in the CF lower airways.[5,42] Further, the categorization of microbiota into oropharyngeal and airway compartments is somewhat artificial in that these compartments are contiguous, with the contents of one being reflected in the composition of the other. Venkataraman et al.[48] employed an ecological "neutral biodiversity model" to conclude that dispersal of microbes from the oral cavity is the primary driver of the composition of the healthy lung microbiome. Bassis et al.[49] used deep sequencing to compare microbiota in sets of mouthwash, BAL fluid, nasal swab, and gastric aspirate samples from 28 healthy subjects. Their analysis similarly showed that microbial migration from the oral cavity was a significant source of the lung microbiota during health. Similarly, Hansen

et al.[50] have presented data showing that the sinuses in people with CF provide bacterial pathogens with a niche for adaptation to the host and a reservoir for seeding of the lower respiratory tract. Finally, Whiteson et al.[51] applied an island biogeography ecological model to conclude that the detection of oral microbes in CF sputum or BAL samples more likely represents immigration from the oropharynx to the airways, rather than mere contamination of respiratory samples.

As expectoration of sputum necessitates its passage through the non-sterile oropharynx, it is expected that oral bacteria will be detected in sputum samples. However, several lines of evidence indicate that oral bacteria have only a marginal impact on measures of airway microbiota. Direct comparisons of oral and lower airways samples have found differences in the microbiota between these sites. Rogers et al.[52] compared bacterial community structures in sets of expectorated sputum and mouthwash samples obtained from 19 adult CF patients. Overall, the great majority of species detected in mouthwash were not detected in any sputum samples. Conversely, nearly one-third of the species detected in the sputum samples were not detected in any of the mouthwash samples. In the majority of patients, species detected in both sputum and mouthwash differed in their relative abundances between the two sample types. Similarly, Zemanick et al.[53] compared induced and expectorated sputum with OP and saliva samples, and found distinct lower and upper airway communities in many individuals. In a study by Filkins et al.,[54] deep sequencing showed significant differences between CF inpatients and outpatients with respect to the abundance of *Streptococcus*, a frequent member of oral bacterial communities. Since both patient groups provided expectorated sputum, potential contamination of samples with oro-pharyngeal microbiota would have affected both groups equally, suggesting that the differences seen reflected true differences in the abundance of *Streptococcus* in the lower airways.

Finally, sputum samples are frequently dominated by species not typically considered major components of "oral flora," arguing against significant oral contamination. In a longitudinal study, Zhao and colleagues[10] observed dramatic changes in bacterial community structure in serial sputum samples obtained from adult CF patients over the course of 8–9 years. Reproducible community profiles, allowing differentiation of individual patients, were found. Communities were most often dominated by species that are known lower airway pathogens in CF, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Achromobacter sp*. In patients with advanced lung disease, communities in sputum were highly constrained, often being heavily dominated by a single non-oral species, as has been observed in CF lung explant specimens.[17]

IS USING OP SWAB SAMPLES IN NON-EXPECTORATING INDIVIDUALS WORTHWHILE?

OP swabs have also been criticized as being poorly representative of microbiota present in the lower airways. In an analysis of the microbiota in resected lung from a child with CF, Brown et al.[42] observed that none of the most abundant species detected by deep sequencing were recovered in routine culture of the child's OP swab sample or by sequence analysis of the swab sample, suggesting that OP swabs may poorly reflect species in diseased lower airways.[42] Goddard et al.[17] similarly identified discordance between the microbiota identified from throat swabs and lung explants. Recently, Zemanick et al.[53] assessed the airway microbiota in concurrently collected saliva, OP swab, induced sputum, and expectorated sputum samples from 16 children with CF. Higher relative abundances of *Pseudomonas* and *Staphylococcus* were detected in induced and expectorated sputum than in the respective OP and saliva samples. Increased divergence between microbiota in induced or expectorated sputum compared to microbiota in saliva or OP swab samples was associated with increased markers of airway inflammation, suggesting that as lung disease (and inflammation) worsens, OP swab samples more poorly reflect lower airway microbiota. Nevertheless, differences in bacterial communities were greater between patients than were differences between sampling methods within individual patients, indicating that in the absence of expectorated sputum, OP swab samples may provide a tractable model by which to assess airway microbial community dynamics within individuals.

CAN LIVE VERSUS DEAD BACTERIA BE DIFFERENTIATED AND DOES IT MATTER?

Another technical consideration in designing and interpreting studies of the CF lung microbiome involves the viability of the bacteria identified. Analyses of CF airway microbiota using deep sequencing most often have not differentiated viable and nonviable bacteria.[5,10,14,27,34,43–47,51,53–61] That is, typically, DNA from both dead and alive organisms is extracted from a respiratory specimen and sequenced to provide a profile of the bacterial community in the airway. This has raised concern that culture-independent approaches do not accurately reflect metabolically active bacterial communities residing in the airways. The use of propidium monoazide (PMA) to block sequencing of DNA that is not associated with living bacterial cells has been proposed.[62–66] PMA is able to penetrate only damaged or dead bacterial cells, where it binds to DNA upon photoactivation, preventing the DNA from being amplified and sequenced. However, the accuracy of PMA in differentiating viable from nonviable bacterial cells is dependent on cell type, and on several experimental

conditions, including PMA concentration, light exposure time, specimen quality, and incubation time and temperature.[67] The use of PMA also tends to overestimate the number of viable bacteria by detecting nonviable but membrane intact cells.[68,69] Of particular note for studies of CF airway microbiota is that freezing and thawing of respiratory specimens is likely to lyse viable bacteria therein.[62] Thus, unless samples are processed immediately, PMA treatment would be expected to underestimate the viable bacteria found in the sample prior to freeze-thaw.

The limitations of PMA (or other methods) in differentiating viable from nonviable bacteria notwithstanding, the degree to which the detection of extracellular bacterial DNA confounds interpretation of analyses of CF airway microbiome is not clear. Airway bacterial communities show marked changes in their structures shortly after the initiation of antibiotic therapy, an observation that would not be expected if DNA from nonviable bacterial cells was present in appreciable quantities.[10,12,30] One may also argue that extracellular DNA, which is not biologically inert and may be important in estimating the "burden" of some species (e.g., extracellular DNA in *P. aeruginosa* biofilm), should not be eliminated from microbiome analyses. Ultimately, the decision to attempt to limit measuring extracellular DNA hinges on the specific question being addressed.

CONCLUSIONS

The use of culture-independent molecular methods has expanded our understanding of CF airway microbiology and has highlighted several areas of opportunity to reconsider management of airway infection in CF (Table 1). Although the bacterial species included in the small suite of opportunists associated with CF lung disease remain the dominant pathogens, we now recognize that the CF airways often harbor complex microbial communities, and that anaerobic species are frequently detected in relatively high abundances in these communities. Change in the structure of these communities correlates with lung disease progression in the long term and, most likely, with clinical status (e.g., exacerbation) in the short term. Ongoing and future research on the CF lung microbiome offers a promising path to translate these findings into improvements in clinical care. Are there treatment approaches that would, for example, better maintain airway microbial communities that seem to be associated with relatively more favorable clinical outcomes? Should consideration be given to the greater use of antimicrobials targeting anaerobic species, particularly in treating pulmonary exacerbations? Will species heretofore not associated with lung disease progression—or combinations of species that correlate with worsening clinical state—be identified?

Further culture-independent study of the CF airway microbiome has considerable potential to address questions such as these. The practical application of this approach in the care of CF patients, however, cannot rely on repeated invasive sampling of the lower airway. The translational utility of microbiome analysis, ultimately, will rely on assessing respiratory specimens obtained non-invasively to identify reliable, reproducible measures that track with clinical features of disease progression and can be used to inform future therapies. Debates over whether or not culture-independent analysis of expectorated sputum or OP swabs provides a *precise* representation of the lower airway community, or whether or not deep sequencing should be performed on DNA from "viable" bacteria only, are less relevant in this context. The knowledge gained regarding the CF lung microbiome over the past ~15 years described here has laid the groundwork for ongoing and future work in this field, with the ultimate goal of continuing to improve outcomes for individuals with CF.

TABLE 1— Future Research Questions

Long-term airway bacterial community dynamics:
What is the causal relationship between decreasing community diversity and advancing age and lung disease?
What are the drivers of the decreasing community diversity associated with advancing age and lung disease?
Would maintaining more diverse communities have a positive impact on lung health? If so, are there treatment options that may enable maintenance of 'healthier' airway communities?
Short-term airway bacterial community dynamics:
Are there reliable changes in community structure and/or activity that are associated with changes in clinical state?
Can biomarkers of impending exacerbation be identified? If so, can these be monitored in real time and exploited to prevent exacerbation?
Are there community changes that predict exacerbation severity and/or recovery from exacerbation? If so, can these be employed to refine therapy of exacerbation?
Role of anaerobes in CF health and disease:
What is the role of obligate and facultative anaerobic species in contributing to lung disease progression?
Would antimicrobial therapy targeting anaerobic species provide better treatment of exacerbation (in the short term) or further decrease community diversity (in the long term)?

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