ABCs of the Lung Microbiome

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Abstract

The lungs of healthy humans have traditionally been considered to be sterile when examined by culture-based techniques. However, molecular identification techniques are now being used to explore the lung microbiome in ways that mirror study of other body sites and organ systems. Familiarity with population definitions and indices of diversity will lead to better understanding of the literature now

coming to publication. Differences in methodology and sampling may contribute significantly to experimental variability, and the field has not coalesced around standard ways to present data or to perform statistical comparisons. This emerging and exciting field of investigation is leading to new ways of thinking about the lung and about lung disease.

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In a recent examination of emerging topics in pulmonary research, Dr. James Kiley of the NHLBI identified study of the lung microbiome as a new field and stated that study of the lung microbiome may lead to important new ways of thinking about lung disease (1). The lungs of healthy humans have traditionally been considered to be sterile when examined by culture-based techniques. However, molecular identification techniques are now being used to explore the lung microbiome in ways that mirror study of other body sites and organ systems (2). Although the National Institute of Health's pioneering Human Microbiome Project did not originally include sampling of the lung, recent work examining healthy and ill humans is now being published. Most recent work has focused on bacterial populations, summarized below, but viral and fungal populations also require further investigation.

In beginning to understand investigations of the lung microbiome,

sharing precise definitions has become increasingly important. A microbe is defined as any microscopic life form. The microbiome is a population of microbes assembled at a particular time and in a particular location. The term microbiota refers to the specific microbes in the population. Finally, microbial communities are populations of microbes that interact functionally and metabolically. To describe and organize these microbial populations, the term organizational taxonomic unit (OTU) is frequently used. OTUs identify DNA sequences, rather than more familiar genera and species, and so this term can lead to some confusion. With more detailed sequencing, OTUs can be resolved into genera and species.

Considering multiple populations of organisms can be complex, particularly when many scientists and clinicians are more familiar with colonization or infection caused by a single type of organism. Comparison of these microbial populations includes evaluation of the following factors: (1) richness, or the number of different

OTUs in the population; (2) evenness, or the relative abundance of different OTUs in the population; and (3) dominance, or the emergence of a single OTU to the exclusion of others (3). Calculated diversity indices express complexity of populations based on their richness and evenness and can be used to perform statistical comparisons among populations.

Most investigations of the lung bacterial microbiome depend on sequencing of the variable regions of the 16S gene encoding bacterial ribosomal RNA (4) (Figure 1). Fortunately, 16S rRNA is not present in mammals, and with proper controls the confounding effects of host DNA can be minimized. The 16S DNA sequences contain nine variable regions, and they can be identified by various techniques, including pyrosequencing, phylogenetic microarrays, terminal restriction length polymorphisms, and others (5, 6). Importantly, the OTUs identified from a single sample may be quite different depending on which region or regions are sequenced. For example, the

Figure 1. Secondary structure model of the 16S rDNA. V1 through V9 indicate major hypervariable regions, which are sequenced to identify organizational taxonomic units; V4 and V6 are unlabeled. Reprinted by permission from Reference 4.

NHLBI's Lung HIV Microbiome Project has demonstrated that OTUs identified by sequencing the V1 through V3 regions are not identical to those identified by sequencing the V3 through V5 regions (7). Additionally, comparison of data from different laboratories may be problematic, until more standardized approaches to data analysis are reached by consensus. Both qualitative and quantitative differences in reported taxa may result.

Focusing on bacterial populations, recent published work demonstrates that diverse microbial communities in the lungs of healthy humans can be detected using molecular techniques. In normal volunteers without intercurrent respiratory diseases, Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria are most commonly identified at the phylum level (7–11). Importantly, there are significant overlaps between these phyla and those identified in the upper airway. At the genus level, the most prevalent organisms identified include *Streptococcus*, *Prevotella*, *Fusobacteria*, and *Veillonella*, with lesser contributions by *Haemophilus* and *Neisseria*. Clinicians and microbiologists

will recognize several of these genera as known pulmonary pathogens, but their presence at very low numbers in asymptomatic hosts does not necessarily have clinical implications. Other authors will discuss disordered microbial communities in disease states, but characterizing and measuring the “normal” lung microbiome seems essential to allow valid comparisons.

Based on quickly expanding literature in this field, we recently discussed controversial issues in the study of the lung microbiome (2). Given the preliminary nature of this field, it is important to realize that significant experimental variability may be introduced at each stage of investigation (4). Methods for DNA isolation, polymerase chain reaction, and sequencing vary widely, and differences in techniques and in manufacturers' kits may produce disparate data.

Samples need to be collected in a rigorously controlled manner to avoid introducing DNA from extraneous microbes. Regardless of the sampling procedure, appropriate negative controls must always be included and reported. For

example, sampling of saline before bronchoscopy, as well as sampling of saline drawn through the suction channel of the bronchoscope before the procedure, are both essential. As in all polymerase chain reaction–based assays, rigorous controls to exclude contamination by laboratory reagents and/or specimen handling must be evaluated. Additionally, handling and preservation methods need to be standardized.

Bronchoalveolar lavage (BAL) has been examined most frequently to describe populations in the lower respiratory tract and has the advantage of sampling relatively large volumes of the lung. It should be noted, however, that communities in the lung are not homogeneously distributed between lobes or even within the individual segments. In an important study that sampled a single patient's lungs immediately after removal before transplant, Erb-Downward and colleagues demonstrated significant differences in the relative abundances of taxa obtained from different sections of the same lobe, a variability that has been termed “microgeography” (8). Whether such spatial diversity exists in the lungs of healthy individuals requires further investigation. Protected specimen brushes might be useful in identifying microgeographic differences, as well as minimizing carryover from the upper airway, but have the disadvantage of sampling very small volumes of lung.

The ability to distinguish upper and lower respiratory samples also needs more attention. Often the lower respiratory tract has been studied in isolation, but we must also consider the influences of the upper gastrointestinal tract and the upper airway on the lower respiratory tract. Microaspiration of gastric contents occurs spontaneously or with the conscious sedation provided to perform bronchoscopy, and so esophageal and gastric contributions need to be considered. The most appropriate methods to evaluate the upper airway's contribution to the lower respiratory tract microbiota remain controversial. Some investigators use a two-bronchoscope method, in which a first bronchoscope is used to sample the upper airway (12). A second bronchoscope is then advanced through the vocal cords into the lower airways

without installation of fluid or suctioning, to minimize contamination by upper airway organisms. Although these investigators concluded that the lower airway microbiota largely reflect those of the upper airway, other groups find divergent results.

For example, investigators in the NHLBI's Lung HIV Microbiome Project compared oral washes to BAL using a single bronchoscope protocol. We used the neutral model to test hypotheses regarding statistical differences between the mouth and the lung (Figure 2). This model assumes that microorganisms are equally likely to be present in all sites sampled (13) and in this case compares the relative abundances of OTUs in oral washes to detection frequency of OTUs in BAL. In recent publications, we identified taxa enriched in the lung compared with the mouth in smokers and nonsmokers (7). Additionally, we identified a unique organism, *Tropheryma whippelii*, that was present in the BAL but was virtually never present in the oral washes (14).

An additional approach is use of surgical specimens obtained under aseptic conditions. Size and colleagues demonstrated differences among microbiota in normal volunteers, patients with chronic obstructive pulmonary disease, and patients

with cystic fibrosis (15). Importantly, separation of populations from these groups differed somewhat depending on the method of analysis (terminal restriction fragment length polymorphism vs. pyrosequencing).

In addition to microgeography within the lung, there is a need to evaluate geographic diversity present in the external environment. Most studies originate from a single center, and the microbial ecology of the environment is often not characterized. Patients with cystic fibrosis who live in different locations may have vastly different populations of organisms in sputum (16), underscoring the need to study patients from different locations and centers. In considering the environment, the microbial populations contained in dust from homes with pets differs from dust obtained from homes without pets (17). This important observation emphasizes the necessity to consider the environmental microbiome as well as that of the host.

Several other issues in study design, sampling, and data analysis are noteworthy. Many studies published to date present sampling at a single time point, but there is a need for longitudinal studies that include repetitive sampling of the same individuals. This approach can be more readily achieved when the respiratory

samples consist of nasal swabs, oral washes, and expectorated or induced sputa (18). The logistics and ethics of performing repetitive bronchoscopies are more problematic, and data from sputa may not adequately reflect simultaneously obtained bronchoscopic data (19). Reproducibility in healthy individuals, as well as in patients, needs to be addressed vigorously. Finally, there is a need to study large and well-characterized groups of normal volunteers and patients, rather than relying on small studies. Only in this way will adequate statistical power be achieved to test hypotheses. This nascent field has not coalesced around accepted ways to present data or to perform statistical comparisons.

An NHLBI Workshop focused on the lung microbiome was conducted in December 2011 and emphasized these issues as targets for future investigation. The consensus recommendations for future research included: (1) procedural details need to be agreed on, including obtaining samples and processing; (2) the gut–lung axis and its role in respiratory disease needs to be considered; (3) the field needs to move beyond describing the microbiome to studies of functional and metabolic interactions among microbes and with the host; (4) noninvasive biomarkers and imaging must be developed because of inherent difficulties with repeated bronchoscopies; (5) the fungal and viral microbiomes must be characterized and related to the bacterial microbiome; and (6) relevant animal and *in vitro* models should be developed to confirm human data.

In conclusion, familiarity with population definitions and indices of diversity will lead to better understanding of the literature now coming to publication. Microbiome data obtained from patients with diverse disease states will be presented in subsequent conference summary articles. Differences in methodology and sampling may contribute significantly to experimental variability, and this new field has not coalesced around standard ways to present data or to perform statistical comparisons. However, this emerging and exciting field of investigation is indeed leading to new ways of thinking about the lung and about lung disease. ■

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Figure 2. Neutral model analysis. In this example, the relative abundance of organizational taxonomic units (OTUs) in oral washes is plotted against the detection frequency of OTUs in the lung. A line of identity can be constructed, with confidence intervals indicated by the shaded areas. OTUs falling outside the confidence intervals are deemed significantly enriched in lung or mouth. Adapted by permission from Reference 7.

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