Lung Microbiota: Genuine or Artifact?

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KEY WORDS: lung, microbiota, diseases, next-generation sequencing, biomass

IMAJ 2013; 15: 731–733

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Analysis of the human microbiota (the collection of microorganisms that are present in a community from a defined body habitat, such as the respiratory tract) implicates global alteration of microbial communities in a wide spectrum of human diseases such as asthma [1], obesity [2,3] and Crohn's disease [4,5]. The ability to accurately characterize the complex structure and rich composition of these microbial communities became possible due to advances in deep sequencing and bioinformatics analyses. Unlike conventional methods that can detect only a single microbe in a sample, the high-throughput, next-generation sequencing allows identification of almost the entire microbiota present in a sample. With 16S ribosomal RNA (16S rRNA) sequencing, the final data set consists of thousands to millions of sequences from a segment of the *16S rRNA* gene. Each sequence is taken to represent an individual microorganism, and the collection of sequences is taken to be representative of the community as a whole in terms of both types of organisms present and their relative abundance to one another.

In 2007 the National Institutes of Health launched the Human Microbiome Project. The article describing the findings of this sentinel study was published only recently [6]. A total of 4788 specimens from 18 female body habitats and 15 male body habitats representing five major body areas (oral cavity and oropharynx, skin, nostrils, gastrointestinal tract, vagina) were collected from 242 healthy adults. These samples were subjected to *16S rRNA* gene pyrosequencing and a subset were shot-gun sequenced for metagenomics. Rich communities in each of the body's habitats were found with strong niche specialization both within and among individuals. Interestingly, oral (considered part of the upper respiratory tract) and stool communities were especially diverse in terms of community membership [6]. The lung can be reached only with invasive means and was traditionally thought to be a sterile site and therefore was not part of the Human Microbiome Project consortium sampling. However, multiple lines of theoretical arguments and investigative evidence call into question the notion that the lower respiratory tract and lungs are truly sterile.

This article presents evidence supporting the concept of a true resident lung microbiota and further discusses the controversial question of whether the lung microbiotia represent a permanent and growing population of the lower respiratory tract/lung with a unique structure and composition or merely constitutes a transient and diluted reflection of aspirated upper respiratory tract communities.

Because open lung biopsy in healthy subjects is not ethically defensible and not indicated in most lung diseases, the search for lung bacterial communities and their characterization is mostly based on sputum and bronchoalveolar lung fluid collected by bronchoscopy. However, reliance on sputum and BALF to determine that unique LRT/lung microbome exists is problematic. The former is obviously an admixture of lower and upper respiratory tract organisms and BALF is contaminated by URT organisms as a result of 'carry over' on the bronchoscope. Several studies, however, have attempted to overcome this obstacle for BALF.

The bronchoscope must pass through the naso- or oropharynx, demonstrated by the Human Microbiome Project to be very rich in microbes, which obviously allows for contamination of the bronchoscope and sampling channel by organisms from the URT. In order to get the cleanest sample possible with this technique, Charlson et al. [7] used two bronchoscope procedures to sample the LRT. The first scope was passed to the level of the vocal cords, and samples from this scope were used to define the overall contamination of the scope passing through the oropharynx. Sequential bronchoalveolar lavage with the second scope was done in two immediately adjacent regions of the right middle lobe, keeping the first aliquot of the initial BAL separate. The first aliquot was considered to be the bronchial aliquot and likely to be more contaminated by aspirated oropharyngeal secretions during passage of the bronchoscope. The lower left lobe bronchial mucosa was then sampled by protected specimen brush. Their analysis focused on comparing the composition and relative abundance of bacterial species present in the second and third BALF aliquot and the protected specimen brush, taken to be representative of the

BALF = bronchoalveolar lavage fluid
LRT = lower respiratory tract
URT = upper respiratory tract
BAL = bronchoalveolar lavage

LRT community, with the URT samples. They found that the LRT does harbor a bacterial community, similar to but 2–4 logs lower in biomass than the URT community [7].

The second piece of evidence for the existence of a bacterial community in the lung comes from identification of sequences in whole-lung samples. Two recently published works bypassed the need for bronchoscopy and obtained whole-lung tissue under sterile conditions. Sze and co-authors [8] concluded that there is a detectable bacterial community within human lung tissue and, consistent with Charlson's findings [7], the total bacterial populations within lung tissue were small. Similarly, Erb-Downward et al. [9] found that all tissues sampled from eight chronic obstructive pulmonary disease lung explants contained readily identifiable bacterial communities.

The final line of investigative evidence supporting a true lung microbiota comes from recent murine studies. Identification of a bacterial population in BALF from mice is not suspected to be contaminated by oral organisms due to the fact that BAL in mice is obtained by sterile surgical tracheal exposure and insertion of an angiocatheter [10]. Shen and colleagues [11] used whole-lung homogenates from the left and right lungs of mice and demonstrated significant bacterial communities in all lung samples by culture-independent molecular analyses.

Assuming this evidence for the existence of genuine LRT/lung microbiota is solid, the next step is to provide answers to two cardinal questions: is it a viable and prosperous community that resides *permanently* in this habitat, and does this bacterial population have a *distinct* structure different from that of the URT community?

Given the anatomical structure of the respiratory tract, together with the well-known fact that many healthy individuals frequently aspirate while sleeping, a plausible hypothesis is that the documented lung microbiota are derived *transiently* from oropharyngeal sources through microaspiration. However, persuasive arguments based on experimental data dispute this theoretical concept.

Sloan et al. [12] showed that for a wide range of prokaryotic communities, the structure of the community in samples can be explained by a neutral community model, a stochastic, birth-death-immigration process. While not a complete or literal description of microbiota community assembly, the neutral community model has the ability to contribute information relevant to the questions raised above. The community structure of bacteria from the lower respiratory tract of 24 patients with and without asthma follows the predictions of a neutral model. This implies that the community comprises a high proportion of viable organisms rather than transients, because at least 80% of the deaths are replaced from within the lung community [12].

To answer the challenging question of whether the structure of URT and LRT communities differs, only studies that sampled the URT and LRT simultaneously from the same person and incorporated strict environmental controls are appropriate.

Charlson et al. [7] sampled respiratory flora intensively at multiple sites in six healthy individuals. Only one organism (*Tropheryma whipplei*) was found in all three BAL and protected specimen brush samples from one individual but not in URT or environmental controls. This isolate, therefore, may represent genuine detection of a bacteria resident only in the LRT of this subject. The authors conclude that bacterial populations in the healthy LRT largely reflect URT organisms, likely resulting from transient entry rather than independent communities with a distinguishable structure [7].

The structure of the bacterial community in the respiratory tract is altered in smokers [13] and in many lung diseases, including asthma [1,14], chronic obstructive lung disease [9], cystic fibrosis [15] and acute lung infection [16]. Nevertheless, the question of whether the profile of URT and LRT bacterial populations significantly differs in these populations remains unresolved.

Charlson et al. [17] sampled the oral and lung microbiota from six individuals evaluated bronchoscopically for lung disease. A single sample of BAL fluid was obtained from each subject along with contemporaneous oral wash to sample the oropharynx. DNA was extracted from three separate aliquots of each. This method of comparing replicates provides high reliability for the identification of authentic lung microbiota. When taking advantage of the replication, they found that the number of significantly lung-enriched lineages varied from 0 for one subject to 6 for a subject with sarcoidosis [17].

The same group recently described the microbial populations in the respiratory tract of transplant patients [18]. By using UniFrac, a method that measures the similarity of bacterial communities based on the extent to which members of the communities are phylogenetically related, they demonstrated that healthy subjects show close similarity between their BAL and oral wash microbiota, as shown in their previous work [7]. Phylogenetic distances were greater for lung transplant subjects, indicating more distinct populations. They concluded that, while transplant subjects' BAL largely shared bacterial profiles with oral wash samples, a subset was clearly dominated by specific lineages absent in oral wash or detected at much lower abundance [18].

Interestingly, the only organism uniquely found in the lung in the previous Charlson paper [7], *T. whipplei* was discovered in a high incidence in BAL in asymptomatic HIV-infected individuals compared to uninfected subjects. Importantly, the organism was never detected in paired oral wash in those subjects in whom both sites were sampled [Submitted for publication].

The study of Iwai and collaborators [19] compared paired oral and airway microbiota present in 15 hospitalized HIV-

HIV = human immunodeficiency virus

infected patients receiving antimicrobial treatment for acute pneumonia. Consistent with other studies, the airway bacterial burden was significantly lower than that of the oral cavity. However, in contrast to the findings of other investigators, oral and airway microbiota exhibited niche specificity. Oral microbiota was characterized by significantly increased relative abundance of multiple species associated with the mouth, including members of the Bacteroides and Firmicutes, while airway microbiota was primarily characterized by a relative expansion of the Proteobacteria. Twenty-two taxa were detected in both niches [19].

Finally, Flanagan and team [20] looked at the bacterial diversity during antibiotic treatment in endotracheally intubated patients. They began their study by comparing endotracheal aspirate to BAL in two patients. While there were distinct differences in community compositions between these two patients, the community compositions of the endotracheal aspirate and BAL samples from each individual were highly similar. In seven patients colonized with Pseudomonas and serially sampled from 4 to 102 days after initial enrolment, the microbiota demonstrated significant loss of bacterial diversity in association with systemic antibiotic treatment. In many cases, a pathogenic species, usually *P. aeruginosa*, became the single dominant species despite antibiotic treatment appropriate for Pseudomonas.

The possibility of an independent, viable and replicating bacterial population residing regularly in the lung is realistic. Additionally, one cannot dispute the fact that in some cases, by definition, the composition (the microbial types present in a community) and/or structure (the combination of the composition and the relative abundance of community members) of the LRT/lung microbiota is distinguishable from the URT microbiota. In order to put things into the correct perspective, it is important to point out that respiratory tract bacterial communities contain hundreds of different bacterial genera and thousands of different bacterial species. Therefore, the presence of one or several organisms found uniquely in BAL and not in oral wash, or found in both but at higher relative abundance in the lung does not conclusively contradict the argument that the LRT bacterial community is basically a diluted URT bacterial community.

In summary, we believe that viable lung microbiota, possibly derived by aspiration from the oropharynx, does exist. It has much lower biomass and weak niche specialization within the respiratory tract, in health as well as in other situations. Needless to say, the lung community structure and composition are subject to change as the lung might be colonized and dominated by bacteria derived from blood during bacteremia or, more commonly, other pathogens derived from the oropharynx. In light of the possible involvement of this bacterial population in lung diseases, designated studies in which the lung microbiota is the focus are called for.

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"A library is thought in cold storage"

Herbert Samuel (1870-1963), British politician and diplomat