Multicenter Comparison of Lung and Oral Microbiomes of HIV-infected and HIV-uninfected Individuals

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Abstract

Rationale: Improved understanding of the lung microbiome in HIV-infected individuals could lead to better strategies for diagnosis, therapy, and prophylaxis of HIV-associated pneumonias. Differences in the oral and lung microbiomes in HIV-infected and HIV-uninfected individuals are not well defined. Whether highly active antiretroviral therapy influences these microbiomes is unclear.

Objectives: We determined whether oral and lung microbiomes differed in clinically healthy groups of HIV-infected and HIV-uninfected subjects.

Methods: Participating sites in the Lung HIV Microbiome Project contributed bacterial 16S rRNA sequencing data from oral washes and bronchoalveolar lavages (BALs) obtained from HIV-uninfected individuals (n = 86), HIV-infected individuals who were treatment naive (n = 18), and HIV-infected individuals receiving antiretroviral therapy (n = 38).

Measurements and Main Results: Microbial populations differed in the oral washes among the subject groups (Streptococcus, Actinomyces, Rothia, and Atopobium), but there were no individual taxa that differed among the BALs. Comparison of oral washes and BALs demonstrated similar patterns from HIV-uninfected individuals and HIV-infected individuals receiving antiretroviral therapy, with multiple taxa differing in abundance. The pattern observed from HIV-infected individuals who were treatment naive differed from the other two groups, with differences limited to Veillonella, Rothia, and Granulicatella. CD4 cell counts did not influence the oral or BAL microbiome in these relatively healthy, HIV-infected subjects.

Conclusions: The overall similarity of the microbiomes in participants with and without HIV infection was unexpected, because HIV-infected individuals with relatively preserved CD4 cell counts are at higher risk for lower respiratory tract infections, indicating impaired local immune function.

Keywords: lung; microbiome; HIV infection; bronchoscopy; bronchoalveolar lavage

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The lung was long considered to be a sterile compartment, but recent molecular investigations from multiple groups demonstrate that the lung contains bacterial DNA sequences, although at low levels relative to the upper respiratory tract (1). However, clarifying the relationship between the microbiome of the lung and the microbiome of other compartments, including the oropharynx, presents a challenge for lung microbiome analysis (2, 3). Because sampling of the lower airways by bronchoscopy depends on insertion of a bronchoscope through the nose or mouth, careful methodologic attention to possible contamination from the upper airway is required (4). Environmental sources that may confound the lung data must be evaluated, particularly in low bioburden samples. Although bacterial taxa in the lung closely resemble those of the upper airway from the oropharynx (5), there seem to be differences between the mouth and the lungs in both health and disease (6, 7). A growing body of literature documents changes in the microbiome that occur during pulmonary disease processes, including chronic obstructive pulmonary disease (8–11), bronchiectasis (12), asthma (13–15), lung transplantation (16–19), bronchopulmonary dysplasia (20, 21), and cystic fibrosis (22, 23).

The Lung HIV Microbiome Project (LHMP) is a multicenter consortium established by the NHLBI to understand the respiratory tract microbiome during HIV infection. Previous work from the LHMP compared smokers and nonsmokers without HIV infection and demonstrated that the oropharyngeal microbiome in smokers differs from that in nonsmokers (6). However, significant differences were not detected in the lung microbiome between smokers and nonsmokers. The LHMP then demonstrated that in HIV-infected individuals there is widespread colonization of the lung by the bacterium Tropheryma whipplei, and the institution of antiretroviral therapy decreases the relative abundance of this organism (7). However, the broader bacterial community characteristics of the upper and lower respiratory tract were not addressed in HIV-infected individuals in this study.

In the current study, we performed a comprehensive analysis of upper and lower respiratory tract bacterial microbiomes from individuals with and without HIV infection. We examined volunteers who were HIV-uninfected, who were HIV-infected but untreated with antiretroviral therapy, and who were HIV-infected and effectively treated with antiretroviral therapy. We chose to study clinically healthy individuals, rather than those with active pulmonary infections or other disease processes, to understand baseline changes in microbial populations that occur during HIV infection and treatment. These studies will help to inform further investigations aimed at understanding HIV-associated lung diseases.

### Methods

#### Study Design

The LHMP performed a prospective, multisite cohort study that was approved by the institutional review boards at each participating site. A second level of institutional review was provided by the Data Coordinating Center and the NHLBI, including an Observational Safety Monitoring Board. All subjects provided written informed consent as approved by the review board at each site.

#### Participants

Participants were healthy individuals with and without HIV infection, recruited from eight cities participating in the LHMP. Men and women aged 18 to 80 years were eligible. We enrolled HIV-negative subjects (referred to as “Negative”), HIV-positive subjects who had not been treated with any antiretroviral medications (referred to as “Naive”), and HIV-positive subjects currently receiving highly active antiretroviral medications (referred to as “HAART”).

Subjects completed questionnaires including medical history and use of medications including nonprescription drugs, illicit drugs, tobacco, and/or marijuana. At the time of microbiome sampling, individuals were asymptomatic for upper respiratory or lung infections and had no reported fever, cough, or other acute respiratory symptoms during the previous 4 weeks. Subjects had not used any antibacterial medications in the previous 3 months and had not used corticosteroids (including inhaled medications) in the previous 6 months. Individuals with known respiratory diagnoses, including chronic obstructive pulmonary disease and asthma, were ineligible for enrollment.

#### Sample Collection

Oropharyngeal and lung samples were collected as previously described (6). Smokers were asked to stop smoking at least 12 hours before collection, and all subjects were instructed to avoid food and water from midnight before the procedure. Subjects gargled with 10 ml sterile 0.9% saline at the start of the procedure, and this oral wash sample was collected and iced immediately. To potentially reduce the presence of bacteria in the upper airway, all subjects then gargled with antiseptic...
mouthwash immediately before bronchoscopy. Bronchoscopy was performed as previously described (6). Using minimal sedation, the bronchoscope was inserted through the mouth and was advanced quickly to the wedge position in the right middle lobe or lingula. Bronchoalveolar lavage (BAL) was collected by instillation and aspiration of 0.9% sterile saline to a maximum instillation volume of 300 ml. Subjects from the University of Pennsylvania cohort underwent bronchoscopy with a two-scope method as previously described (5). Samples obtained by this method did not differ systematically from those obtained at the other sites (data not shown); thus, data were combined for the current analysis.

Controls obtained at the time of sampling included a neat sample of the sterile saline immediately after the container was opened and 10 to 50 ml of the same saline aspirated through the suction channel of the designated bronchoscope before the procedure. Control samples were processed and sequenced in parallel with the subjects’ samples.

Sample Processing, Sequencing, and Curation
DNA was extracted at each site, using consensus protocols. To assure consistency in sequencing, all samples were sequenced at Washington University. Sequencing was performed on a Roche 454 FLX Titanium platform using primers for variable regions 1 through 3 (V1–3) and regions 3 through 5 (V3–5). Successful sequencing was substantially more robust for V1–3 than V3–5. Additionally, V3–5 primers resulted in fewer specific amplifications that included more host sequences (data not shown). Therefore, we focused on the V1–3 sequences for this study. The 16S rRNA sequences were assigned to organizational taxonomic units (OTUs) using the mothur software package according to previously described methods (6, 24, 25).

Neutral Model Analysis for Potential Contaminant Sequences
We previously used the neutral model to examine enrichment of oral versus lung samples (6, 26). In this analysis, frequency describes the percentage of samples in which an OTU was detected, and abundance describes the percentage of sequences assigned to an individual OTU. For the present study, we used the neutral model to identify sequences in BAL samples that may have arisen from contamination introduced by the bronchoscope or from reagents used in sample collection or processing. In the implementation of the model, the control samples (bronchoscope and reagents) were considered as sources contributing DNA sequences to the BAL samples (Figure 1).

Statistics
Demographic characteristics were compared using Chi-square tests or Fisher exact test for categorical variables, analyses of variance for normally distributed continuous variables, and the Wilcoxon rank test for skewed data (for example, in comparing CD4 counts between groups). Alpha diversity was evaluated by comparing the number of observed OTUs and the Shannon Diversity Index by rarefying each sample to 500 sequences with 1,000 randomizations. The Shannon Diversity Index combines measurements of evenness (distribution of OTUs across a population)
and richness (number of different OTUs in a population) to express diversity numerically. After randomly selecting 500 sequences per sample with 1,000 randomizations, the distances between populations were measured using $\Theta_{VC}$ (ThetaYC) as previously described (27). $\Theta_{VC}$ measures dissimilarity between the structures of communities. We tested for differences in the average distance between each group of samples by analysis of molecular variance, a statistical tool analogous to analysis of variance (28). OTUs were compared by the Wilcoxon signed rank test for all OTUs with average relative abundances of greater than 1% across all samples. These tools produce similar results to the weighted and unweighted UniFrac distances (data not shown); we chose the $\Theta_{VC}$ distance metric over the UniFrac metrics because the former is based on the relative abundance of individual OTUs, whereas the latter is based on a phylogenetic tree without the use of OTU designations.

**Results**

**Demographics**

We enrolled 142 subjects from the clinical LHMP sites (Table 1). Eighty-six subjects were HIV negative ("Negative"), 18 subjects were HIV positive but had not received antiretroviral medications ("Naive"), and 38 subjects were HIV positive and treated with highly active antiretroviral medications ("HAART"). The groups were not matched for demographic characteristics. The Negative group contained a higher proportion of women than both HIV-positive groups, and the HAART group was older than the other groups. The HIV-positive groups contained larger proportions of African Americans, and the Naive group contained more Hispanics.

As expected, plasma HIV RNA was significantly lower in the HAART group than in the Naive group. However, the CD4 counts in the Naive and HAART groups did not differ significantly and reflected relatively preserved immune function in this clinically healthy cohort. There were no significant differences in smoking status among the groups, with each cohort containing nonsmokers, current smokers, and former smokers. Analysis of molecular variance revealed no differences in microbiome data by smoking status when the data were corrected for multiple comparisons. Therefore, data were combined for analysis. Although we excluded subjects who reported antibacterial use within 3 months of sampling, we also examined more distant antibacterial use. The Naive group contained a higher proportion of subjects who had used antibacterial medications in the 3 to 6 months before enrollment, but the difference was not statistically significant.

**Neutral Model Application, Sequencing Yield, Observed OTUs, and Diversity Indices**

We applied the neutral model to the BAL samples to identify and to remove sequences that might have resulted from contamination sources (Figure 1). After curation and removal of sequences potentially derived from control sources, samples containing greater than or equal to 500 sequences were subject to analysis. The increased microbial biomass present in oral wash samples, compared with BAL samples, resulted in improved 16S rRNA sequence amplification and successful sequencing of 136 oral wash samples (Table 2). Despite the relatively low biomass present in BAL, DNA recovery was adequate and sequencing was successful for 111 BAL samples. Sequences were binned at the 97% identity levels and were assigned OTUs as described in the METHODS.

<table>
<thead>
<tr>
<th>Table 1. Characteristics of Participants</th>
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<tr>
<td><strong>HIV Negative (n = 86)</strong></td>
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<tr>
<td><strong>Sex, n (%)</strong></td>
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<tr>
<td>Male</td>
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<td>Female</td>
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<td><strong>Age, mean ± SD, yr</strong></td>
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<td><strong>Race, n (%)</strong></td>
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<td>White</td>
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<td>African American</td>
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<td>Other</td>
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<td><strong>Ethnicity, n (%)</strong></td>
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<td>Hispanic</td>
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<tr>
<td>Not Hispanic</td>
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<td>*Decline to state</td>
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<tr>
<td><strong>CD4 count/µl, median (min–max)</strong></td>
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<tr>
<td><strong>Plasma HIV RNA copies/ml, median (min–max)</strong></td>
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<td><strong>Smoking status, n (%)</strong></td>
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<td>Nonsmoker</td>
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<td>Current</td>
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<tr>
<td>Former</td>
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<td>Antibiotics &lt; 6 mo, n (%)</td>
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*Definition of abbreviations: HIV/HAART = HIV-positive subjects treated with highly active antiretroviral therapy; HIV/Naive = HIV-positive but treatment-naive subjects; HIV Negative = HIV-negative subjects; ND = not done.

*Chi-square test.
†Analysis of variance.
‡Fisher exact test.
§Wilcoxon rank test.
Numbers of observed OTUs in the oral washes and BALs did not differ among groups (see online supplement). Similarly, the Shannon Diversity Index did not differ among groups (see online supplement).

Community Structure Comparison by Subject Group
We compared nonmetric multidimensional scaling plots using all V1–3 sequences from oral washes obtained from subjects in the three groups (Figure 2A). Nonmetric multidimensional scaling plots provide two-dimensional visualization of differences between members of a population, with similar members grouping together, and this analysis allows measurement of overall differences in microbial populations among the subject groups. Oral washes from the Negative subjects differed significantly from those obtained from Naive subjects ($P < 0.01$) and also differed significantly from those obtained from HAART subjects ($P = 0.01$). In comparison of the two HIV-infected groups, there were also significant differences between the oral washes obtained from Naive and HAART subjects ($P = 0.01$). Thus, oral microbial populations differ significantly between HIV-uninfected and HIV-infected subjects and also differ significantly according to HIV treatment status.

We then compared all sequences from BALs from the subjects in the three groups (Figure 2B). Unlike the oral washes, we did not detect statistically significant differences among the groups. Therefore, our findings do not support differences in the microbial populations sampled in BAL according to HIV infection status or by HIV treatment status.

**Table 2. **Samples Sequenced Successfully

<table>
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<tr>
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<td>38</td>
</tr>
<tr>
<td>BAL</td>
<td>67</td>
<td>14</td>
<td>30</td>
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**Definition of abbreviations:** BAL = bronchoalveolar lavage; HIV/HAART = HIV-positive subjects treated with highly active antiretroviral therapy; HIV/Naive = HIV-positive but treatment-naive subjects; HIV Negative = HIV-negative subjects. Samples considered successfully sequenced contained at least 500 sequences after curation and contaminant removal.

Differences in Taxa by Subject Group
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**Figure 2.** Nonmetric multidimensional scaling (NMDS) plots for all subjects showing oral wash (A) and bronchoalveolar lavage (BAL) (B). Samples were obtained as detailed from HIV-negative subjects (‘Negative,’ red circles), HIV-positive but treatment-naive subjects (‘Naive,’ blue squares), and HIV-positive subjects treated with highly active antiretroviral therapy (‘HAART,’ green triangles). Analysis is based on organizational taxonomic units (OTUs) from sequences of the V1–3 regions of the 16S rRNA. Centroids are indicated by crosses. For oral washes, NMDS analysis demonstrated significant differences in OTUs between Negative and Naive subjects ($P < 0.01$), and between Negative and HAART subjects ($P = 0.01$), and between Naive and HAART subjects ($P = 0.01$). There were no significant differences in the BALs.

Community Structure Comparison by Body Site
We next examined the relationships in microbial communities between oral washes and BALs in each subject group. There were significant differences in communities between oral wash and BAL in the Negative group (Figure 3A; $P < 0.01$), in the Naive group (Figure 3B; $P = 0.03$), and in the HAART group (Figure 3C; $P < 0.01$). These results support our previous investigations (6) and provide further evidence that the lung compartment, as sampled by BAL, does not completely mirror the oropharyngeal compartment.

Differences in Taxa by Body Site
Comparison of oral washes and BALs from the Negative subjects demonstrated significant differences in abundances of 11 OTUs, including increased abundances of *Streptococcus, Veillonella, Prevotella, Fusobacterium, Rothia, Porphyromonas, Granulicatella,* and *Gemella* OTUs in oral washes than in BAL (Figure 6A). In contrast, a *Rothia* OTU was significantly more abundant in oral washes from the HAART group than in the other groups. In contrast to the oral washes, there were no significant differences in the most common taxa identified in BALs from the three subject groups (Figure 5).

**Figure 2.** Nonmetric multidimensional scaling (NMDS) plots for all subjects showing oral wash (A) and bronchoalveolar lavage (BAL) (B). Samples were obtained as detailed from HIV-negative subjects (‘Negative,’ red circles), HIV-positive but treatment-naive subjects (‘Naive,’ blue squares), and HIV-positive subjects treated with highly active antiretroviral therapy (‘HAART,’ green triangles). Analysis is based on organizational taxonomic units (OTUs) from sequences of the V1–3 regions of the 16S rRNA. Centroids are indicated by crosses. For oral washes, NMDS analysis demonstrated significant differences in OTUs between Negative and Naive subjects ($P < 0.01$), and between Negative and HAART subjects ($P = 0.01$), and between Naive and HAART subjects ($P = 0.01$). There were no significant differences in the BALs.

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a unique pattern in the taxa differing in oral washes and BALs.

**Effect of CD4 Count on Microbiome**
Finally, we examined whether CD4 count influenced oral wash or BAL microbiome composition in the two groups of HIV-positive subjects. When CD4 count was evaluated as a dichotomous value, there were no significant differences regardless of the nominal value of CD4 used as a cutoff (data not shown). We also determined whether CD4 count, evaluated as a continuous variable, might modulate the microbiome, and again determined that there were no overall differences, including examination of specific OTUs (see Figures E1 and E2 in the online supplement).

**Discussion**
This large, multicenter study conducted at sites across the United States contributes unique information about the oral and lung microbiomes in healthy HIV-uninfected and HIV-infected individuals and represents the first data directly comparing these subject groups. We did not detect overall differences in numbers of OTUs or in the Shannon diversity index. Microbial populations in oral washes differed between HIV-uninfected and HIV-infected subjects, but BAL populations did not differ significantly. The most surprising finding in our study was the overall similarity of the microbiota in subjects with and without HIV infection. This result was unexpected, since HIV-infected individuals (even with relatively preserved CD4 counts) are at higher risk for lower respiratory tract bacterial infections, including bacterial pneumonia and tuberculosis, indicating significantly impaired local immune function. We did identify greater differences in oral washes than in BALs. It is possible that these differences reflect the greater complexity of the oropharyngeal compartment and/or increased exposure to the environment, but we did observe significant differences in our subject groups related to HIV infection and treatment status. Alterations in the oral microbiome have important implications for disease processes (29), such as periodontal diseases (30), but may precede changes in the lung. Previous work by one of the LHMP collaborators identified increased abundances for a large number of taxa in oral samples from HIV-infected patients with pneumonia, in comparison to HIV-uninfected control subjects (31). A culture-based study identified abundant *Streptococcus* spp. and *Staphylococcus* spp. in oral samples from HIV-infected subjects, but uninfected subjects were not examined.
in this study (32). A study of the lingual microbiome associated increased proportions of *Veillonella*, *Prevotella*, *Megasphaera*, and *Campylobacter* with HIV viremia (33).

The current study extends earlier observations from the LHMP cohorts. We previously compared HIV-uninfected smokers and nonsmokers and found that the lung microbiome in HIV-uninfected individuals was largely derived from the oral microbiome, except for some unique lung species such as *Tropheryma whipplei* (6). The mouth microbiome differed in smokers and nonsmokers, whereas smoking did not alter the lung microbiome significantly. The LHMP then conducted a targeted analysis of colonization of the lung by *T. whipplei* in the lungs of HIV-infected individuals and also demonstrated that the use of antiretroviral therapy decreased the relative abundance of this organism (7). In the current, larger cohort, we adopted a different approach by combining smokers and nonsmokers for analysis (as there were no significant differences in smoking status between subject groups), by analyzing all OTUs, and by approaching potential environmental controls conservatively using the neutral model. In comparing relative abundances of OTUs in oral washes and BALs, we found more OTUs that differed in the Negative and HAART subjects than in the Naive subjects. Interestingly, we observed differences in *T. whipplei* in Negative and HAART subjects but not in Naive subjects.

Our BAL results agree closely with published data obtained from subjects without respiratory disease and further support the current consensus regarding communities of bacteria identified in the lungs (6, 10, 13, 15, 34). The possible pathogenic, or protective, effects of these taxa require further investigation. However, it is likely that groups of microbes establish lung environments that may encourage or inhibit growth of pathogens or inflammatory reactions. It is important to note that all subject groups in this study were clinically well at the time of enrollment and sampling. Very different results are observed during episodes of clinical pneumonia and with decreased CD4 counts (35).

There are several unique aspects of the current study that advance the literature in this field. First, we collected samples from individuals at eight different clinical sites, whereas most of the existing literature examines subjects enrolled from a single center. Previous, small studies have suggested geographic variation, for example in sputa obtained from patients with cystic fibrosis in the United States and the United Kingdom (36). We did not detect systematic variation among our sites. The current results support our previous multicenter investigation in which patterns of diversity were not driven by cohort location (7). Next, none of the patients received oral or inhaled corticosteroids. The role of inhaled corticosteroids in modulation of the upper and/or lower airway microbiota remains unclear (14). Several limitations of the present study require comment. Our groups were not matched demographically, and it is possible that differences influenced the results. However, the statistical power to analyze these factors post hoc is problematic given the small numbers in the subgroups. We focused on bacteria and did not analyze viruses or fungi. We also did not analyze gut microbiota or the subjects’ diets, but it is likely that the gut and diet have significant influence on the upper and lower airway microbiota (11). In mice, the gut microbiota is intimately associated with pulmonary phenotypes, including allergic and asthma-like conditions (37, 38). Next, the samples were obtained at a single point in time, although several of the LHMP sites are conducting serial sampling. A recent analysis of the Human Microbiome Project data demonstrated that samples from oral communities were the least stable over time, in comparison to the relative stability of gut samples (39). We chose oral washes as integrative samples of the mouth, realizing that it was impractical to control accurately for differences in oral hygiene. Thus, small differences in composition of bacterial communities in different regions of the oropharynx may impact the most appropriate comparator against which to assess BAL specimens (40). Bronchoscopy, although providing the only practical method to sample the lung in large numbers of subjects, runs an inherent risk of contamination from the oropharynx and upper airway during the procedure. The lower biomass of BAL samples from clinically well individuals necessitated the sequencing depth we reported, and future investigations using higher biomass samples should consider deeper sequencing. It also appears that the lung microbiome in individuals who are clinically well and without lung disease is largely derived from the oral microbiome, making it challenging to distinguish carry-over from true members of the community (6). Although sampling during surgery can approach a gold standard (9, 10), this methodology is...
In conclusion, this study compares the oropharyngeal and lung microbiomes in a healthy population of HIV-uninfected and HIV-infected individuals in a large, multicenter cohort. Oral bacterial communities differed significantly depending on HIV status, whereas lung communities sampled by BAL were similar. We identified specific taxa in oral samples that differed among the subject groups. Within each subject group, we identified a unique signature of taxa differing between the mouth and the lung; the patterns in the Negative and HAART subjects were similar to one another but differed from the pattern in the Naive subjects. These LHMP data serve as a basis for further investigations of the role of the lung microbiome during health and disease in HIV-infected individuals (3).

Author disclosures are available with the text of this article at www.atsjournals.org.

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Figure 6. Relative abundances of the most common organizational taxonomic units (OTUs) identified in oral wash versus bronchoalveolar lavage (BAL) for each subject group. Samples were obtained as detailed in METHODS, and V1–3 regions were sequenced. OTUs were compared by the Wilcoxon signed-rank test for all OTUs with average relative abundances of greater than 1% across all samples. The OTUs indicated by asterisks demonstrated significant differences between BAL and oral wash in HIV-negative subjects (Negative) (A), HIV-positive but treatment-naive subjects (naive) (B), and HIV-positive subjects treated with highly active antiretroviral therapy (HAART) (C). Some genus designations appear more than once because multiple OTUs have the same consensus taxonomy.
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