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Oral and Airway Microbiota in HIV-Infected Pneumonia Patients

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Despite the increased frequency of recurrent pneumonia in HIV-infected patients and recent studies linking the airway bacterial community (microbiota) to acute and chronic respiratory infection, little is known of the oral and airway microbiota that exist in these individuals and their propensity to harbor pathogens despite antimicrobial treatment for acute pneumonia. This pilot study compared paired samples of the oral and airway microbiota from 15 hospitalized HIV-infected patients receiving antimicrobial treatment for acute pneumonia. Total DNA was extracted, bacterial burden was assessed by quantitative PCR, and amplified 16S rRNA was profiled for microbiome composition using a phylogenetic microarray (16S rRNA PhyloChip). Though the bacterial burden of the airway was significantly lower than that of the oral cavity, microbiota in both niches were comparably diverse. However, oral and airway microbiota exhibited niche specificity. Oral microbiota were characterized by significantly increased relative abundance of multiple species associated with the mouth, including members of the *Bacteroides*, *Firmicutes*, and TM7 phyla, while airway microbiota were primarily characterized by a relative expansion of the *Proteobacteria*. Twenty-two taxa were detected in both niches, including *Streptococcus bovis* and *Chryseobacterium* species, pathogens associated with HIV-infected populations. In addition, we compared the airway microbiota of five of these patients to those of five non-HIV-infected pneumonia patients from a previous study. Compared to the control population, HIV-infected patients exhibited relative increased abundance of a large number of phylogenetically distinct taxa, which included several known or suspected pathogenic organisms, suggesting that recurrent pneumonia in HIV-infected populations may be related to the presence of these species.

Even in the presence of a dominant organism, multiple other bacterial species exist in the airways of patients with underlying pulmonary disease (10, 22–24). Recent studies have demonstrated that microbiota composition defines the behavior and virulence gene expression of primary pathogens (17, 30) and host susceptibility to infection (16, 30). Hence, a first step toward understanding the contribution of the airway microbiome to recurrent pneumonia in HIV-infected patients (12, 27, 43) is the identification of organisms present, despite antimicrobial treatment, both in the airway and in the oral cavity, since the latter has long been recognized as a potential microbial reservoir for reinfection of the airways (32, 36). Identification of members of the microbiota that is present despite therapeutic intervention provides valuable insights into the efficacy of such treatments and identifies species that may contribute to subsequent pulmonary episodes.

Culture-independent phylogenetic profiling approaches based on genetic biomarkers such as the 16S rRNA gene have permitted more detailed assessments of mixed-species consortia and identified specific families or species that are responsible for particular host immune phenotypes (25) or associated with particular disease states (40, 41). Recent studies have demonstrated that despite interpersonal variation (42), characteristic microbial communities exist at specific human host sites (31, 34) and that the majority of healthy subjects exhibit a relatively low, if any, bacterial burden in the lower airways (24). Whether this is also the case in HIV-infected patients who are immunocompromised and at increased risk of developing opportunistic airway infections is currently unknown.

Here, we describe an initial study of paired oral and airway microbiota profiles from 15 hospitalized HIV-infected patients with acute respiratory infections receiving antimicrobial therapy for pneumonia. Our study objectives were to determine whether,

as in healthy individuals, niche specificity exists in the oral and airway assemblages of HIV-infected patients and to define the microbiota members that were common to or distinguished these niches. In an effort to begin to examine the impact of HIV infection on the airway microbiota, we also compared five of our HIV-infected pneumonia patients with five non-HIV-infected pneumonia patients from a previous study (20) to define the airway microbiota-based differentials associated with HIV infection.

MATERIALS AND METHODS

Subjects. HIV-infected subjects ($n = 15$) were admitted to San Francisco General Hospital for acute pneumonia and underwent bronchoscopic bronchoalveolar lavage (BAL) for clinical diagnosis from July 2008 through October 2009 (Table 1). Non-HIV-infected subjects ($n = 5$) were enrolled in a separate study of ventilator-associated pneumonia (20) (Table 1). The University of California at San Francisco Committee on Human Research approved the study protocols, and all subjects provided written informed consent.

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TABLE 1 Clinical characteristics of samples^a

Patient group and ID	Age	Gender	CD4 ⁺ cell count ^b	HIV RNA level ^c	ART	Antimicrobial therapy (no. of days on therapy prior to sampling) ^d
HIV-infected patients enrolled in this study						
SFGH012	47	F	101	74	+	PCP Tx: atovaquone (3), then clindamycin and primaquine (3) BP Tx: vancomycin (6), cefepime (4) Disseminated MAC Tx: clarithromycin and ethambutol (6)
SFGH021	43	M	49	500,001	–	PCP Tx: TMP-SMX (1), then clindamycin and primaquine (4) Doxycycline (4) MAC prophylaxis: weekly azithromycin
SFGH041	39	M	38	405,002	–	PCP Tx: TMP-SMX (3) BP Tx: ceftriaxone, doxycycline (3) MAC prophylaxis: weekly azithromycin
SFGH071	44	M	305	72,851	–	BP Tx: ceftriaxone, doxycycline (3) PCP Tx: TMP-SMX (3)
SFGH081	42	M	145	408,204	–	PCP Tx: clindamycin and primaquine (4) BP Tx: ceftriaxone, doxycycline (4)
SFGH091	31	M	36	429	+	PCP Tx: TMP-SMX (2) CMV pneumonitis (no pre-BAL CMV therapy) MAC prophylaxis: weekly azithromycin
SFGH111	36	M	2	80,732	–	PCP Tx: TMP-SMX (5) BP Tx: ceftriaxone, doxycycline (5), vancomycin (4) MAC prophylaxis: weekly azithromycin
SFGH121	33	M	28	<40	+	BP Tx: vancomycin, cefepime (5); necrotizing BP Disseminated MAC Tx: clarithromycin and ethambutol (5) PCP prophylaxis: dapsone (5)
SFGH161	27	F	45	277,614	–	PCP Tx: TMP-SMX (1), then clindamycin and primaquine (6) BP Tx: ceftriaxone, doxycycline (2), stopped
SFGH171	50	F	2	24,507	+	Fungal Tx: posaconazole (37); Aspergillus pneumonia PCP prophylaxis: daily TMP-SMX MAC prophylaxis: weekly azithromycin
SFGH231	37	M	178	705,753	–	PCP Tx: TMP-SMX (3) BP Tx: ceftriaxone, azithromycin (3)
SFGH241	48	M	24	20,557	–	BP Tx: vancomycin (1 dose—renal failure), cefepime (3) PCP Tx: TMP-SMX (2) MAC prophylaxis: weekly azithromycin
SFGH251	50	F	29	84,815	–	PCP Tx: TMP-SMX (3) BP Tx: doxycycline (3) TB Tx: RIPE (1) MAC prophylaxis: weekly azithromycin
SFGH271	46	M	6	336,899	–	BP Tx: ceftriaxone, doxycycline (3) PCP Tx: clindamycin and primaquine (2) Fungal Tx (oral thrush): fluconazole (2)
SFGH291	45	M	5	69,885	–	BP Tx: ceftriaxone (7), doxycycline (2), switched to azithromycin (4) PCP Tx: TMP-SMX (6) MAC prophylaxis: weekly azithromycin
Non-HIV-infected patients ^e						
1-(2)	57	F	NR	NR	–	Cefazolin, fluconazole, levofloxacin (<6) ^f Piperacillin-tazobactam (<6) ^f
2-(2)	79	M	NR	NR	–	Antifungal, cefazolin, ceftazidime, ciprofloxacin (<10) ^f Piperacillin-tazobactam, vancomycin (<10) ^f
3-(2)	54	F	NR	NR	–	Ciprofloxacin (<4) ^f
4-(2)	55	M	NR	NR	–	Piperacillin-tazobactam, vancomycin (<6) ^f
5-(3)	85	F	NR	NR	–	Ciprofloxacin, clindamycin, piperacillin-tazobactam (<14) ^f Vancomycin (<14) ^f

^a Abbreviations: ART, antiretroviral therapy; PCP, *Pneumocystis carinii* pneumonia; Tx, treatment; BP, bacterial pneumonia; MAC, *Mycobacterium avium* complex; TMP-SMX, trimethoprim-sulfamethoxazole; CMV, cytomegalovirus; RIPE, rifampin, isoniazid, pyrazinamide, and ethambutol; NR, no record.

^b Measured between 103 and 3 days before the BAL sampling date.

^c Measured between 155 days before and 1 day after the BAL sampling date.

^d The final clinical and/or microbiological diagnosis is in bold.

^e IDs and clinical characteristics of samples are from the work of Flanagan et al. (20).

^f No number of days was recorded; however, patients were given antimicrobial therapy during the indicated period.

Sample and clinical data collection. Paired oral and airway samples from HIV-infected patients with acute pneumonia were collected for this study. Oral samples were collected immediately prior to bronchoscopy by scraping the patient's tongue 6 to 8 times using a sterile tongue depressor; scraping was repeated twice more. Oropharyngeal wash was performed by gargling with 10 ml of sterile saline for 60 s prior to swishing the fluid vigorously in the mouth for 5 s. Tongue scrapings and oropharyngeal wash fluids were combined for oral microbiota analyses.

As part of clinical care, bronchoscopy was performed on spontaneously breathing patients by bronchoscope passage through the mouth. To remove loosely bound microbes and minimize oral bacterial contamination of the BAL specimen, patients rinsed with 10 ml of 0.12% chlorhexidine gluconate for 60 s immediately prior to bronchoscopy. BAL fluid was obtained from a subsegment of the lobe that was most involved, as determined by chest imaging, or the right middle lobe (if imaging revealed diffuse pneumonia). A 3- to 5-ml aliquot of BAL fluid was immediately placed on ice following collection for subsequent airway microbiota analysis. Clinical data were collected using standardized forms, including patient demographics, habits, prior or underlying lung disease, CD4 cell count, HIV RNA level, antiretroviral therapy (ART), antimicrobial therapy, and steroid administration (Table 1; also, see Table S1 in the supplemental material).

DNA extraction, 16S rRNA gene amplification, and PhyloChip processing. Bacterial genomic DNA from airway and oral samples was extracted using an AllPrep DNA/RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with the following modifications. Cell pellets obtained by centrifugation of either BAL fluid or oral wash samples were resuspended in RLT Plus buffer (provided by the kit) with beta-mercaptoethanol according to the manufacturer's instructions, and resuspended material was transferred to a lysing matrix B tube (Qbiogene, Carlsbad, CA). Cells were lysed by bead-beating using a FastPrep system (Qbiogene) for 30 s at 5.5 m/s. Supernatant was then transferred to the DNA column provided by the kit. All buffers in the kit were tested by PCR using universal primers (see below) for any bacterial contamination. The universal 16S rRNA primers 27F (5'-AGAGTTTGA TCCTGGCTCAG-3') and 1492R (5'-GGTTACCTGTTACGACTT-3') (29) were used to amplify the 16S rRNA gene using 12 PCRs per sample run across a gradient of annealing temperatures (47°C to 58°C) to maximize the diversity recovered (10, 11, 23, 24). Each PCR mixture contained 100 ng of template DNA, 0.025 U/μl of Ex Taq (TaKaRa Bio, Shiga, Japan), 1× buffer, 0.8 mM deoxynucleoside triphosphate (dNTP) mixture (TaKaRa Bio), 1 μg/μl of bovine serum albumin (Roche, Basel, Switzerland), and a 0.3 μM concentration of each primer. PCR conditions were 1 cycle of 3 min at 95°C followed by 30 cycles of 95°C for 30 s, the gradient annealing temperature for 30 s, and 72°C for 1 min and a final extension at 72°C for 7 min. PCR products for each sample were pooled and gel purified, and 500 ng of purified product was processed for PhyloChip analysis as previously described (4, 13). All PCRs were performed with parallel no-template control reactions in which no amplification product was observed. Relatively conservative detection and quantification criteria were applied for each taxon as previously described (4, 13). Briefly, probe pairs, which consisted of perfectly matched (PM) and mismatched control (MM) probes, were scored as positive if they met two criteria: (i) the fluorescence intensity of the PM probe was ≥ 1.3 times greater than that of the MM probe, and (ii) the difference in intensity in each probe pair was 130 times greater than the squared noise value for that array. The positive fraction of probe sets (minimum of 11, median of 24 probe pairs per taxon) was calculated, and a taxon was considered present if the positive fraction was ≥ 0.9.

PhyloChip validation. The presence of target taxa was confirmed by PCR amplification and sequencing. PhyloChip probes for taxa 5249 (*Prevotellaceae*) and 3446 (*Streptococcaceae*) were used to perform nested PCR using two primer sets for each taxon to obtain specific amplification of the target (see Table S3 in the supplemental material). The optimal PCR conditions for each primer pair were determined by running reactions with a

gradient of annealing temperatures (55 to 70°C). Each PCR mixture contained 20 ng of template DNA, 0.025 U/μl of Ex Taq (TaKaRa Bio), 1× buffer, 0.8 mM dNTP mixture (TaKaRa Bio), 1 μg/μl of bovine serum albumin (Roche) and a 0.3 μM concentration of each primer. PCR conditions were 1 cycle of 3 min at 95°C followed by 30 cycles of 95°C for 30 s, the gradient annealing temperature for 30 s, and 72°C for 1 min and a final extension at 72°C for 7 min. The product from the reaction performed at the highest annealing temperature (most specific amplification) was gel purified and underwent bidirectional sequencing to confirm identity. The PhyloChip data used in this study are available in Table S8 in the supplemental material.

To confirm that the reported array fluorescence intensities reflected the relative abundance of the target taxa, quantitative-PCR (Q-PCR) was also performed with 11 paired samples that had sufficient DNA for the analysis. A pair of primers (5249_2F and 5249_qR) which targets taxon 5249 was chosen for this validation (see Table S3 in the supplemental material). An optimal annealing temperature of 60°C was determined by performing PCR with the range of annealing temperatures. Q-PCR was performed in triplicate 25-μl reaction mixtures containing 1× QuantiTect SYBR green PCR master mix (Qiagen), 20 ng of extracted DNA, and each primer at a final concentration of 0.3 μM under the following conditions: 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, and final extension at 72°C for 10 min. Resulting inverse cycle threshold (C_T) values were used in correlation analyses against array-reported fluorescence intensity to determine concordance between the two approaches. No-template control reactions run in parallel did not produce any detectable signal.

Quantification of 16S rRNA. 16S rRNA gene copy number was assessed by Q-PCR for 11 paired samples that had sufficient remaining DNA following PhyloChip analysis using the 16S rRNA universal primers 338F (5'-ACTCCTACGGGAGGAGCAGCAG-3') and 518R (5'-ATTACCGCGG CTGCTGG-3') (18). To calculate total 16S rRNA gene copy numbers, standard curves at serial log concentrations of 1×10^2 to 1×10^9 copies per reaction were generated from PCR products obtained using the universal 16S rRNA primers 27F and 1492R. Regression coefficients (r^2) for all standard curves were >0.99. Q-PCR conditions were as described above, except that annealing was performed at 53°C. No-template control reactions were performed in parallel. Melting curve analyses demonstrated that later C_T signals observed in these reactions were derived from primer dimer products and not from 16S rRNA amplification.

Statistical analyses. Statistical analyses were performed in the R environment (35) using the Vegan package (33). Hierarchical cluster analysis (HCA), an exploratory statistical tool that groups communities based on compositional (dis)similarity, and nonmetric multidimensional scaling (NMDS), an unconstrained ordination method to plot samples based on community (dis)similarity, was performed using a Bray-Curtis distance matrix (3). The function Envfit of the Vegan package, which fits environmental (clinical) vectors or factors onto an ordination plot, was used to examine relationships between clinical variables and bacterial composition. A paired or two-tailed Welch's t test followed by adjustment for false discovery using q values (39) was used to identify taxa that were significantly altered in relative abundance between groups.

Microbiota profiles of non-HIV-infected pneumonia patients. In a previous study, we demonstrated that endotracheal aspirate (EA) microbiota were compositionally similar to those of paired BAL samples by clone library and sequence analyses (20). Thus, PhyloChip-derived microbiota profiles of EA from five non-HIV-infected intubated *Pseudomonas aeruginosa*-positive patients, generated in our previous study (20), were used in the reported study for comparative and exploratory analyses.

Nucleotide sequence accession numbers. The nucleotide sequences described in this study have been submitted to DDBJ/EMBL/GenBank under the accession numbers HQ728321 to HQ728323.

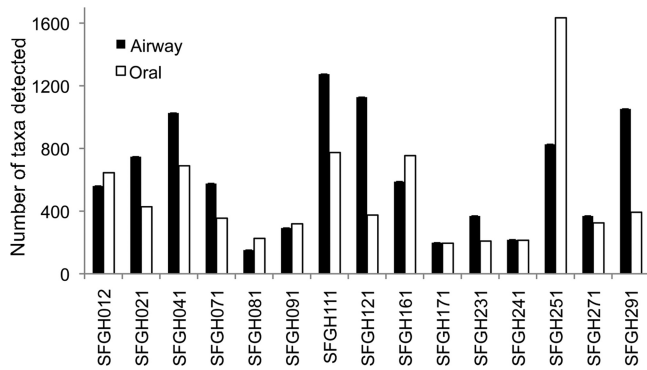


FIG 1 Bacterial community richness (number of taxa detected) in paired oral and airway samples of HIV-infected patients with pneumonia.

RESULTS

Oral and airway microbiota of HIV-infected patients. Paired oral and airway samples from 15 HIV-infected patients hospitalized for acute pneumonia and given a variety of antimicrobial therapies were used for this study (Table 1). Total bacterial burden was significantly higher ($P < 0.01$ by the Wilcoxon signed-rank test) in oral than in airway samples (2.80×10^6 [median, 1.37×10^6] versus 5.43×10^3 [median, 1.22×10^3] copies per 20 ng total DNA, respectively). However, despite a lower airway bacterial burden, oral and airway community richness (number of taxa detected) was comparable (Fig. 1; also, see Fig. S1 in the supplemental material); median richness (range) was 576 (151 to 1,274) and 375 (195 to 1,634), respectively. In addition, community evenness (distribution of taxa within a community) and Shannon diversity (37) (a metric based on both richness and evenness indices) were also comparable across the two niches (see Fig. S1).

In oral cavities, 1,754 taxa, representing 42 phyla and 153 families, were detected in at least one of the 15 individuals (see Table S2 and Fig. S2 in the supplemental material). The six most abundant phyla detected in an independent study of oral cavities of healthy individuals by sequence-based approaches (*Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, and *Cyanobacteria*) as well as four rarer phyla (TM7, *Tenericutes*, *Spirochaetes*, and SR1) detected in that study (9) were also detected in at least 1 of the 15 oral samples from our HIV-infected cohort. In addition, well-known respiratory pathogens in HIV-infected patients were detected in the oral cavities of multiple patients in this study. For example, 13 (87%) and 10 (67%) of the 15 oral samples examined demonstrated the presence of taxon 3290 (includes *Streptococcus pneumoniae*) and 3258 (includes *Staphylococcus aureus*), respectively. Additionally, taxa that include other well known pulmonary pathogens, such as *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Chlamydomphila pneumoniae*, were detected in multiple oral samples examined.

Airway samples from these patients exhibited a total of 1,654 taxa representing 41 phyla and 152 distinct families detected in at least one of the 15 samples (see Table S2 and Fig. S2 in the supplemental material). Of those, 194 taxa were detected in $\geq 80\%$ of patients (≥ 12 of our 15 patients), including members of the *Sphingomonadaceae*, *Campylobacteraceae*, and *Helicobacteraceae*, which were also detected in a previous study as organisms common to a population of COPD patients with acute pneumonia (23). This suggests commonalities in airway microbiota member-

ship across pneumonia patients with various underlying health issues. Twenty-two taxa belonging to the phyla *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, and *Natronoanaerobium* were common to both niches in all patients and included *Streptococcus bovis* and *Chryseobacterium* species.

A recent study of healthy airway microbiota profiled by 454 sequencing identified seven airway-specific bacterial taxa (5), though their prevalence was inconsistent across the six subjects profiled in that study. Our profiling efforts identified four of these genera, *Paenibacillus*, *Fusobacterium*, *Microbacterium*, and *Virgibacillus*, in our HIV-infected cohort, though at much higher frequencies than those reported for healthy subjects (in 5, 5, 7, and 10 of our 15 patients' airways, respectively). *Tropheryma whippelii*, found in BAL fluid from a single healthy individual (5), was also detected in one of our subjects. Interestingly, despite the depth of community coverage afforded by the phylogenetic array used in this study, a genus previously detected in healthy airways, *Terrabacter* (5), was not detected in any of our HIV-infected patients, suggesting that its presence may be indicative of good health. The seventh airway-specific taxon, the family *Peptostreptococcaceae* incertae sedis, is not detected by the G2 PhyloChip; hence, it was not possible to determine its presence in our cohort. Although this taxon is not well defined, this illustrates a limitation of all phylogenetic arrays: they can detect only those taxa represented on the array.

Comparative analyses of oral and airway microbiotas of HIV-infected patients. HCA analysis was performed to examine community compositional (dis)similarity. Fourteen of the 15 paired oral and airway samples clustered closely together, with a distance less than 0.13 (<13% compositional dissimilarity), indicating a high degree of microbiota similarity at these two distinct anatomical sites (Fig. 2A). Interestingly, we noted that patients receiving ART exhibited more closely related oral and airway microbiota profiles (median distance, 0.055 [range, 0.023 to 0.10]; 5.5% dissimilar) than patients not receiving ART (median distance, 0.10 [range, 0.037 to 0.13]; 10% dissimilar), indicating an even higher degree of oral and airway microbiota similarity in patients receiving ART. Though the numbers are admittedly small, this observation suggests that ART may exert a selective pressure on oral and airway mucosal microbiota. We also verified that four samples that did not have sufficient material for bacterial burden analysis (see above) did not cluster together, indicating that there was no particular microbiota composition associated with the total DNA extracted.

Comparative analyses of taxa in oral and airway samples revealed that, as observed for healthy individuals (9), characteristic niche-specific microbiota members exist in these niches (see Table S5 in the supplemental material). In the oral cavity, members of characteristic oral phyla, such as the *Bacteroides* (*Prevotellaceae* and *Porphyromonadaceae*), *Firmicutes* (*Streptococcaceae*), and TM7 members (associated with subgingival plaque), were detected in significantly ($P < 0.05$; $q < 0.05$) higher abundance than they were in airway samples (Fig. 2B), as determined by paired t test. In comparison, taxa exhibiting significantly ($P < 0.05$; $q < 0.05$) higher abundance in the airways primarily belonged to the main divisions of the *Proteobacteria* and included members of the *Alcaligenaceae*, *Campylobacteraceae*, *Enterobacteriaceae*, *Pasteurellaceae*, and *Pseudomonadaceae* (Fig. 2B) by paired t test. In addition to the *Proteobacteria*, other phyla present in significantly

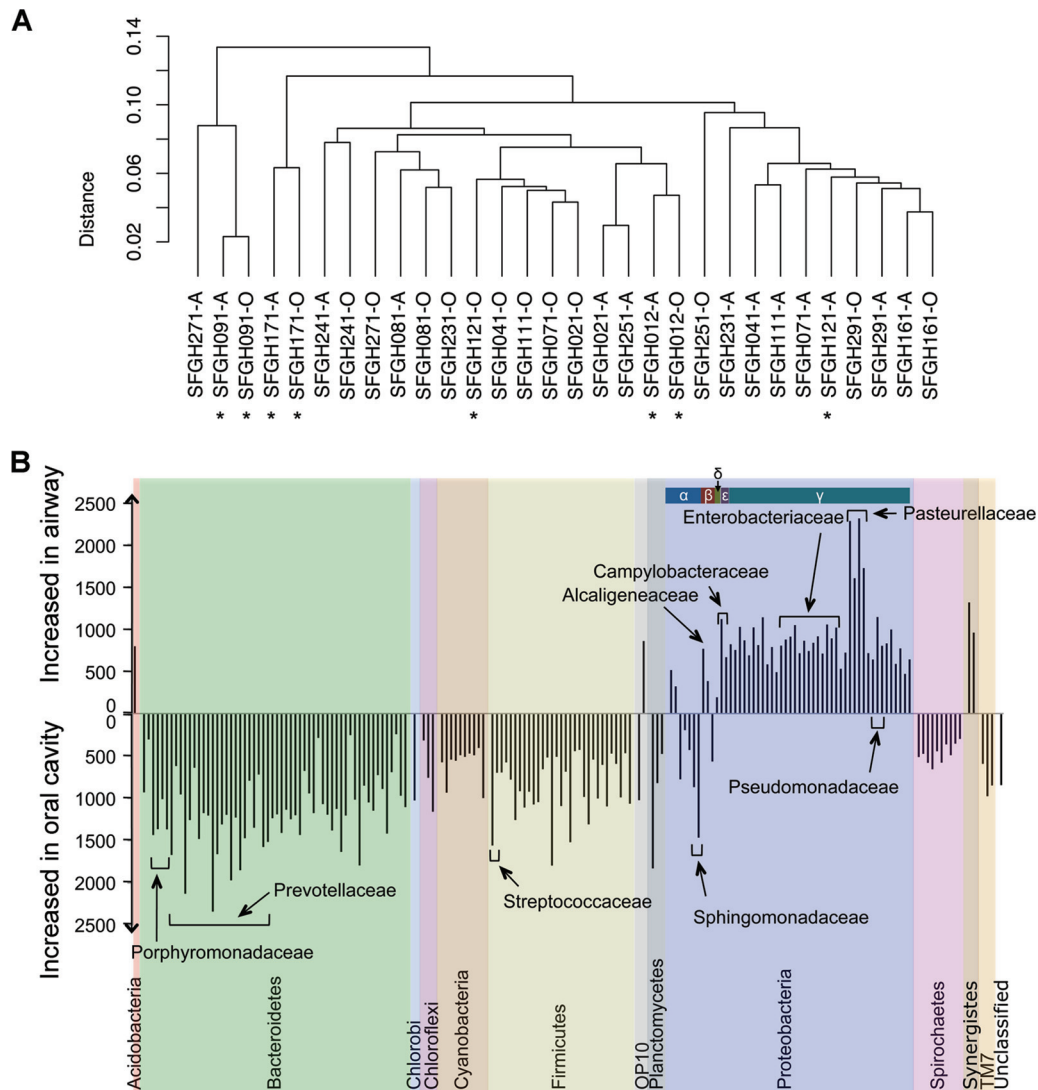


FIG 2 Comparison of oral and airway microbiota of HIV-infected patients with pneumonia. (A) Hierarchical cluster analysis of oral (O) and airway (A) samples. Samples from patients receiving ART are indicated with an asterisk. (B) Taxa exhibiting significant ($P < 0.05$) changes in relative mean fluorescence intensity values between oral and airway samples. The background is colored based on phylum; specific *Proteobacteria* classes are indicated.

higher relative abundances in the airways included *Acidobacteria* (*Acidobacteriaceae*) and unclassified *Synergistes*.

Validation of findings. To validate the array-based findings, we sequenced target-specific PCR products for two selected taxa (see details in the supplemental material; also see Tables S3 and S4). The resulting sequences exhibited $>97\%$ identity with the target genera (*Prevotella* and *Streptococcus*). In addition, we also performed correlation analysis between array-reported fluorescence intensity and independent Q-PCR data for taxon 5249, targeting *Prevotellaceae*, which exhibited good concordance ($r^2 = 0.61$; $P < 0.0001$) (see Table S4 and Fig. S3 in the supplemental material).

Relationships between airway microbiota and clinical variables. Though the microbiota profiles in our cohort are undoubtedly primarily influenced by on-going antibiotic administration, we nonetheless examined all clinical variables (Table 1; also, see Table S1 in the supplemental material) for potential association with airway microbiota composition. Though none

of the measured variables exhibited significant (i.e., $P < 0.05$) relationships with airway microbiota composition (see Table S6 in the supplemental material), the use of particular antimicrobial classes trended toward significance ($P = 0.12$ to 0.13) as expected. Perhaps in larger cohorts, the influence of specific antibiotic classes on airway microbiome composition may be more apparent.

We also compared clinical laboratory culture results with that of the microbiota profiles and demonstrated that these distinct approaches were not particularly concordant. Three patients were *Mycobacterium avium* complex (MAC) positive, as determined by mycobacterial culture (see Table S1 in the supplemental material), and two of them were also positive by the array for MAC-related taxa (taxon 1650 or 1860). However, six patients who were MAC negative according to clinical culture were positive by the array.

Over 6 months of follow-up, three of the 15 HIV-infected patients in our cohort developed a subsequent episode of pneumonia. For one patient who had available clinical laboratory culture

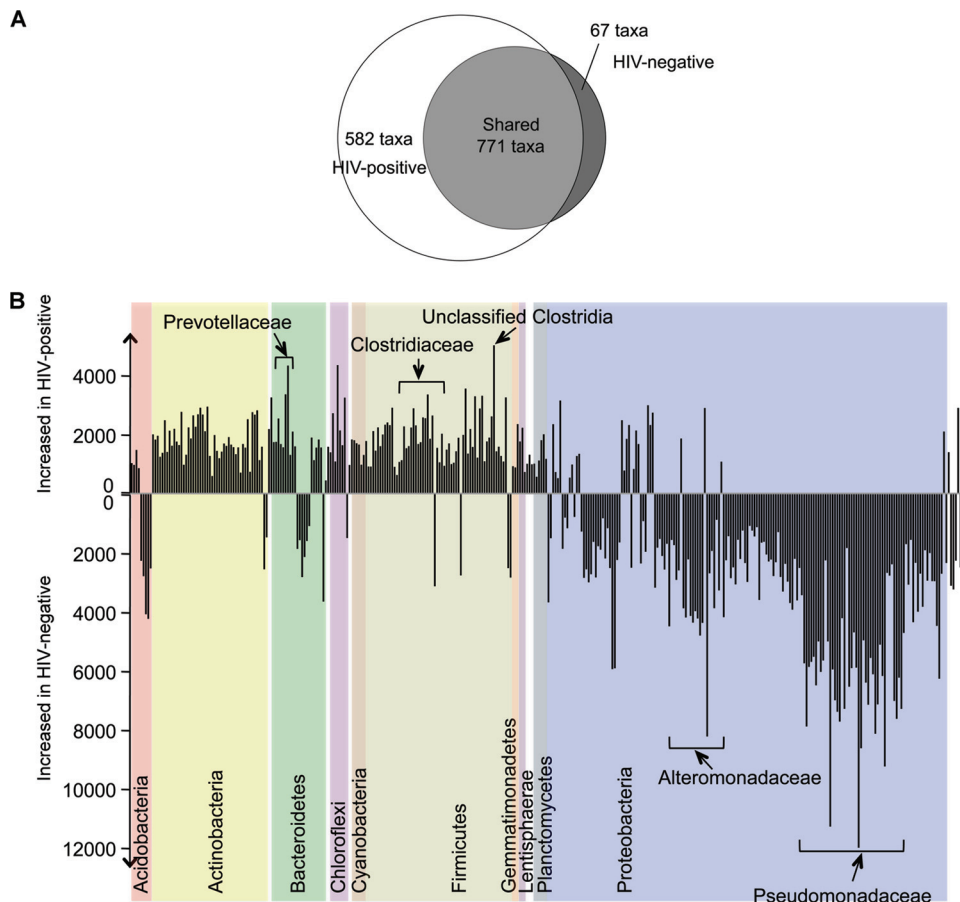


FIG 3 Comparison of airway microbiota detected in HIV-infected and non-HIV-infected patients with pneumonia. (A) Venn diagram depicting the number of taxa shared (light gray) and the numbers exclusive to HIV-infected (white) and non-HIV-infected (gray) patients. (B) Taxa exhibiting significant ($P < 0.05$) changes in relative mean fluorescence intensity values between HIV-infected and non-HIV-infected airway samples. The background is colored based on phylum (only phyla possessing at least three taxa are labeled).

results, *Streptococcus pneumoniae* was indicated as the causative agent. Examination of the microbiome profile of the original BAL fluid collected for our study from this patient also demonstrated the presence of *Streptococcus* species. In a second patient readmitted with clinically suspected hospital-acquired pneumonia, the microbiota profile for this patient demonstrated presence of several pathogens commonly acquired during hospitalization. Thus, in these few cases, the presence of specific bacterial species despite antibiotic administration may predict future recurrent pneumonia with these organisms, though clearly a larger temporal study would be necessary to develop predictive algorithms for such bacterial-based prognostics.

Comparison of HIV-infected and -uninfected airway microbiota during acute pulmonary exacerbation. As relatively high numbers of taxa were detected in our HIV-infected airway samples despite antimicrobial therapy, we hypothesized that this may represent an HIV infection-associated phenomenon. We therefore compared five non-HIV-infected patients with bacterial pneumonia who had received antimicrobial therapy for a comparable length of time (20) to five HIV-infected patients in our cohort with bacterial pneumonia as the final diagnosis (patients SFGH071, SFGH121, SFGH241, SFGH271, and SFGH291). A substantially greater number of taxa were detected in the airways

of the HIV-infected patients than the uninfected subjects. Seven hundred seventy-one taxa were detected in both non-HIV-infected and HIV-infected patients; 67 and 582 taxa were exclusively detected in non-HIV-infected and HIV-infected patients, respectively (Fig. 3A). This may be due to several factors, including HIV infection, associated immunosuppression (all patients in our cohort exhibited $CD4^+$ cell counts of $< \sim 300$), and/or therapies associated with treatment of this patient population. The non-HIV-infected patients were primarily characterized by significantly higher relative abundance of members of the *Proteobacteria* and some *Bacteroidetes* ($P < 0.05$; $q < 0.05$), including *Pseudomonas* species that were the cause of pneumonia. On the other hand, HIV-infected patients exhibited significant ($P < 0.05$; $q < 0.05$) increases in taxa belonging to a diversity of phyla (Fig. 3B; also, see Table S7 in the supplemental material). These included members of the *Actinobacteria*, *Chloroflexi*, *Cyanobacteria*, *Bacteroidetes* (including *Prevotellaceae*), and *Firmicutes* (including *Clostridiaceae*). Although our numbers are small, these data hint that different pathogenic processes occurring in HIV-infected patients and the susceptibility of this population to recurrent pneumonia may be due to the presence of a compositionally distinct and substantially more diverse airway microbiota.

DISCUSSION

Here, we profiled and compared the oral and airway microbiotas of HIV-infected patients treated with antimicrobial therapies for acute pneumonia. The oral cavity was characterized by rich microbiotas, including hallmark organisms characteristic of the mouth microbiota. Many taxa, including several known respiratory pathogens, were detected at this site despite antimicrobial therapy, further supporting a role for this niche as a potential bacterial reservoir for subsequent airway infections. The airway communities, which have been understudied compared to oral microbiota, exhibited significantly higher relative abundance of multiple members of the *Proteobacteria* in our cohort, including several known pathogens, such as *Klebsiella pneumoniae* and *Pseudomonas* spp. The presence of these organisms, despite antimicrobial therapy, may contribute to the high rate of recurrent pneumonia in HIV-infected patients. We have noted in other DNA-based airway microbiota studies that a 10-fold decrease in bacterial richness can be detected within 24 h of antimicrobial administration, suggesting that DNA turnover is relatively rapid in this niche (W. Kong, M. Allgaier, M. J. Cox, B. D. Dill, N. C. Verberkmoes, and S. V. Lynch, unpublished data). Thus, we believe that the majority of organisms detected by this approach in this niche are likely viable.

We and others have demonstrated microbiota niche specialization in healthy individuals (9, 34), e.g., in the nares and oropharynx (31), demonstrating that selective pressures, such as distinct epithelial cell surfaces at these sites, result in differential bacterial colonization patterns. Since the specific families distinguishing the oral and airway samples in this study are considered characteristic colonizers of each of these particular niches (1, 10, 11, 23, 24, 28), these data presumably provide a relatively accurate reflection of the microbiota present at these sites in HIV-infected patients. It also suggests that niche specialization, which is a feature of healthy individuals (9, 31), exists in our patient cohort despite antimicrobial therapies and HIV infection.

The number of phyla detected in this study appears to be inflated compared to the numbers obtained via sequence-based efforts by other studies of these niches (5, 9, 19), for a number of reasons. First, the scheme used to define taxa for the PhyloChip is based on Greengenes classification, which divides bacteria into 71 phyla (14). In comparison, RDP phylogeny (8), commonly used for sequence data classification, has 35 phyla. Hence, PhyloChip-generated data commonly detect seemingly large numbers of phyla due to differential classification schemes to define phylogeny. Second, due to its parallel nature, the array can detect lower-abundance community members as easily as high-abundance organisms, thus increasing the resolution of the microbiota profile generated. This feature is of great utility when microbiome profiles are being compared, i.e., between niches or between samples from diseased and healthy patients. However, it should be cautioned that, as for all PCR-based microbiome profiling approaches, the results of such studies are relative, not absolute, and are biased both by the primers used and by the amplification process.

Our observation that a greater proportion of patients on combination ART exhibited compositionally similar oral and airway microbiota is provocative. ART impacts both HIV RNA level (38), and CD4⁺ T-cell and mucosal immunity, as has been demonstrated in simian models of HIV infection (21). Indeed, treat-

ments that influence mucosal immunity would undoubtedly have an impact on the microbiome colonizing these sites, which, as has been established in recent studies of the gastrointestinal tract, plays a key role in defining local and putatively systemic immune responses (25). Though based on small patient numbers, these observations underscore the complexity of interactions within the HIV airway microbiota, the putative impact of a targeted therapeutic on other microbial species, and the fundamental need to understand this dynamic at a functional level.

In comparing our findings to those of a recent study of healthy airway microbiotas (5), we observed that four of the genera detected in healthy airways were found with much higher frequency in our HIV-infected populations, whereas one genus detected in healthy airways was not detected in the airways of any of our patients. While genera detected in healthy airways but absent from HIV-infected airways may well constitute markers of pulmonary health, the significance of increased prevalence of a number of genera in airways of HIV-infected subjects compared to healthy subjects is not yet apparent. However, it should be cautioned that the increased identification of these genera in our HIV-infected populations may also be due to detection of very low abundance species by the array that are below the level of detection of the sequencing technique used in the study of airways of healthy subjects.

Two of the more notable representative species of taxa present in both sites sampled (at similar relative abundance) in all patients included *Streptococcus bovis* and *Chryseobacterium* species. *Streptococcus bovis* is a member of the Lancefield group D streptococci and is associated with the lower gastrointestinal tract microbiome but also with bacteremia and meningitis in HIV patients (15, 26). *Chryseobacterium meningosepticum* is historically associated with meningitis in premature and newborn infants and causes infection among adults, usually with severe underlying illness, including immunocompromised HIV-infected patients (2, 6). Both species are commonly resistant to antimicrobial agents. Though we focus here on two species with established links to pathogenesis in HIV-infected populations, any of the 22 taxa present in this core community common to both oral and airway microbiota of our HIV-infected cohort may contribute to recurrent opportunistic infection, though further studies are necessary to determine such roles.

Our comparison of clinical laboratory culture results with array-based detections demonstrated that findings were not particularly concordant. This may be due to detection by the culture-independent approach of DNA from nonviable MAC species (false positive) or because of culturing difficulties due to the notoriously fastidious nature of MAC species (false negative). Discordance between culture-based and molecular approaches has also been reported previously for viral species (7). In that study, the improved array-based viral detection was confirmed by additional assays, indicating the enhanced ability of molecular methods to detect infectious agents.

Limitations of this study include the administration of antimicrobial therapy, which may reduce the relative abundance of primary pathogens associated with acute pneumonia and likely masks associations between clinical variables and airway microbiota composition. Also, controlling for clinical variables was challenging, due to the heterogeneity of the population. Though in the nature of a pilot study, this study demonstrated the presence of distinct oral and airway bacterial communities in HIV-infected

patients despite antimicrobial therapy. Although the numbers are small and the non-HIV-infected samples used in this study were from *Pseudomonas aeruginosa*-colonized pneumonia patients and thus not ideal controls, the data generated are provocative and underscore a clear need for further studies. Such studies will improve our understanding of the collective mechanisms that contribute to HIV-associated recurrent pneumonia, a fundamental step toward developing approaches for treatment and management of these polymicrobial infections.

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