Title: A Metagenomic Comparison of Tracheal Aspirate and Mini-Bronchial Alveolar Lavage for Assessment of Respiratory Microbiota

Running Title: Sequencing of Tracheal Aspirate and Mini-BAL Specimens

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Abstract (220 / 250 words):

Accurate and informative microbiologic testing is essential for guiding diagnosis and management of pneumonia in critically ill patients. Sampling of tracheal aspirate (TA) is less invasive compared to mini-bronchoalveolar lavage (mBAL) and is now recommended as a frontline diagnostic approach in mechanically ventilated patients, despite the historical belief that TA was suboptimal due to contamination from oral microbes. Advancements in metagenomic next generation sequencing (mNGS) now permit assessment of airway microbiota without a need for culture, and as such provide an opportunity to examine differences between mBAL and TA at a resolution previously unachievable. Here, we engaged shotgun mNGS to quantitatively assess the airway microbiome in matched mBAL and TA specimens from a prospective cohort of critically ill adults. We observed moderate differences between sample types across all patients (Pearson correlation of 0.72, 95% CI: 0.68 – 0.76), however we found significant compositional similarity in patients with bacterial pneumonia, whose microbial communities were characterized by a dominant pathogen (Pearson correlation of 0.92, 95% CI: 0.88 – 0.95). In addition, we found that both mBAL and TA were similar in terms of microbial burden, abundance of oropharyngeal taxa, and microbial diversity. Our findings suggest that TA sampling provides a similar assessment of airway microbiota as more invasive testing by mBAL, and that this similarity is most significant in the setting of bacterial pneumonia.

Importance (150 / 150 words):

Less invasive tracheal aspirate (TA) sampling has historically been considered inferior to mini-bronchial alveolar lavage for both diagnostic microbiology and for investigations of the lung microbiome. Recent studies demonstrating that sample type does not affect patient outcomes has led to shift in clinical practice guidelines, which now recommend TA. Despite the implications of this shift for both patient care and lung microbiome research, differences in microbial composition between fluid types had not been assessed using metagenomic methods. To address this gap, we employed metagenomic sequencing to characterize the microbial composition of matched mBAL and TA specimens. While modest differences in taxonomic composition were observed, mBAL and TA were highly similar with respect to microbial burden and diversity, and in patients with bacterial pneumonia. Our findings provide metagenomic evidence that TA is an acceptable replacement for more invasive mBAL sampling, which has implications both for clinical microbiology and lung microbiome research.

Observation (997 / 1200 words):

Pneumonia causes more deaths each year in the United States than any other type of infectious disease (1). The ability to accurately detect etiologic pathogens and distinguish them from background commensal microbiota is essential for guiding optimal antimicrobial treatment. In patients requiring mechanical ventilation, less invasive tracheal aspirate (TA) sampling has historically been considered inferior to specimen collection by mini-bronchoalveolar lavage/telescoping catheter (mBAL) due to the potential for oropharyngeal microbiota contamination (2, 3). This idea has been challenged, however, by studies demonstrating a lack of clinically significant differences between sample types, (2–5) and a greater acceptance of TA sampling is now reflected in recent updates to clinical practice guidelines (6). Despite the broad potential implications of this shift in diagnostic sampling approach, relatively little information exists regarding microbial composition differences between mBAL and TA specimens and the potential implications of such differences for both clinical diagnostic testing and airway microbiome studies.

To address this gap in knowledge, we used shotgun metagenomic next generation sequencing (mNGS) to evaluate the microbial compositions of matched mBAL and TA specimens from 52 adults who were admitted to the intensive care unit with acute respiratory failure and enrolled under University of California San Francisco protocol 10-02701 as detailed in (**Table S1**) and in supplemental materials . Two-physician adjudication based on retrospective medical record review (blinded to mNGS results) and the United States Centers for Disease Control surveillance case definition of pneumonia was used to identify 15 subjects with culture-confirmed bacterial

pneumonia (PNA-pos) (8). Adjudication also identified 12 patients with a clear alternative non-infectious etiology of acute respiratory failure (PNA-neg), and 25 patients with acute respiratory illnesses of indeterminate etiology (PNA-ind), which included patients with negative bacterial cultures but suspected pneumonia based on clinical criteria alone (**Table S2**). Patients with PCR-confirmed viral etiologies were also included in the PNA-ind group because occult bacterial co-infection could not be excluded.

Excess mBAL and TA specimens collected on the same day, within 72 hours of intubation, underwent DNA extraction and sequencing library preparation according to previously described methods (8). Following paired-end Illumina sequencing, we employed a custom bioinformatics pipeline (8) to detect and profile airway microbes (**Figure S1**) . We sequenced no-template water control samples and restricted analyses to taxa present at > 1% of the microbial population by abundance, as previously described (2, 9). No microbe was universally present in every sample, suggesting that systematic contamination across TA or mBAL sampling methods was unlikely.

We began by evaluating the correlation between genus abundance in mBAL and TA specimens and identified a Pearson correlation of 0.72 (95% CI: 0.68 – 0.76), suggesting moderate differences (**Figure 1A**). Next, we examined microbial burden (total genus alignments per million reads sequenced) and found no significant differences between TA and mBAL specimens (Median = 43.30 (IQR: 6.69 – 327.93) versus 26.89 (5.66 - 167.63), p = 0.66 by Wilcoxon rank sum). Oropharyngeal microbiota have historically been suspected to compromise TA specimens, and thus we next evaluated for differences in the abundance of common oropharyngeal microbiota

(2). Surprisingly, we found no statistically significant differences between mBAL and TA specimens with respect to *Prevotella, Veillonella, Streptococcus, Fusobacterium, Rothia,* or *Neisseria* abundance (**Table 1B**). We also identified no significant inter-specimen type differences in microbial β-diversity based on Bray-Curtis dissimilarity index ($p = .31$ by PERMANOVA).

We reasoned that differences in microbial composition between mBAL and TA specimens would be most clinically significant if they impacted diagnostic accuracy in patients with pneumonia, and thus assessed taxonomic similarity between the PNA-pos and PNA-neg groups. We found significantly greater similarity in PNA-pos subjects as compared to the PNA-neg subjects (Pearson correlation of 0.92 (0.88 – 0.95) versus 0.39 (0.28 – 0.49), $p < 1.00 \times 10^{-3}$), suggesting that pathogen dominance of the lung microbiome during infection may drive compositional similarity (9, 10) (**Table 1A, Figure 1B**). For both sample types, a culture-confirmed pathogen was the most abundant microbe detected by mNGS in 14 (93%) of PNA-pos subjects, and the second most abundant in the remaining subject (**Table S3**). mNGS of mBAL specimens detected all 23 culture-identified microbes, while mNGS of TA samples identified 22. The discrepant microbe was from a polymicrobial culture and was detected by mNGS in the TA specimen, but present at < 1% relative abundance and therefore indistinguishable from background using our bioinformatic approach (**Table S3**).

Reduced alpha diversity of the human respiratory microbiome has been described as an ecological marker of infection(9, 10), and thus we next asked whether Shannon's Diversity Index (SDI) differed by specimen type. We found that SDI differed significantly between PNA-pos and PNA-neg patients within mBAL samples, and a

similar, albeit less significant difference, was also observed within TA samples (Wilcoxon rank sum $p = 5.2 \times 10^{-6}$ and 4.7×10^{-2}, respectively) (**Figure S2**). Community richness (total number of different genera identified in each sample) was significantly decreased in PNA-pos compared to PNA-neg subjects when assessed by either specimen type, although the difference was again more significant for mBAL than for TA (Wilcox rank sum $p = 1.2 \times 10^{-3}$ versus 6.5×10^{-2}, **Table 1**).

Advances in genome sequencing have revealed that the lung, previously considered sterile, supports diverse microbial communities that play a role in both health and disease (2). Using shotgun mNGS to compare mBAL and TA samples, we found that while some differences exist in microbial composition between these two sample types, the dissimilarities become insignificant in the setting of a pathogen-dominated infection. As such, our data indicate that from a metagenomic perspective, TA sampling is an effective surrogate for more invasive testing by mBAL, a conclusion consistent with findings of prior clinical studies (4, 5) and the Clinical Practice Guidelines from the Infectious Diseases Society of America and the American Thoracic Society (6). These results may help inform both culture-independent clinical microbiologic testing, and research on the lung microbiome.

Data availability: Data are available publicly via BioProject Accession ID PRJNA445982. Analyses of primary sequencing data including pre-processed data files and an R markdown file with documentation are available on GitHub: (https://github.com/katrinakalantar/mBALvTA_comparison).

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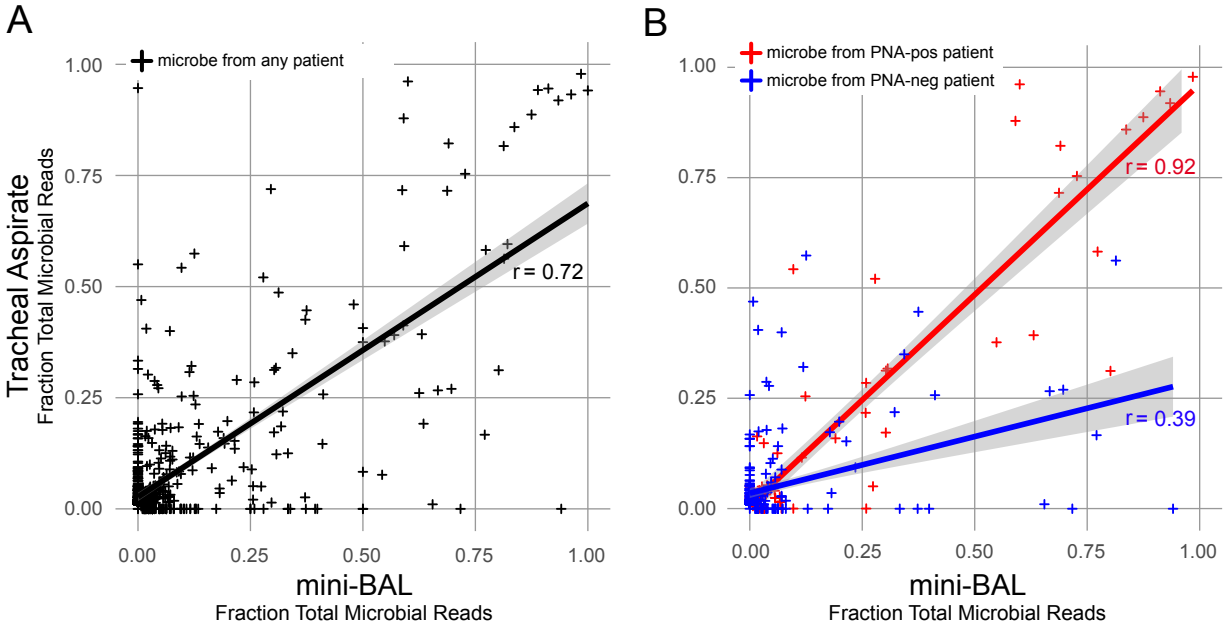
Figure Legends and Tables

Figure 1. A. Pearson correlation demonstrates taxonomic similarity by genus in mBAL versus TA specimens (r = 0.72 overall, (95% CI: 0.68 – 0.76)). **B.** Pearson correlation was stronger among microbes from PNA-pos subjects (red plus, r = 0.92 (95% CI: 0.88 – 0.95)) than PNA-neg subjects (blue plus, r = 0.39 (95% CI: 0.28 – 0.49)).

Table 1. A) Microbial community metrics in tracheal aspirate (TA) and mini-bronchial alveolar lavage (mBAL) samples, including differences between subjects with pneumonia (PNA-pos) and those with respiratory failure due to etiologies other than respiratory infection (PNA-neg). **B)** Abundance differences (reads per million reads mapped, rpM) for common oropharyngeal microbes by genus rpm between TA and mBAL samples calculated using Wilcoxon Rank Sum.

A.	**mBAL**		**TA**		
Metric	Median (IQR)	PNA-pos vs. PNA-neg	Median (IQR)	PNA-pos vs. PNA-neg	mBAL vs. TA
Richness	6.00 (4.00-9.00)	$p = 6.5 \times 10^{-2}$	8.00 (4.00-13.50)	$p = 1.2 \times 10^{-3}$	$p = 5.0 \times 10^{-2}$
Shannon Diversity	1.05 (0.71-1.55)	$p = 4.7 \times 10^{-2}$	1.45 (0.74-2.05)	$p = 5.2 \times 10^{-6}$	$p = 6.0 \times 10^{-2}$

B.	**mBAL**	**TA**	
Genus	**mean rpM (IQR)**	**mean rpM (IQR)**	mBAL vs. TA
Prevotella	0.07 (0.00 - 0.06)	0.02 (0.00 - 0.03)	p = 0.15
Veillonella	0.03 (0.00 - 0.02)	0.03 (0.00 - 0.02)	p = 0.99
Streptococcus	0.20 (0.00 - 0.33)	0.18 (0.00 - 0.34)	p = 0.88
Fusobacterium	0.02 (0.00 – 0.00)	0.01 (0.00 - 0.00)	p = 0.53
Rothia	0.03 (0.00 - 0.03)	0.01 (0.00 - 0.00)	p = 0.31
Neisseria	0.04 (0.00 - 0.02)	0.03 (0.00 - 0.02)	p = 0.72


A
microbe from any patient
r = 0.72
Tracheal Aspirate
Fraction Total Microbial Reads
mini-BAL
Fraction Total Microbial Reads
0.00
0.25
0.50
0.75
1.00
B
microbe from PNA-pos patient
microbe from PNA-neg patient
r = 0.92
r = 0.39
mini-BAL
Fraction Total Microbial Reads