## Research Background

### Introduction

Mechanically ventilated children are at high risk for ventilator-associated pneumonia (VAP). Children who develop VAP have an increased risk of mortality and morbidities such as prolonged intubation and pediatric intensive care unit (PICU) stays, and the need for extensive rehabilitation. Suspected VAP is the most common indication for antibiotic use in the PICU, accounting for almost half of all antibiotic days. Limited understanding of the microbial and host factors associated with VAP has precluded the development of effective prevention, diagnostic, and treatment strategies.

The prevailing theory behind the pathogenesis of pneumonia, including VAP, is that a pathogen enters the respiratory tract and multiplies until it overwhelms endogenous microbiota and the host defense. Endogenous bacteria are likely critical regulators of both pathogen behavior and host responses in the airways. As such, factors that impact airway microbiota or the host response are key risk factors for development of VAP. Yet, the typical culture methodology employed in the clinical environment lacks the sensitivity to assess changes in the microbiota over time.

The objectives of this prospective multi-center cohort study of mechanically ventilated children were to determine whether 1) compositional differences at the time of intubation, and 2) decreasing lower airway bacterial alpha diversity, increasing bacterial burden, and compositional change of the microbiome (increasing pathogen abundance) over time, are associated with development of VAP. Further, we sought to determine whether these patterns are evident prior to the clinical determination of VAP, allowing for earlier detection and more effective treatment strategies.

### Methods

*Clinical Data*

We conducted a prospective cohort study of mechanically ventilated children admitted to the 8 PICUs in the National Institute of Child Health and Human Development’s Collaborative Pediatric Critical Care Research Network (CPCCRN) from February 2015 to December 2017. Children ages 31 days – 18 years who were expected to require mechanical ventilation (MV) via endotracheal tube (ETT) >72 hours were eligible. Exclusion criteria included: children in whom an ETT aspirate was not obtained within 24 hours of intubation, those with a tracheostomy tube or with plans to place one, conditions in which deep tracheal suctioning was contraindicated, a previous episode of MV during the hospitalization, previous enrollment into this study; and limitations of care.

Eligible patients and their legal guardians were approached for consent within 96 hours of intubation. Delayed consent was granted, allowing for tracheal aspirate (TA) samples collected from standard of care suctioning of the ETT via sterile specimen trap and stored at −80°C until informed consent could be obtained. Specimens from non-consenting patients were destroyed. The study was approved by the University of Utah central IRB.

Initial specimens were collected within 24 hours of intubation, and subsequent samples were collected daily until the first attempted extubation or for up to 14 days. Only subjects undergoing MV for >72 hours were included in the final analyses.

VAP Diagnosis Subjects were screened daily to identify VAP defined by the pediatric 2008 Center for Disease Control (CDC) criteria.

*Antibiotics*

Physicians (attending or fellow) were also surveyed daily to determine whether they initiated antibiotics for suspected or physician diagnosed VAP. They were asked: “Is the subject receiving antibiotics today for a suspected or diagnosed hospital acquired lower respiratory tract infection?” If the question was answered in the affirmative, they were then asked whether the antibiotics were being administered for “rule out of infection” (suspected VAP) or for a “dedicated treatment course” (physician diagnosed VAP). Antibiotic data were collected for each patient and included the drug name, administration route, and start and stop date. These data were summarized in two ways, 1) the number of drugs given on each day of intubation and 2) total coverage score that was based on whether the antibiotic included coverage against gram-positive bacteria, gram-negative bacteria and/or anaerobic bacteria. Coverage in each area was graded on a 3-point scale, 0 for no activity, 1 for narrow activity, and 2 for broad activity. For patients treated with multiple antibiotics simultaneously, the score for each antibiotic received on a given day was summed. Cumulative scores were summed across days.

*Protocol for Specimen Collection*

The first tracheal aspirate specimens were collected with routine suctioning of the endotracheal tube (ETT) as soon as possible after intubation, but within 24 hours. Subsequent collections were obtained with the first morning routine suctioning performed by the clinical caregiver (nurse or respiratory therapist).

*Microbiome Methods DNA extraction and Quantitative PCR*

DNA extractions were performed using the Qiagen EZ1 Advanced automated extraction platform (Qiagen Inc., Valencia, CA) with the bacterial card and tissue extraction kit per manufacturer’s instructions. Total bacterial load was measured using a quantitative real-time PCR assay that has been previously published and evaluated for use in human airway samples. Fidelity of the molecular biology preparation was monitored using triplicate blanks in each plate. 16S rRNA sequencing: Bacterial profiles were determined by broad-range amplification and sequence analysis of 16S rRNA genes. Amplicons were generated using primers that target approximately 300 base pairs of the V1/V2 (27F/338R) variable region of the 16S rRNA gene. Illumina MiSeq paired-end sequences were sorted by sample via barcodes in the paired reads with a python script. Sorted paired end sequence data were deposited in the NCBI Short Read Archive under accession number PRJNA533819 (data generated from methods development [PRJNA436139] and comparison of gastric and tracheal aspirates [PRJNA508231] were also utilized by this study). The sorted paired reads were assembled using phrap. Pairs that did not assemble were discarded. Assembled sequence ends were trimmed over a moving window of 5 nucleotides until average quality met or exceeded 20. Trimmed sequences with more than 1 ambiguity or shorter than 200 nt were discarded. Potential chimeras identified with Uchime (usearch6.0.203\_i86linux32) using the Schloss Silva reference sequences were removed from subsequent analyses. Assembled sequences were aligned and classified with SINA (1.2.11) using the 418,497 bacterial sequences in Silva 115NR99 as reference configured to yield the Silva taxonomy. Sequences with identical taxonomic assignments were clustered into Operational taxonomic units (OTUs). This process yielded 182,567,651 sequences from 2,202 samples (average size: 82,948 sequences/sample; min: 6,266; max: 494,800). The median Goods coverage score was ≥ 99.4% at the rarefaction point of 6,266 with 100 resamplings. The software package Explicet (v2.9.4, www.explicet.org) (18) was used to calculate rarefied Good’s coverage, alpha diversity measures (Shannon diversity and evenness index) and beta diversity (Morisita-Horn index).

**\*Disclaimer: Introduction and Methods are subsets from the full Aim 1 paper for this study. To see the full paper please visit the following citation:**

**“**Mourani PM, Sontag MK, Williamson KM, Harris JK, Reeder R, Locandro C, Carpenter TC, Maddux AB, Ziegler K, Simões EAF, Osborne CM, Ambroggio L, Leroue MK, Robertson CE, Langelier C, DeRisi JL, Kamm J, Hall MW, Zuppa AF, Carcillo J, Meert K, Sapru A, Pollack MM, McQuillen P, Notterman DA, Dean JM, Wagner BD; Eunice Kennedy Shriver National Institute of Child Health and Human Development Collaborative Pediatric Critical Care Research Network (CPCCRN); Eunice Kennedy Shriver National Institute of Child Health and Human Development Collaborative Pediatric Critical Care Research Network (CPCCRN) members are as follows:. **Temporal airway microbiome changes related to ventilator-associated pneumonia in children. Eur Respir J. 2021 Mar 18;57(3):2001829. doi: 10.1183/13993003.01829-2020. PMID: 33008935; PMCID: PMC7979474.”**

## Research Questions

Analytic questions of Interest:

1. Are antibiotics associated with microbiome metrics?
   1. Microbiome metrics include alpha diversity, beta diversity, and bacterial load.
   2. Measures for alpha diversity include: Shannon diversity, richness, evenness, good’s coverage.
      1. Potential analyses include: T-tests, linear regression
   3. Measures for beta diversity include: individual taxa and overall community composition
      1. Potential analyses include Ordination methods (PCA/PCoA), heirarchical clustering, feature selection (RF), negative binomials, beta-binomials, Wilcoxon Rank Sum.
2. Create a graphic to show antibiotic administration.
   1. Because the children are in the ICU, they’ll all be on antibiotics. Many children are on multiple antibiotics at the same time. The antibiotic start and end time is different for each antibiotic. We would like to see figures that can answer questions such as: Which antibiotic were you on and for how long?
3. Are there specific microbiome phenotypes?
   1. For any person, you could have > 1000 different bacteria in your lungs. Do certain groups of people have certain groups of bacteria?
   2. If you have a higher X, do you see a certain grouping of bacteria?
   3. Potential analyses include:
      1. Potential analyses include Ordination methods (PCA/PCoA), heirarchical clustering, feature selection (RF), negative binomials, beta-binomials, Wilcoxon Rank Sum.
4. What is the association between antibiotics and culture data?
   1. There are two types of bacterial measures: culture data and 16 S data. For this question, we want participants to focus on the presence or absence of certain bacteria in the cultures in order to answer the question: Were the bugs suspectable to the antibiotics prescribed
   2. Potential analyses include:
      1. chisq

Data management questions of interest

1. Clean up antibiotic log
   1. The antibiotic log is a bunch of text columns. There is one observation per antibiotic per study day per person. We would like this antibiotic log to be in analysis-ready format.
2. Create dataset including antibiotic log and culture data
   1. Culture data is clean