Relationship of the Middle Ear Effusion Microbiome to Secretory Mucin Production in Pediatric Patients With Chronic Otitis Media

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INTRODUCTION

Chronic otitis media (COM) is characterized by the presence of thick viscous fluid in the middle ear persisting for 3 or more months and generally lacks signs of acute infection.1,2 COM, affecting as many as 80% of children at some point during childhood, is the commonest cause of hearing loss in the developed world and frequently results in speech delay and educational problems.3 Surgery is the typical method of treatment for this condition, making myringotomy with tympanostomy tube insertion the most common childhood surgery in the developed world.4,5 An estimated 667,000 myringotomy procedures are performed each year in the United States.6

After multiple acute bacterial or viral exposures, the single layer epithelium in the middle ear gradually transforms into pseudostratified epithelium7,8 capable of secreting heavily glycosylated proteins called mucins, which are predominant macromolecular components of middle ear effusions (MEEs) resulting from COM. To date, more than 20 human mucin (MUC) genes have been identified (reviewed in reference Val†). The secreted mucins MUC5AC and MUC5B have been noted in MEEs, and though both often occur together, MUC5B was present in 94.5% and MUC5AC in 65.5% of MEEs.9 A recent study has shown that mouse Muc5b, but not Muc5ac is required for infection control of the middle ear and respiratory tract.10 In this study, we found that Muc5b−/− mice died because of bacterial infection and inflammation in the respiratory tract and middle ear, while Muc5ac−/− mice survived and showed normal acute mucociliary clearance and infection control, suggesting the importance of MUC5B over MUC5AC in defense against upper airway and middle ear pathogens.10

Streptococcus pneumoniae, nontypable Haemophilus influenzae and Moraxella catarrhalis are 3 of the most frequent etiologic agents of acute otitis media (AOM),11,12 with S. pneumoniae, H. influenzae and M. catarrhalis present in 20%–35%, 20%–30% and 20% of acute infections, respectively, as determined through clinical culture methods.13 Less is known about the global microbial communities and their involvement in the pathogenesis of COM. Utilization of next-generation sequencing, specifically 16S rRNA amplicon sequencing, is a relatively new technique used to classify prokaryotes in laboratory and clinical settings.14,15 This technique can detect and classify nonculturable, poorly described, rarely isolated or novel bacteria, making it more accurate and less subjective than other commonly used methods.16

To our knowledge, there have been no attempts to analyze and compare differences in the microbiome to other COM-related variables, such as presence of mucin in MEEs, clinical outcomes and patient demographics. Here we use 16S rRNA high-throughput sequencing to characterize the MEE microbiome of effusion samples from 55 pediatric patients. We hypothesize that MEEs containing MUC5B will exhibit a microbiome largely devoid of typical AOM bacteria.

MATERIALS AND METHODS

Sample Collection

Fifty-five MEE samples from children with COM undergoing myringotomy with tympanostomy tube placement at Children’s National Health System in Washington DC were collected under Institutional Review Board approval and parental consent. Bilateral effusions were combined into one sample per patient. Recovered MEEs were frozen in phosphate-buffered saline at −80°C.
Western Blot Analysis

Effusion samples containing on average 360 μg of total protein in 20 μL were dissolved in Laemmli buffer containing 0.1 mM Dithiothreitol (DTT) and subjected to 1-dimensional sodium dodecyl sulfate (SDS) gel electrophoresis fractionation at 200 V for 50 minutes as previously described.22 After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). Human saliva was used as the MUC5B positive control. Calu3 or A549 lung cell lysates were used as the MUC5AC positive control. The primary polyclonal antibody for MUC5B (Santa Cruz, CA), H-300, was used at a 1:300 dilution. LUM5-1, a rabbit polyclonal anti-MUC5AC antibody generously provided to us by Dr. Mehmet Kesimer, University of North Carolina at Chapel Hill, was used at a 1:4000 dilution. Blots were developed using the horseradish fluoro-illuminescence detection protocol using SuperSignal West Pico Chemiluminescent Substrate (Thermofisher, Rockford, IL) to visualize protein signal.

DNA Isolation and Molecular Analyses

DNA from the same MEEs was purified using the QiaAmp mini kit (Qiagen, Valencia, CA) before electrophoresis on 1% agarose gels run at 100 V in 1× TAE buffer containing ethidium bromide for visualization under ultraviolet light. Quantification of relative DNA concentrations in each effusion sample was performed by the Qubit double stranded DNA kit (Invitrogen, Carlsbad, CA). All extractions yielding >50 ng of total DNA (as indicated by NanoDrop 2000 UV–Vis Spectrophotometer measurement) were further processed. DNA extractions were prepared for sequencing using the Schloss’ MiSeq_WetLab_SOP protocol (09.2015) in Koizich et al.15 Each DNA sample was amplified for the V4 region (≈250 bp) of the 16S rRNA gene, and libraries were sequenced using the Illumina MiSeq sequencing platform at University of Michigan Medical School.

Data Analysis

Raw FASTQ files were processed in mothur v1.35.1.16 Default settings were used to minimize sequencing errors as described in Schloss et al.16 Clean sequences were aligned to the SILVA-based bacterial reference alignment at http://www.mothur.org. Chimeras were removed using UCHIME,17 and nonchimeric sequences were classified using the naïve Bayesian classifier of Wang et al.18 Sequences were clustered into operational taxonomic units (OTUs) at the 0.03 threshold. OTU sequence representatives and taxonomy were imported into QIIME19 for subsequent analyses. The mothur OTU table was filtered to a minimum of 2 observations (sequences) per OTU. Samples were subsampled (rarefaction analysis) to the smallest sample size (1332 sequences) to remove the effect of sample size bias on community composition.

Trees for phylogenetic diversity calculations were constructed using FastTree.20 Taxonomic alpha-diversity was estimated as the number of observed OTUs, but also by the Chao1 and Shannon indexes. Phylogenetic alpha-diversity was calculated by the Faith’s phylogenetic diversity index.21 Similarly, both taxonomic (Bray–Curtis and Euclidean) and phylogenetic unifrac (unweighted and weighted) beta-diversity metrics were calculated. For beta-diversity, we estimated relative mean proportions, that is, the mean proportion of sequences assigned to a feature relative to the total number of sequences. For this purpose, we used White’s nonparametric t test and calculated 95% confidence intervals as described by White et al.22 Sample dissimilarity was assessed using principal coordinates analysis and unifrac distances. Alpha- and beta-diversity estimates (dependent variables) were compared between and within samples grouped by the following independent binomial variables: age (≤2 versus >2 years old), gender, hearing loss (≤30 versus >30 dB), presence of only MUC5B versus both MUC5B and MUC5AC using nonparametric t tests. Taxonomic and phylogenetic distances were also compared among the above groups using permutational multivariate analysis of variance (adonis) as implemented in the vegan R package.23 Significance was determined through 10,000 permutations. Finally, phyla and genera abundances (dependent variables) were compared between samples grouped according to the independent variables listed above using the White’s nonparametric t test.22 Taxa showing significantly different proportions were further assessed using logistic regression, including all the independent variables listed above in the model. All analyses were performed in mothur, QIIME, STAMP24 and RStudio.25

RESULTS

Patient Demographics

MEEs were collected from 55 patients with COM undergoing myringotomy at Children’s National Health System in Washington, DC. The mean age of the patients was 40.2 months (range, 3–176 months). The majority of patients were male (38/55; 69.1%). Significant hearing loss, defined by a lower limit threshold of 30 dB or higher as determined through an audiogram, was present in 72.4% of patients (Table 1).

Western Blot Analysis

Western blot analysis for the detection of MUC5B and MUC5AC was performed on 55 MEEs collected from a total of 55 pediatric patients. Representative blots are shown in Figure 1A and 1B, respectively. Before analysis, 44 of the 55 samples (80%) were identified as mucoid according to clinical impression of viscosity, with the remaining 11 classified as serous. Results revealed that a greater proportion of the samples contained MUC5B (94.5%) than MUC5AC (65.5%). Thirty-five out of 55 samples (63.6%) contained both MUC5B and MUC5AC, while only 1 sample (1.82%) contained MUC5AC alone. Two samples (3.63%) did not contain either MUC5AC or MUC5B (Table 2).

Microbial Composition

Fifty-five MEE samples corresponding to 55 children with COM were analyzed using the Illumina MiSeq platform. A total of 50 samples surpassed the cutoff of 1332 sequence reads and were further analyzed. Reads ranged from 1332 to 49,647 per sample (mean, 16,399.8; median, 16,323.5). From this we identified a total of 62 OTUs belonging to 20 phyla and 62 genera. Only 39 OTUs had at least a 0.1% relative abundance (Fig., Supplemental Digital Content 1, http://links.lww.com/INF/C653), while only 6 bacterial genera had a relative abundance of ≥5% in our samples. These genera included Haemophilus (22.54%), Moraxella (11.11%), Turicella (7.84%), Alcaligenaceae unclassified (5.84%), Pseudomonas (5.40%) and Alloicococcus (5.08%; Table 3).
Microbial Diversity

Alpha-diversity indices (observed OTUs, Shannon diversity, phylogenetic diversity and Chao1 richness) did not show significant differences between samples grouped by listed variables, including gender, age, hearing loss and mucin type. Principal coordinates analyses, however, showed dissimilarity between microbiomes grouped by these same variables. Our adonis analyses revealed significant differences in the beta-diversity of microbiomes from samples grouped by age (P = 0.029) and hearing loss (P = 0.019) for unifrac weighted distances and by both mucins (P = 0.013) for unifrac unweighted distances (Fig., Supplemental Digital Content 2 and 3, http://links.lww.com/INF/C654 & http://links.lww.com/INF/C655). Four taxa showed (P = 0.05–0.018) significant differences in relative abundance of sequences grouped by age (months; Fig. 2A), hearing loss (Fig. 2B and 2C) and presence of both MUC5AC and MUC5B (Fig. 2D) based on the White’s t test. After adjusting for multiple confounders, all these taxa remained significant (P = 0.05–0.037). The microbiome in MEEs from children over 24 months of age contained a higher proportion of Turicella (P = 0.032) and Pseudomonas (P = 0.041) than those from children 24 months of age and younger (Fig. 2A). Actinobacteria (P = 0.018; Fig. 2B) and Turicella (P = 0.039; Fig. 2C) were in greater abundance in MEEs from children without hearing loss as compared with those from children with hearing loss. Haemophilus (P = 0.025) was detected at a significantly higher proportion in patients with hearing loss as compared with those without hearing loss (Fig. 2C). Haemophilus was also found in greater abundance in MEEs that contained both MUC5AC and MUC5B as compared with samples containing MUC5AC only, MUC5B only, or neither mucin (P = 0.025; Fig. 2D). Interestingly, the 1 sample containing only MUC5AC was found to have the highest level of Pseudomonas species. No significant differences in abundance were observed when samples were grouped by gender or effusion type.

DISCUSSION

Consistent with previous findings, MUC5B was found to be present in significantly more of the MEEs (94.5%) than was MUC5AC (65.5%), supporting the notion that MUC5B plays a more substantial role in long-term homeostasis than MUC5AC, which in turn is thought to act primarily as an acute responder. To our knowledge, only 2 previous studies have also used 16S rRNA gene sequencing to describe the microbiome of MEE samples. Indigenous Australian children were analyzed by Jervis-Bardy et al. Using this technique, they found that Alloiococcus otitidis and H. influenzae were the most common bacterial species, with 13 total OTUs identified. Liu et al. described the microbiome of the MEE of 1 patient with chronic serous otitis media (OM), in which they found expected OTUs in addition to some that had yet to be described in OM. In line with these studies, our findings uncovered previously undescribed OTUs along with common AOM pathogens. However, our work is unique in that it analyzes a larger number of samples and considers other clinical variables in the microbiome analysis.
Using 16S rRNA amplicon sequencing, we observed that genera corresponding to the most commonly identified pathogens in AOM (Haemophilus, Moraxella and Streptococcus)\textsuperscript{3,11} were also some of the most relatively abundant bacteria within our MEEs. When averaging across all 50 samples, Haemophilus was found to make up 22.54\% of the bacterial reads. Moraxella was the second most abundant genus (11.11\%), while Streptococcus accounted for 4.21\% of the MEE bacterial reads.

In addition to these common AOM pathogens, we detected a relatively high abundance of other previously isolated or identified bacterial genera associated with COM. Turicella, the third most relatively abundant genus (7.81\%), was originally identified within OM samples,\textsuperscript{29} leading to the naming of the only species—Turicella otitidis.\textsuperscript{30} Another species of the genus Stenotrophomonas, Stenotrophomonas maltophilia, was also previously isolated from OM samples and is comprised of only one known species, A. otitidis.\textsuperscript{30} Pseudomonas and Staphylococcus have also been previously found in COM samples through the use of multiple techniques, including culture, polymerase chain reaction and 16S rRNA gene sequencing.\textsuperscript{27,28,31,32}

Interestingly, Stenotrophomonas, members of the Chitinophagaceae and Alcaligenaceae for whom we detected reads of unclassified genera, and multiple other genera found in varying abundance within our samples have not previously been described in MEEs. The identification of bacteria not previously found within these samples is unsurprising as traditional technologies used for bacterial detection were unable to detect unculturable or undefined bacteria. However, species within many of these previously unreported genera have been implicated in human pathogenesis or as commensal bacteria within the human microbiome, including Bordetella species of the Alcaligenaceae family and Stenotrophomonas maltophilia of the genus Stenotrophomonas.\textsuperscript{33,34} The role all these genera associated with OM may play (or not) in the pathogenesis of COM remains to be determined.

No significant differences in alpha-diversity were observed between samples grouped by any of the clinical variables or the presence of mucins, suggesting similar microbial intra-sample composition (richness and evenness) across these population groups. On the other hand, when analyzing beta-diversity, that is, inter-sample variation in community structure across the cohort group, significant differences were detected when comparing samples grouped by presence of hearing loss (greater than 30 dB), age (≤24 or >24 months) and presence of MUC5AC. D. Significant differences (White’s t test) in relative abundance of taxa between patients grouped by age (≤2 or >2 years old). By this analysis, Turicella and Pseudomonas were found to be significant. Bar graphs depict the relative mean proportions of taxa for each of these categories. B. Significant differences (White’s t test) in relative abundance of taxa between patients grouped by hearing loss presence (dB=decibels). By this analysis, Actinobacteria was found to be significant. Bar graphs depict the proportion of taxa for each hearing loss category. C. Significant differences (White’s t test) in relative abundance of taxa between patients grouped by hearing loss presence (dB=decibels). By this analysis, Haemophilus and Turicella were found to be significant. Bar graphs depict the proportion of samples positive for each of these by hearing loss category. D. Significant differences (White’s t test) in relative abundance of taxa between patients grouped by presence of both MUC5AC and MUC5B or only those with MUC5B present. By this analysis, Haemophilus was found to be significant. Bar graphs depict the proportion of samples positive for each of these by mucin presence category.

**FIGURE 2.** A, Significant differences (White’s t test) in relative abundance of taxa between patients grouped by age (≤2 or >2 years old). By this analysis, Turicella and Pseudomonas were found to be significant. Bar graphs depict the relative mean proportions of taxa for each of these categories. B, Significant differences (White’s t test) in relative abundance of taxa between patients grouped by hearing loss presence (dB=decibels). By this analysis, Actinobacteria was found to be significant. Bar graphs depict the proportion of taxa for each hearing loss category. C, Significant differences (White’s t test) in relative abundance of taxa between patients grouped by hearing loss presence (dB=decibels). By this analysis, Haemophilus and Turicella were found to be significant. Bar graphs depict the proportion of samples positive for each of these by hearing loss category. D. Significant differences (White’s t test) in relative abundance of taxa between patients grouped by presence of both MUC5AC and MUC5B or only those with MUC5B present. By this analysis, Haemophilus was found to be significant. Bar graphs depict the proportion of samples positive for each of these by mucin presence category.
This finding supports the fact that *H. influenzae* has long been shown to influence the genetic expression of MUC5AC.26,35–37 When MUC5B was present alone in the samples, the dominance of *Haemophilus* species, or other AOM associated bacteria, was no longer noted. This would seem to indicate that MUC5AC, but not MUC5B, occurs in tandem with *Haemophilus* species in the middle ear. Whether MUC5B is protective against these pathogens is not clear.

Additionally, Actinobacteria and *Turicella* were found to be at a significantly higher abundance in MEEs from children without hearing loss as compared with those from children with hearing loss. We also found that both *Turicella* and *Pseudomonas* occurred in significantly higher proportions of the microbiome in MEEs from children over 24 months of age as compared with those 24 months of age and younger at the time of surgery. We believe that these differing microbiomes potentially suggest a distinct pathophysiology for OM susceptibility in older children or those with effusions not causing hearing loss.

Limitations of our study include the inability to directly quantify specific mucins (ie, MUC5AC and MUC5B). Antibody-based techniques typically used for protein quantification, such as enzyme-linked immunosorbent assays, have often proven erroneous at distinguishing MUC5B from MUC5AC mucin because of lack of the assay’s specificity, presumably because the extensive mucin O-glycosylation masks amino acid epitopes under the nondenaturing conditions used in enzyme-linked immunosorbent assays, which predictably blocks accessibility of antibodies.39 In our hands, the best way of distinguishing these 2 mucins remains Western blot analysis using polyclonal antibodies raised against the nonglycosylated domains of each mucin. For all samples, we loaded the maximum amount of protein possible onto the agarose gels. As such, the primary goal was not to quantify the mucins per se, but to detect whether they were present (yes/no) in each sample. A second limitation in our analysis was the fact that we were only able to compare the samples that had both mucins present (n=35) versus those with only MUC5B present (n=16). The samples with no mucins at all (n=2) or with only MUC5AC (n=1) were not considered for statistical analysis because microbial results in these outlier samples are anecdotal. Notably, we only conclude and report on differences among the samples with both mucins versus those with MUC5B only present—showing a statistical increase in the proportion of *Haemophilus* species present when both mucins were detected by Western blot assays. A final limitation was that 4 samples showed low MiSeq sequencing yields of less than 2000 (each of these 4 between 1332 and 2000 reads). However, it is known that MiSeq sequencing yields usually vary greatly among samples. Importantly, all the other samples showed >2000 reads. Oral and nasal microbiomes are considered to have low diversity; hence, rarefaction depths of n=1000 reads are usually considered sufficient to assess microbiomai composition and structure.39

Results from our study suggest that the microbiome of MEEs from children with COM is significantly associated with clinical features, such as hearing loss. In addition, we found a significant association between mucin content and bacterial proportions, specifically of *Haemophilus*. As mentioned earlier, *H. influenzae* is often implicated in AOM infections. This finding suggests that this bacterial species likely plays a role in mucin overproduction, specifically of the acute responding mucin MUC5AC, possibly contributing to the progression of OM from acute to chronic.

REFERENCES


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