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Original Article

The Adenoid Microbiome in Recurrent Acute Otitis Media and Obstructive Sleep Apnea

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OBJECTIVE: To compare the microbial flora of adenoids from patients with recurrent acute otitis media (AOM) and patients with obstructive sleep apnea (OSA).

MATERIALS and METHODS: This study was prospective and controlled. Adenoids were obtained from children undergoing adenoidectomy for recurrent AOM (n=7) or OSA (n=13). Specimens were processed for total deoxyribonucleic acid (DNA) isolation. 16s DNA 454-pyrosequencing was performed on AOM (n=5) and OSA (n=5) specimens. All specimens were analyzed by real-time polymerase chain reaction for the quantification of the oral commensal bacteria, *Streptococcus salivarius*.

RESULTS: All adenoid specimens had evidence of microbes. Haemophilus *influenzae, Moraxella catarrhalis, Streptococcus pneumoniae, Pseudomonas aeruginosa*, and *Staphylococcus aureus* were among the dominant species in all samples. *S. salivarius, Prevotella* sp. and *Terrahaemophilus aromaticivorans* were more common on adenoids from OSA patients ($p<0.05$). *Bradyrhizobium* sp. was more common on adenoids from patients with recurrent AOM ($p<0.05$). The microbial profiles associated with recurrent AOM were different from, but overlapped with OSA. *S. salivarius* quantified by real-time PCR was not different between the two groups.

CONCLUSION: Microbes are present on all adenoid specimens, though the microbial profile differs between recurrent AOM and OSA. The clinical significance of these differences remains to be determined.

KEYWORDS: Adenoids, recurrent acute otitis media, obstructive sleep apnea, pyrosequencing, microbiome

INTRODUCTION

Acute otitis media (AOM) is one of the most common infections in children, with approximately 50 to 85% of children experiencing at least one episode of AOM by two to three years of age [1, 2]. By three years, about 46% of children experience three or more episodes of AOM. Treatment of recurrent AOM (RAOM) includes tympanostomy tube insertion, adenoidectomy, and oral administration of broad spectrum antibiotics, accounting for 25-90% of antibiotics prescribed for young children [2, 3]. Similar to other acute upper respiratory tract infections, AOM is a self-limited disease in the majority of cases [4]. However, inadequately treated acute infections are thought to lead to chronic suppurative otitis media, antibiotic resistance, and development of sequelae [5-7]. The economic and social burden of AOM and its sequelae is considerable, particularly in the first five years of life, hence AOM remains the focus of much investigation [8].

It is well known that the bacterial pathogens most commonly associated with AOM are *Streptococcus pneumoniae, Haemophilus influenza,* and *Moraxella catarrhalis* [9, 10]. Current literature also supports the presence of microbial biofilms in AOM which impart microbial resistance to antibiotic treatments and may lead to the recurrence of AOM [11, 12]. Although biofilms are commonly associated with disease, certain biofilms can be beneficial or harmless [13, 14]. Thus, understanding the composition and diversity of the microbial population in normal and diseased tissue is equally important. Elucidating the composition and diversity of a polymicrobial population is now possible with the advent of molecular diagnostic techniques, such as gene pyrosequencing. The use of these techniques has shown far greater microbial diversity than previously shown with conventional microbiology techniques [15-17].

Gene pyrosequencing has been utilized to study the prevalence of bacterial families in nasopharyngeal swab specimens from children with AOM and to compare nasal microbial communities in children with and without otitis media [18, 19]. In the present study, we utilized 16S DNA 454-pyrosequencing to compare the microbial flora, at the species level, in adenoid specimens from

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patients with RAOM against patients with obstructive sleep apnea (OSA). The adenoid tissue is regarded as an important reservoir of pathogenic bacterial biofilms in children with recurrent infections [11, 20, 21]. Adenoidectomy is associated with a decrease in recurrence of otitis media. Therefore, adenoid tissue removed from patients with RAOM is ideal for studying the microbiology pertinent to the pathogenesis of AOM.

MATERIALS and METHODS

Subjects and Tissue Collection

This study was approved by the Institutional Review Board. The patient and/or the parent or guardian consented to participate in this study. Adenoid specimens were obtained from children between 2 and 11 years old who were undergoing routine adenoidectomy for RAOM (n=7) or OSA, without otitis media (n=13). The diagnoses of RAOM and OSA were based on conventional criteria [22, 23]. Subjects were otherwise healthy with no other significant co-morbidities, such as craniofacial disorders or immunologic deficiencies.

Adenoids were obtained using curettage and were immediately transferred into sterile specimen containers. Specimens were promptly transported on ice to the Otolaryngology Laboratory and stored at -80°C until batch analysis.

Adenoids were thawed in 1 molar phosphate buffered saline (PBS) at 4°C and cut with sterile disposable scalpels into cubes, approximately 4 mm^3. Since the presence and abundance of microbes may vary depending on sampling location, the adenoid tissue was carefully sectioned to include the surface, middle and bottom layer. Approximately 25 milligrams of tissue from each section were combined into a 2-mL sterile microcentrifuge tube and homogenized for DNA isolation. Due to cost, a subset of ten adenoid samples (5 OSA and 5 RAOM) were randomly selected and transferred to 2-mL sterile microcentrifuge tubes for microbial analysis by 454 pyrosequencing. This number of specimens was deemed to be the minimum number of samples per group for the 454 pyrosequencing to yield representative results. Real-time PCR analysis was performed on all specimens (7 RAOM and 13 OSA) after liberating microbes from biofilms by sonication.

Microbial Identification by Gene Pyrosequencing

DNA isolation was performed using the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN) following a modified manufacturer's protocol. Each tissue sample was placed in a 2-mL screw cap tube containing 200 µL of binding buffer and 200 uL of tissue lysis buffer. The extraction procedure was modified to include a bead beating step for tissue and cell disruption using 5-mm steel beads (Amazon, Seattle, WA), 0.5 mm Zirconium oxide beads (Next Advance, Averill Park, NY), and a Qiagen Tissuelyser II instrument (Qiagen, Valencia, CA) running at 30Hz for five minutes. The lysate was then run through the glass fiber fleece column following the manufacturer's protocol provided with the Roche High Pure PCR Template Preparation Kit.

Amplicon pyrosequencing (bTEFAP) was performed as previously described [24]. The 16S universal eubacterial primers 28F 5'GAGTTTGATCNTGGCTCAG and 519R 5'GTNTTACNGCGGCKGCTG and a single-step, 35-cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) were used under the following conditions: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s; 54°C for 40 s and 72°C for 1 min; after which a final elongation step at 72°C for 10 min was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using AgencourtAmpure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing Roche 454 FLX titanium instruments and reagents (Branford, CT) and following the manufacturer's guidelines for analysis. Sequence data derived from the sequencing process were processed using a proprietary analysis pipeline (Research and Testing Laboratory, Lubbock, TX). Sequences were first trimmed back to Q25 and then run through a proprietary denoiser based upon USEARCH in order to correct ambiguous base calls and sequencer noise [25]. Sequences were then chimera checked using UCHIME and all chimeras as well as any sequences less than 250 base pairs were removed from the data set [26]. Sequences were then stripped of their barcodes and clustered into de-replicated clusters at 100% subsequence identity (0% divergence) using USEARCH [25]. For each cluster the seed sequence was queried against an internal database of high quality sequences derived from NCBI using BLASTN+. Results were then compiled into their appropriate taxonomic levels.

Determination of *S. salivarius* and Total Bacterial Load by Quantitative PCR

Total DNA from the adenoid specimens (7 RAOM and 13 OSA) were purified using DNEasy Blood and Tissue Kit (Qiagen, Valencia, CA), following the manufacturer's protocol for DNA isolation in Gram-positive bacteria, with modifications. Briefly, lysis buffer (270 µL) with 30 µL proteinase K was added to a microcentrifuge containing approximately 25 mg of adenoid tissue. The samples were incubated at 56 °C until lysis of the tissue was achieved (approximately 2 h). Following incubation, the specimens were sonicated (Branson Ultrasonics, Branson 2510, Danbury, CT) for a total of 7.5 min, with serial 1.5-min sonication exposures separated by a 1-min rest. DNA isolation was then carried out per the manufacturer's recommendation. DNA was also isolated from a laboratory strain of *S. salivarius* (*S. salivarius* 57.1, kindly provided by Dr. Robert Burne, University of Florida) for use as positive control in the real-time PCR runs. After DNA extraction, DNA quantity ($A_{260\,nm}$) and quality ($A_{260\,nm}$ /$A_{280\,nm}$) were determined using a spectrophotometer (Synergy™ HT, BioTek Instruments, Inc., Winooski, VT) with Gen5 software.

Total bacterial load was assessed using a 16S primer set: 16S Forward, 5'-ACTCCTACGGGAGGCAGCAG3' and 16S Reverse, 5'-TTACCGCGG CTGCTGG-3'. The specific assay for *S. salivarius* was performed using the following primer set: *S. salivarius* Forward, 5'-CACGCCATGCTG GAAGTG-3' and *S. salivarius* Reverse, 5'-GCGATGAGCC AAGCTGAAG-3' [27]. Detection of DNA by real-time PCR was carried out with the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). DNA was amplified in triplicate for each specimen and the mean values were used. The PCR reaction was performed in a 20-µL total volume using MicroAmp™ Fast Optical 96-well Reaction Plate, with Fast SBYR Green Master Mix (Applied Biosystems, Foster City, CA), and 200 nM of each of the forward and reverse primers (Integrated DNA Technologies, Coralville, Iowa 52241). The PCR reaction conditions for amplification of DNA were: 95°C for 15 s and 40 cycles of 95°C for 3 s and 58.5°C for 30 s. Dissociation (or melting) curves were generat-

ed immediately after the real-time PCR run to check for non-specific product formation.

Statistical Analysis

Power analysis was conducted using G*Power statistical analysis, and using the criteria that at least 25% difference in a species abundance between RAOM and OSA will be observed (based on a previous similar study), a 20% standard deviation in each group, an α of 0.05 and power of 0.80, we determined that at least 7 samples is needed for each group for the pyrosequencing analysis.

Differences in the relative abundance of all operational taxonomic units (OTUs) among groups were evaluated using distance based redundancy analysis (dbRDA) [28]. For the dbRDA, distances among samples were calculated using the Bray-Curtis dissimilarity measure, which was based on the relative abundances of all OTUs in each sample. An ANOVA-like simulation was then conducted to test for group differences. Differences in individual species were examined using ANOVA. Prior to analysis, relative abundances were transformed using a logit transformation [29].

Data for *S. salivarius* qPCR were normalized with the 16S threshold cycle (C_t) and were compared between the OSA and RAOM groups using t-test (JMP™ Pro 11, SAS Institute Inc., Cary, NC). A $p \leq 0.05$ was considered significant.

RESULTS

Adenoid specimens were obtained from 13 patients with OSA and 7 patients with RAOM. The ages of the subjects ranged from two to 11 years, with a mean age of 5.6 years (Table 1). The age range of

Table 1. Study subjects

Subject	Age	Gender	Indication
1	7	M	OSA
2	11	F	OSA
3	8	F	OSA
4	6	F	OSA
5	3	M	OSA
6	5	M	RAOM
7	5	F	OSA
8	9	M	RAOM
9	3	F	RAOM
10	6	F	OSA
11	2	F	RAOM
12	5	M	OSA
13	5	F	OSA
14	8	F	OSA
15	4	M	RAOM
16	4	M	OSA
17	9	F	OSA
18	3	F	RAOM
19	5	M	RAOM
20	5	F	OSA

M: male; F: female; OSA: obstructive sleep apnea; RAOM: recurrent acute otitis media
Age is in years

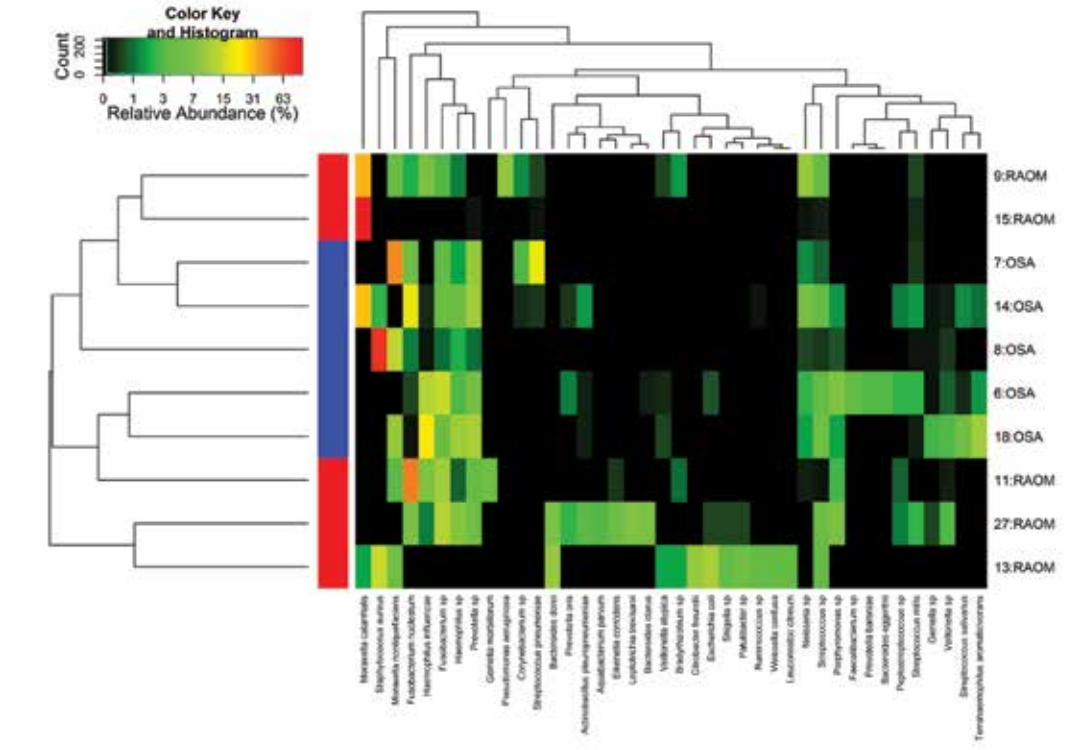

Figure 1. Heat map summarizing the relative abundance (represented by color) of the 40 most dominant species in adenoid specimens from patients with recurrent acute otitis media (RAOM) or obstructive sleep apnea (OSA). Clustering shows that the predominant species associated with RAOM tend to differ from those associated with OSA

the pediatric population used in this study was similar to the ranges in previous microbiological studies of the adenoids [20, 30]. There were more females (n=9) than males (n=4) in the OSA group while males and females was about even in the RAOM group.

All adenoid specimens analyzed with 454 pyrosequencing (n=5 each for OSA and RAOM) had evidence of microbes (Figure 1). Using OTU proportions of ≥1% as cut-off for considering clinically relevant species (J. White, Research and Testing Laboratory, personal communication), there were 42 and 64 different species in the OSA and RAOM groups, respectively. *Haemophilus influenzae, Moraxella catarrhalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Streptococcus pneumoniae* were among the most dominant species in all samples (Figure 1, Table 2) [28]. The levels of the known AOM pathogens such as *Moraxella catarrhalis*, *Pseudomonas aeruginosa,* and *Streptococcus pneumoniae* were higher on the RAOM specimens, though this was not statistically significant (Table 2). The relative abundance of *Fusobacterium nucleautum*, a key pathogen in periodontitis, also tended to be higher in the RAOM group. *S. salivarius, Prevotella sp.* and *Terrahaemophilus aromaticivorans* were more common on adenoids from OSA patients ($p<0.05$; Table 2). In contrast, *Bradyrhizobium sp.* was more common on adenoids from patients with recurrent AOM ($p<0.05$). Other microbes, such as *Fusobacterium sp.*, were also among the most prevalent species in both groups (Table 3).

Table 2. Comparison of the predominant microbes identified by pyrosequencing on adenoid specimens from patients with RAOM or OSA

Species	RAOM (%)	OSA (%)
Moraxella catarrhalis	43.8	28.7
Streptococcus pneumoniae	18.9	0.11
Haemophilus influenzae	5.03	18.02
Haemophilus sp	4.48	4.32
Fusobacterium nucleatum	17.8	8.8
Staphylococcus aureus	14.5	25.4
Escherichia coli	10.9	0
Citrobacter freundii	9.1	0
Pseudomonas aeruginosa	8.79	0
Fusobacterium sp	8.3	5.7
Bacteroides dorei	7.65	0
Leptotrichia trevisanii	7.3	0
Bacteroides clarus	6.1	0
Gamella morbillorum	5.5	0
Patulibacter sp	5.4	0
Neisseria sp	4.7	2.8
Eikenella corrodens	4.5	0
Porphyromonas sp	4.3	3.5
Moraxella nonliquefaciens	4.2	21.1
Actinobacillus pleuropneumoniae	3.7	1.6
Shigella sp	3.6	0
Ruminococcus sp	3.6	0
Weisella confusa	3.6	0
Leuconostoc citreum	3.6	0
Streptococcus sp	3.0	4.0
Prevotella sp	**3.0***	**9.0**
Veillonella sp	2.8	2.8
Aquabacterium parvum	2.8	0
Prevotella oris	2.2	1.1
Veillonella atypica	1.8	0
Bradyrhizobium sp	**1.74***	**0**
Corynebacterium sp	1.4	2.8
Streptococcus mitis	1.3	2
Peptostreptococcus sp	1.12	1.81
Faecalibacterium sp	0	3.7
Prevotella baroniae	0	2.8
Bacteroides eggerthii	0	2.8
Gemella sp	0	2.6
Streptococcus salivarius	**0***	**3.5**
Terrahaemophilus aromaticivorans	**0***	**6**

Bold values with an asterisk represent significant differences ($p<0.05$)
OSA: obstructive sleep apnea; ROAM: recurrent acute otitis media

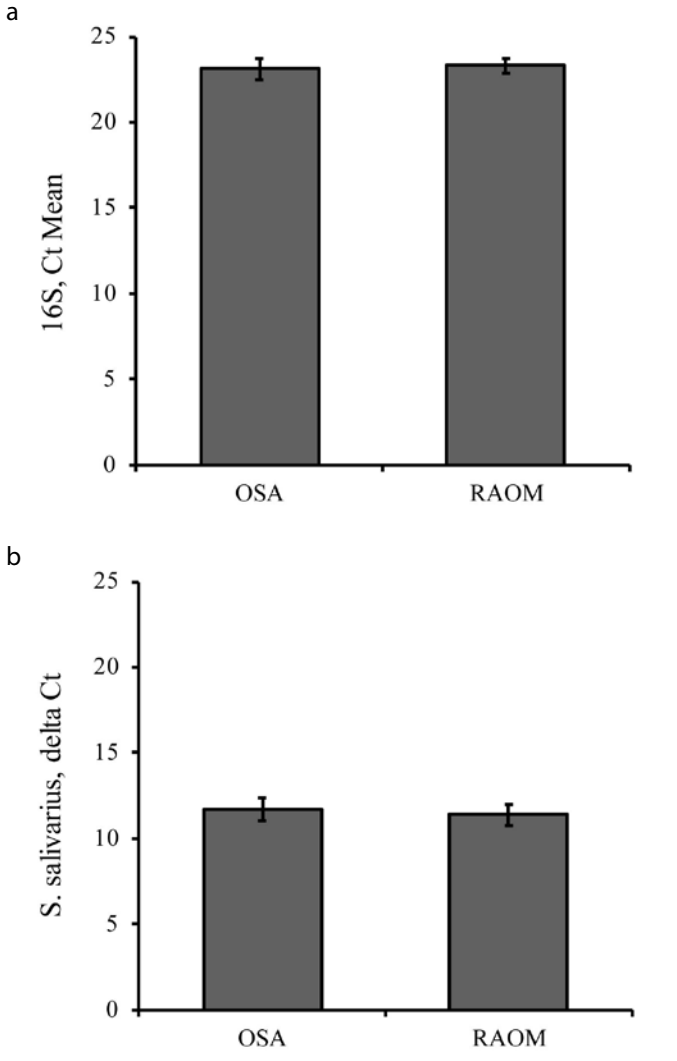

Figure 2. a, b. Real-time PCR detection of total bacterial load (a), and S. salivarius (b) in adenoid specimens from OSA (n=13) and RAOM (n=7) patients. The threshold cycle (Ct) is inversely proportional to the amount of target DNA in the sample
OSA: obstructive sleep apnea; ROAM: recurrent acute otitis media

Table 3. Comparison of the most prevalent bacterial species for each specimen identified by pyrosequencing*

Subject	Indication	Most predominant, %		Second most predominant, %		Third most predominant, %	
1	OSA	*Moraxella nonliquefaciens*	40.1	*Streptococcus pneumoniae*	18.9	*Prevotella sp*	9.7
2	OSA	*Staphylococcus aureus*	71.6	*Moraxella nonliquefaciens*	13.3	*Staphylococcus lugdunensis*	2.3
7	OSA	*Fusobacterium sp*	14.6	*Haemophilus influenzae*	12.8	*Porphyromonas sp*	7.3
14	OSA	*Haemophilus influenzae*	23.3	*Prevotella sp*	10.9	*T. aromaticivorans*	10.4
17	OSA	*Moraxella catarrhalis*	28.7	*Fusobacterium nucleatum*	19.7	*Prevotella sp*	9.9
6	RAOM	*Moraxella catarrhalis*	31.3	*Neisseria sp*	9.3	*Pseudomonas aeruginosa*	8.8
11	RAOM	*Fusobacterium sp*	11.8	*Haemophilus sp*	7.9	*Leptotrichia trevisanii*	7.3
15	RAOM	*Staphylococcus aureus*	14.5	*Escherichia coli*	10.9	*Bacteroides dorei*	9.1
18	RAOM	*Fusobacterium nucleatum*	45.2	*Fusobacterium sp*	10.5	*Haemophilus influenzae*	7.4
19	RAOM	*Moraxella catarrhalis*	98.4	*Streptococcus mitis*	0.4	*Streptococcus sp*	0.2

*Relative abundance of bacterial species are shown in percentage (%)
OSA: obstructive sleep apnea; ROAM: recurrent acute otitis media

Using qPCR and primers specific for 16S rRNA (total bacteria) and *S. salivarius*, we found that the threshold cycle (C_t; the number of cycles required for the fluorescent signal to cross a threshold; i.e., exceeds background level, and is inversely proportional to the amount of target DNA in the sample) of 16S was not different between the OSA and RAOM group (Figure 2a). Similarly, the 16S-normalized C_t (delta C_t) for *S. salivarius* was not different between the two groups (Figure 2b). Melting curve analysis showed no non-specific product formation (data not shown).

DISCUSSION

New sequencing technologies, such as 16S rDNA pyrosequencing, are now increasingly used as an alternative to the standard culture technique for microbial identification. These techniques provide a sensitive means of identifying diverse bacteria in various specimens [15-19, 31-33]. In our effort to gain a better understanding of what could be different in the microbial composition between a healthy and disease state, we have also begun using 454 pyrosequencing to assess microbial flora [32]. Although it is widely recognized that *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* are the commonly isolated pathogens in AOM, a complete representation of the various organisms involved in AOM and their clinical significance is not definitive at this time. Using bacterial culture technique, Subtil and colleagues have also shown that *Haemophilus influenzae, Staphylococcus aureus*, and *Streptococcus pneumoniae* were the most representative species in adenoids from children with infectious or non-infectious indications [30]. An earlier study by Hilty and colleagues, which also utilized 16S rRNA pyrosequencing, identified 58 bacterial families with *Moraxellaceae, Streptococcaceae, and Pasteurellaceae* as the most frequent families in nasal swab specimens from patients with AOM. As differences at the species level may be clinically significant, the goal of the present study was to compare the microbiome of adenoids, at the species level, from patients with RAOM or OSA [18].

Not surprisingly, we found evidence of microbial DNA on all adenoids. The relative abundance of microbial DNA for the common pathogens associated with AOM such as *S. pneumoniae* and *M. catarrhalis* tend to be higher in the RAOM group, though the differences were not statistically significant. On the other hand, *Haemophilus influenzae* and *Moraxella nonliquefaciens* tend to be more prevalent in the OSA group. The assembly of these bacteria and other microbes into biofilms has also been implicated for the recalcitrance of AOM [12]. Biofilms are microbial communities where bacterial interactions (e.g., symbiotic, antagonistic) influence the composition and stability of biofilms. Microbial DNA for *P. aeruginosa*, a robust biofilm former and a pathogen associated with chronic suppurative otitis media, was detected on RAOM adenoids but not in OSA adenoids [34]. The presence of chronic pathogens such as *P. aeruginosa* in the middle ear has long been linked to the clinical disease [34]. Additionally, our observation that adenoids from patients with RAOM have higher prevalence of *S. pneumoniae* in conjunction with the presence of *P. aeruginosa* may lend support to earlier findings that the risk of *P. aeruginosa* otitis media is increased by prior acute otitis media due to *S. pneumoniae* in the chinchilla model [35].

One factor that can contribute to the transition from a healthy to a disease state is the disequilibrium of the indigenous microflora or commensal bacteria. Commensal bacteria are considered beneficial to the host by defending against the colonization of invading pathogens. For example, Abreu et al. [33] suggested a protective role of *Lactobacilli* in chronic rhinosinusitis. Nasal swab specimens from patients with AOM also have less frequent commensal families [18]. In the present study, the relative abundance of *Streptococcus salivarius* was significantly higher in the OSA compared to the RAOM group. *S. salivarius* is a predominant commensal bacterium of the oral cavity and also used as probiotics to control diverse bacterial infections including otitis media and dental caries [36]. However, using slightly more specimens, *S. salivarius* levels did not differ between adenoid specimens from OSA (n=13) and RAOM (n=7) when qPCR was used to detect and quantify these bacteria. As we noted previously, even sensitive molecular techniques, such as gene pyrosequencing, are not completely foolproof, possibly failing to detect microbes that are shown to be present through other techniques [32]. Furthermore, our results show only that the relative quantities of these commensals are not significantly different. Tano and colleagues have shown that the alpha-hemolytic streptococci from children with RAOM are less effective at inhibiting middle ear pathogens than those from children without otitis media [37]. More

detailed genotypic and phenotypic assessment of these commensals may be necessary to explain differences between otitis-prone and non-otitis prone subjects.

The relative abundance of *Fusobacterium nucleautum* microbial DNA was also higher in the RAOM group, though the difference was not statistically significant. *F. nucleautum* is a Gram-negative anaerobe that is commonly found in periodontal plaque and a key pathogen in the development of periodontitis as well as other human infectious diseases [38]. Whether this potentially opportunistic pathogen contributes to the pathogenesis of AOM remains to be determined. The other microbes that were found to be different between the RAOM and OSA groups include *Prevotella sp.* and *Terrahaemophilus aromaticivorans,* which were more common on adenoids from OSA patients, and *Bradyrhizobium sp.*, which was more common on adenoids from RAOM patients. We are not aware of any role these organisms may play in preventing RAOM.

There are some limitations of the present study. These include the relatively small number of specimens used in the 454 pyrosequencing and qPCR analysis, the lack of bacteria-host response evaluation, as well as evaluation of viral pathogens. Therefore, future microbiome analysis should use a larger number of specimens. Additionally, the interaction between bacteria and viral pathogens should be evaluated. The host response should be evaluated as the interactions between bacteria or viruses and the host is an important factor that contributes to the balance of the microflora.

CONCLUSION

Our findings indicate that diverse communities of bacteria are present in adenoids from both OSA and RAOM patients. Although the relative abundance of certain bacteria differs between the two groups, the clinical significance of these differences remains to be determined. Further studies are warranted.

Ethics Committee Approval: Ethics committee approval was received for this study from the Institutional Review Board of University of Florida (Approval Date: 29.10.2012/Approval No: IRB # 201200082).

Informed Consent: Written informed consent was obtained from parents of the patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - C.O.D., P.J.A.; Design - C.O.D., P.J.A.; Supervision - C.O.D.; Resource - C.O.D., R.C.S., W.O.C., P.J.A; Materials - C.O.D., R.C.S., W.O.C., P.J.A; Data Collection and/or Processing - C.O.D., R.C.S., W.O.C., P.J.A; Analysis and/or Interpretation - C.O.D., P.J.A.; Literature Search - C.O.D., P.J.A.; Writing - C.O.D., P.J.A.; Critical Reviews - C.O.D., R.C.S., W.O.C., P.J.A.

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