The Laryngoscope

The Microbiome of Otitis Media With Effusion

Chun Ling Chan, MD; David Wabnitz, MD, MS, FRACS; Jake Jervis Bardy, MD; Ahmed Bassiouni, MBBCh, PhD; Peter-John Wormald, MD, FRACS; Sarah Vreugde, MD, PhD; Alkis James Psaltis, MD (Hons), PhD, FRACS

Objectives/Hypothesis: The adenoid pad has been considered a reservoir for bacteria in the pathogenesis of otitis media with effusion. This study aimed to characterize the middle ear microbiota in children with otitis media with effusion and establish whether a correlation exists between the middle ear and adenoid microbiota.

Study Design: Prospective, controlled study.

Methods: Middle ear aspirates adenoid pad swabs were collected from 23 children undergoing ventilation tube insertion. Adenoid swabs from patients without ear disease were controls. Samples were analyzed using 16S rRNA sequencing on the Illumina MiSeq platform.

Results: Thirty-five middle ear samples were collected. The middle ear effusion microbiota was dominated by *Alloiococcus otitidis* (23% mean relative abundance), *Haemophilus* (22%), *Moraxella* (5%), and *Streptococcus* (5%). *Alloiococcus* shared an inverse correlation with *Haemophilus* ($P = .049$) and was found in greater relative abundance in unilateral effusion ($P = .004$). The microbiota of bilateral effusions from the same patient were similar ($P < .001$). However, the otitis media with effusion microbiota were found to be dissimilar to that of the adenoid ($P = .01$), whereas the adenoid microbiota of otitis media with effusion and control patients were similar ($P > .05$) (permutational multivariate analysis of the variance).

Conclusions: Dissimilarities between the local microbiota of the adenoid and the middle ear question the theory that the adenoid pad is a significant reservoir to the middle ear in children with otitis media with effusion. *A otitidis* had the greatest cumulative relative abundance, particularly in unilateral effusions, and shares an inverse correlation with the relative abundance of *Haemophilus*.

Key Words: Microbiome, microbiota, otitis media, effusion, middle ear, *Alloiococcus otitidis*, *Haemophilus influenzae*, pediatric.

Level of Evidence: NA

***Laryngoscope*, 126:2844–2851, 2016**

INTRODUCTION

Otitis media with effusion (OME) is the most common cause of hearing loss in childhood.[1] It has been estimated that the point prevalence of OME at age 2 years is one in five children, with four in every five children affected at least temporarily by the age of 10 years.[1]

The pathogenesis of OME remains unclear, although the eustachian tube (ET) is thought to play a central role.[1] It has been postulated that the ET's short length, horizontal position, and reduced rigidity in the pediatric population may permit the reflux of naso- and oropharyngeal microbes into the middle ear cavity, explaining the higher incidence of OME in children compared to adults.[1]

Traditional culture-based techniques have isolated *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis* as the most common pathogens in OME. With the advancement of culture-independent techniques such as 16S rRNA pyrosequencing, diverse and previously unknown bacterial communities have now been demonstrated within the middle ear.[2,3] One such emerging potential pathogen is *Alloiococcus otitidis* (AO), which has been isolated from middle ear aspirates with increasing frequency in the last decade.[4–7]

The aim of this study was to use culture-independent techniques to compare the local microbial population of the adenoids in patients with and without OME and test the hypothesis that this region acts as a bacterial reservoir, predisposing to OME.

MATERIALS AND METHODS

Ethics approval was obtained from the institutional research boards of the Women and Children's Hospital and the University of Adelaide.

From the Department of Otolaryngology–Head & Neck Surgery (C.L.C., J.J.B., A.B., P.-J.W., S.V., A.J.P.), Adelaide University, Adelaide, South Australia, Australia; Department of Otolaryngology–Head & Neck Surgery (D.W.), The Women's and Children's Hospital, Adelaide, South Australia, Australia

Editor's Note: This Manuscript was accepted for publication May 12, 2016.

This work was supported by an Adelaide University Post-Graduate Scholarship to Chun Ling Chan, MD. Alkis James Psaltis, MD, is a consultant to ENT Technologies and Aerin Technologies, and is on the speakers bureau for Smith and Nephew. Peter-John Wormald, MD, is a consultant to ENT Technologies, receives royalties from the sale of surgical equipment from Medtronic, and is supported by a bequest donation to The University of Adelaide from the estate of the late Yvonne Carna Laura Ives Thomas.

The authors have no other funding, financial relationships, or conflicts of interest to disclose.

Send correspondence to Alkis Psaltis, MD, Department of Otolaryngology–Head & Neck Surgery, The Queen Elizabeth Hospital, Woodville South, SA 5011, Australia. E-mail: alkis.psaltis@adelaide.edu.au

DOI: 10.1002/lary.26128

Selection Criteria

Children wait-listed for ventilation tube (grommet) insertion with adenoidectomy for chronic OME (>3 months) were enrolled. Control patients were approached if they had no history of otitis media in the last 12 months and were undergoing other forms of surgery requiring general anesthetic and endotracheal intubation that was anatomically distant from the nasopharynx. Written parent/guardian consent was obtained prior to enrolment. Exclusion criteria included previous adenoidectomy, acute otitis media, resolved effusions, upper respiratory tract infection at time of surgery, and antibiotic or steroid use in the 4 weeks preceding surgery. Demographic data including age, gender, and parental smoking status as well as clinical data including presenting symptoms, previous tympanometry, medication history, and previous medical and surgical history were collected.

Sample Collection

Samples were collected while the patient was under general anesthetic, prior to commencement of the planned procedure. All specimens were placed on ice immediately after collection and transferred for storage in a −80° freezer.

Middle ear effusion collection. Following aural toilet of the external canal, all instruments were replaced with new sterile equipment. Myringotomy was performed and the effusion aspirated with microsuction attached to an Argyle specimen trap (Covidien, Mansfield, MA). The circuit was then flushed with 2 μL of sterile normal saline. Caution was exerted to ensure that no instrument touched the canal wall skin. If this occurred the instruments were exchanged for sterile ones.

Adenoid swab. To avoid contamination, all swabs were taken transorally under direct vision. A sterile flocked swab (Copan Italia S.p.A., Brescia, Italy) was guided transorally, avoiding contact with the oropharyngeal mucosa. The swab was then brushed over the surface of the adenoid pad five times and transferred immediately into a cryotube.

DNA Extraction

DNA extraction was carried out using PowerLyzer PowerSoil DNA Isolation Kit (MoBio Laboratories, Salona Beach, CA). Swab heads and tissue were thawed on ice and placed directly onto the beads for homogenization. Total DNA was extracted from all clinical samples and two DNA extraction negative controls containing extraction reagents only. The remainder of the extraction protocol was performed as per the manufacturer's protocol. Extracted DNA was stored at −80°C until sequencing.

Polymerase Chain Reaction Amplification of the 16S rRNA Gene and Sequencing

Polymerase chain reaction (PCR) amplification and sequencing was performed by the Australian Genome Research Facility. Libraries were generated by amplifying the V3–V4 (341F – 806R) hypervariable region of the 16S rRNA gene. PCR amplicons were generated using the primers CCTAYGGGRBGCASCAG in the forward sequence and GGACTACNNGGGTATCTAAT in the reverse sequence, using AmpliTaq Gold 360 mastermix (Life Technologies, Mulgrave, Australia) following local protocol. The resulting amplicons were measured by fluorometry (Invitrogen Picogreen; Thermo Fisher Scientific, Waltham, MA) and normalized. The equimolar pool was then quantified by quantitative PCR (qPCR) (KAPA biosystems, South Africa) and set up for sequencing on the Illumina MiSeq (Illumina Inc., San Diego, CA) with paired end chemistry. Reads from Illumina sequencing were used as raw data for bioinformatic analyses.

Bioinformatics Pipeline

The Paired-End reAd mergeR (PEAR)[8] version 0.9.5 was used to pair forward and reverse reads in each sample, and quality filter the paired reads. Open-reference operational taxonomic unit (OTU) picking strategy with prefilter threshold of 0.80 was used to cluster OTUs.[9] Within the open-reference method, UCLUST[10] version 1.2.22 was used to cluster OTUs at 97% similarity. SILVA (release 111)[11] was used for the reference-based step of the OTU picking strategy and for taxonomic assignment at 95% similarity.

Biostatistical Analysis

Prior to downstream analysis, artefactual OTUs arising from possible reagent contamination were identified by analyzing the OTU distribution relative to amplicon concentration obtained after library preparation of each sample. OTUs with cumulative relative abundance ranked in the top 10 of all OTUs in the dataset were filtered from the data if the relative abundance distribution was inversely correlated with amplicon concentration.[12]

Pursuant to rarefaction, all samples were subsampled to 2,000 reads. Diversity estimates were performed on subsampled data. Simpson's index of diversity was used to estimate diversity. The Simpson's index of diversity takes into account the number of species present, and their relative abundance, providing a value between 0 and 1, with higher numbers representing a more diverse bacterial community. A Mann-Whitney *U* test was used to determine significance of variation in diversity across sample types. GraphPad version 6 (GraphPad Software Inc., La Jolla, CA) was used to generate box plots and calculate the statistical tests used.

Mean relative abundance of top genera were calculated for the middle ear fluid (MEF), adenoid, and control groups. The mean of top genera in the MEF, adenoid, and control groups were compared using a Wilcoxon signed ranks test, two-sample Student *t* test, and independent-sample *t* test as applicable. Bray-Curtis (BC) similarity matrices were generated, providing a value from 0 to 1, estimating the similarity between samples (with higher numbers representing greater similarity). The BC matrix was used to create a hierarchical group-average cluster analysis in PRIMER version 6 (PRIMER-E Ltd., Plymouth, United Kingdom).[13] The BC matrix was also used to summarize the distance between MEF samples from the same patient (within patient), and the MEF samples from all patients (between patient) in QIIME (Quantitative Insights Into Microbial Ecology; http://qiime.org/index.html). A two-sided Student two-sample *t* test was used to test significance between the within-patient and between-patient summaries. A permutational multivariate analysis of the variance (PERMANOVA) test was also performed in PRIMER to test whether there was a statistically significant difference between the bacterial communities in the MEF and the adenoids samples (as a fixed factor), with samples from the same patients grouped in the analysis (as a random factor). Microsoft Excel version 14.4.5 (Microsoft Corp., Redmond, WA) was used to generate bar plots of community composition. Cytoscape version 2.8.2[14] was used to create a co-occurrence model.

Species-level presumptive identification was performed on OTUs classified as *Alloiococcus*, using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information database.[15] Taxonomic assignment for all

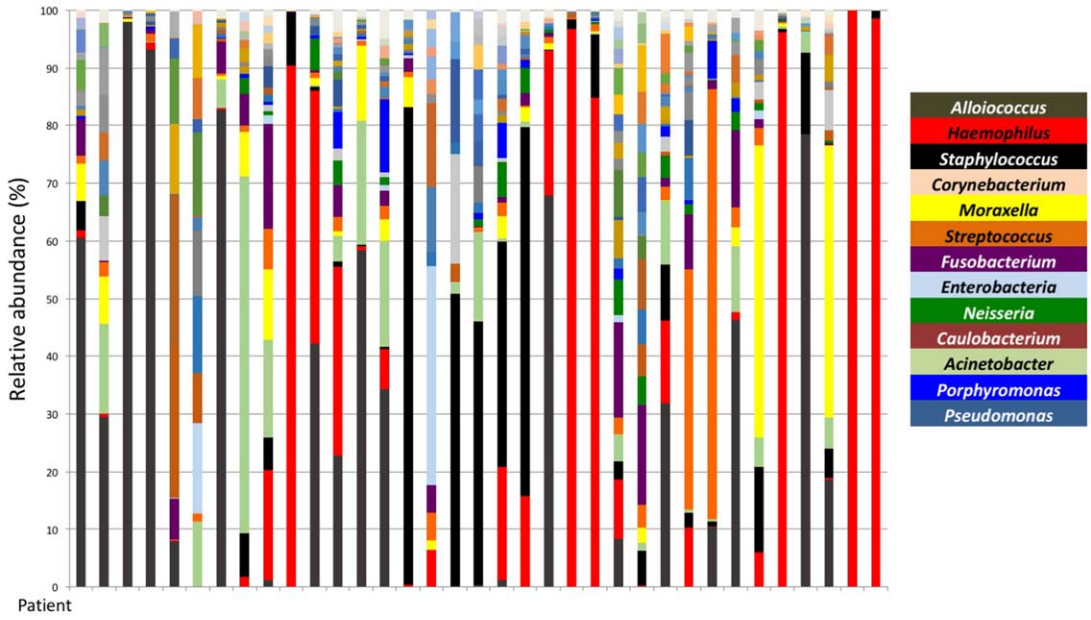

Fig. 1. Microbiome of middle ear fluid represented in relative abundance. Data are presented for genera that were present at ≥1% relative abundance in at least one sample. The 13 most abundant genera are identified (see key).

other OTUs remained at genus or family (if genera not possible) level during taxonomic assignment.

RESULTS

Demographic Data

Twenty-three patients (14 male) with OME, aged 1 to 8 years (mean = 40 months, standard deviation [SD] = 21 months), and 10 control patients aged 1 to 12 years (mean = 73 months, SD = 56 months), were recruited. Thirty-five middle ear effusion samples were collected, with 12 (60%) OME patients presenting with bilateral middle ear effusions. The control group included seven patients undergoing laryngobronchoscopy for investigation of laryngomalacia and/or aspiration, with the remaining three undergoing general anesthetic thyroidectomy, preauricular sinus repair, and airway foreign body retrieval, respectively. There was no significant difference between the groups with univariate analysis of age, sex, and exposure to a member of the same household smoking.

Middle Ear Microbiome

The bacterial composition of MEF was predominated by aerobic bacteria, but relative abundances were highly variable between patients (Fig. 1). The genera with the highest cumulative relative abundance was *Alloiococcus* (presumptive identification of OTUs classified to genus level as *Alloiococcus* using BLAST, confirmed >99% similarity to AO), with otogenic genera (*Moraxella*, *Haemophilus,* and *Streptococcus*) also well represented (Table I). In addition, seven MEF samples demonstrated a dominant genus (relative abundance >90%, *Alloiococcus,* 2/7 and *Haemophilus,* 5/7).

Inverse correlation was demonstrated between relative abundances of *Alloiococcus* and *Haemophilus* (P = .049). In addition, subgroup analysis demonstrated that *Alloiococcus* was more abundant in unilateral effusions than in bilateral effusions (P = .004, Mann-Whitney U test).

TABLE I.
Prevalence and Relative Abundance of Bacterial Genera From Adenoid Swabs and MEF of Patients With Otitis Media With Effusion.

	Prevalence		Relative Abundance		
Bacterial Genera	MEF (%)	Adenoids (%)	MEF (%)	Adenoids (%)	*P* Value
Aerobes					
Alloiococcus	69	78	23	<1	<.001
Haemophilus	100	100	22	25	.271
Staphylococcus	66	87	11	<1	.004
Corynebacteria	66	70	6	<1	.008
Streptococcus	97	96	5	13	.028
Moraxella	89	96	5	14	.271
Neisseria	80	100	1	7	.001
Pseudomonas	68	Not found	1	Not found	.017
Anaerobes					
Fusobacteria	100	100	4	11	.002
Porphyromonas	74	100	1	5	.031
Prevotella	69	100	<1	3	.005
Enterobacteria	66	Not found	2	Not found	.149
Gemella	63	100	<1	3	<.001
Paraprevotella	46	100	<1	2	.001
Veillonella	43	100	<1	1	.02
Caulobacteria	29	Not found	1	Not found	.096

MEF = middle ear fluid.

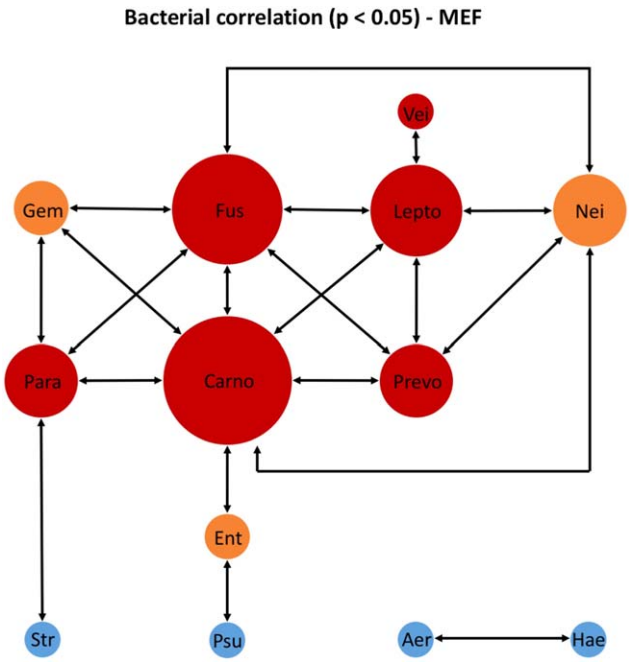

Fig. 2. Graphic representation of the correlation between bacterial genera (family if unspecified) within the middle ear fluid (MEF) ($P < .05$, two-sample t test). Red: anaerobic bacteria, orange: facultative anaerobic bacteria, blue: aerobic bacteria. The size of the circle is relative to the number of correlations with other bacteria. Aer = Aerococcus; Carno = *Carnobacterium;* Ent = *Enterobacteriaceae* (family); Fuso = *Fusobacteria*; Gem = *Gemellaceae* (family); Hae = *Haemophilus*; Lepto = *Leptotrichiaceae* (family); Nei = *Neisseria*; Para = *Paraprevotella*; Prevo = *Prevotella*; Psu = *Pseudomonas*; Str = *Streptococcus*; Vei = *Veillonella*.

TABLE II.
Correlations Between Bacterial Genera (Family if Unspecified) Found in Middle Ear Fluid.

Correlating Bacteria		*P* Value
Fusobacterium	*Neisseria*	.012
	Gemellaceae (family)	.02
	Prevotella	.002
	Leptotrichiaceae (family)	.001
	Paraprevotella	.021
	Carnobacterium	.007
Carnobacterium	*Neisseria*	.031
	Gemellaceae (family)	.013
	Prevotella	<.001
	Leptotrichiaceae (family)	<.001
	Paraprevotella	<.001
Leptotrichiaceae (family)	*Veillonella*	.001
	Prevotella	<.001
	Neisseria	.004
Paraprevotella	*Streptococcus*	.042
	Gemella	<.001
Alloiococcus	*Haemophilus*	.049
Pseudomonas	*Enterobacteriaceae* (family)	.023
Neisseria	*Prevotella*	.002

All *P* values <.05.

Correlation was observed between the relative abundance of different bacteria within the middle ear microbiome (Fig. 2, Table II). In summary, there was a core of anaerobic and facultative anaerobic bacterial genera that were consistently abundant across all MEF samples.

Bilateral effusions were similar within the same patient (two-tailed Student t tests, $P < .05$) (Figs. 3 and 4), as well as between the effusions of different patients with bilateral OME (PERMANOVA analysis, $P < .001$). Specifically, there were strong correlations in relative abundance between with common otopathogenic genera: *Haemophilus* ($P = .007$), *Streptococcus* ($P < .001$), *Staphylococcus* ($P = .025$), and *Corynebacteria* ($P = .006$) using paired t tests.

Adenoid Microbiome Versus Middle Ear Microbiome

The microbiome of the middle ear cavity and the adenoid were dissimilar (Fig. 5, Table I). Alpha diversity between adenoid swabs from patients with OME and from control adenoid swabs were similar. Thirteen of the 17 most abundant bacterial genera demonstrated a significant difference in relative abundance between the different sites, with three bacterial families (*Pseudomonas*, *Enterobacteria,* and *Caulobacteria*) only demonstrated in MEF (Table I). This was confirmed with PERMANOVA multivariate analysis (controlled for paired design) testing β diversity ($P = .001$) and was modeled with co-occurrence (Fig. 6).

Adenoid tissue showed an increase in the abundance of anaerobes, accounting for five out of the 10 most abundant genera found in the adenoid, representing almost half of the total abundance of genera from adenoid samples (48% vs. 6%, $P < .001$).

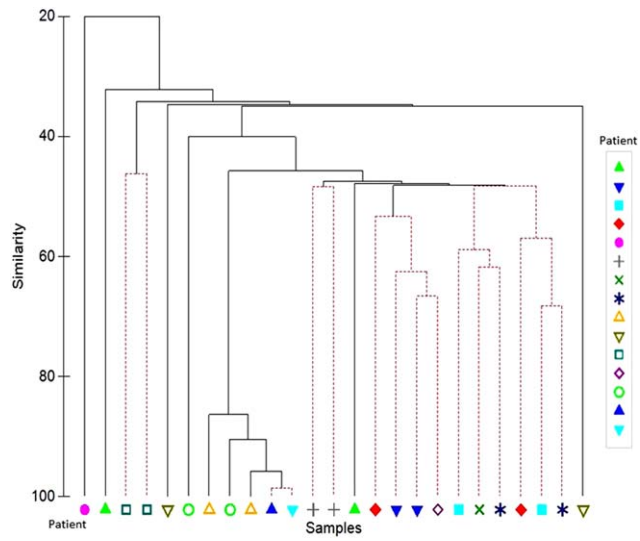

Fig. 3. Cluster analysis of the similarities between middle ear fluid samples. Hierarchical group average cluster analysis based upon Bray-Curtis similarity of genus level data comparing left and right ear effusions from the same patient. Percent similarity is indicated on the y-axis.

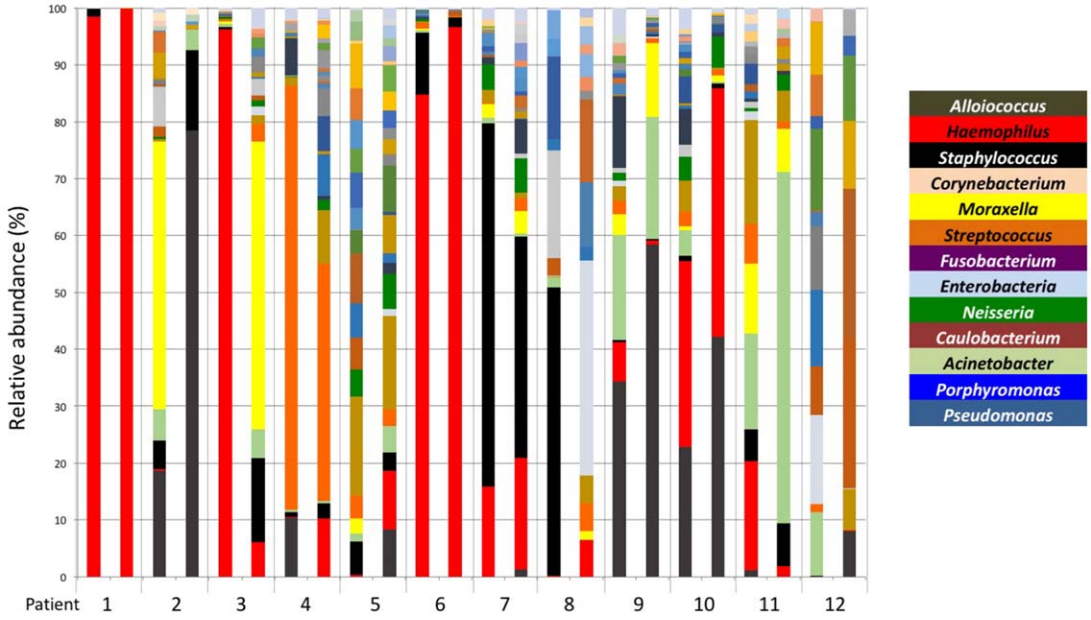

Fig. 4. Microbiome of bilateral middle ear fluid, represented in relative abundance. Data are presented for genera that were present at ≥1% relative abundance in at least one sample. The 13 most abundant genera are identified (see key).

MEF Adenoid Microbiome Versus Control

Adenoid samples from OME patients and control patients were similar in regard to diversity (Mann-Whitney U test, $P = .1$) and relative abundance (independent-sample t test, $P > .05$ throughout) (Table III). PERMANOVA again confirmed this, with similarity demonstrated ($P = .4$). Notably, there was a greater (nonsignificant) abundance of genera associated with middle ear disease (*Haemophilus, Streptococcus, and Moraxella*) observed in the OME adenoids. In addition, the same anaerobic and facultative anaerobic bacteria that were highly correlated in abundance in MEF were found to have similar relationships on the adenoid (see Supporting Figure 1 in the online version of this article).

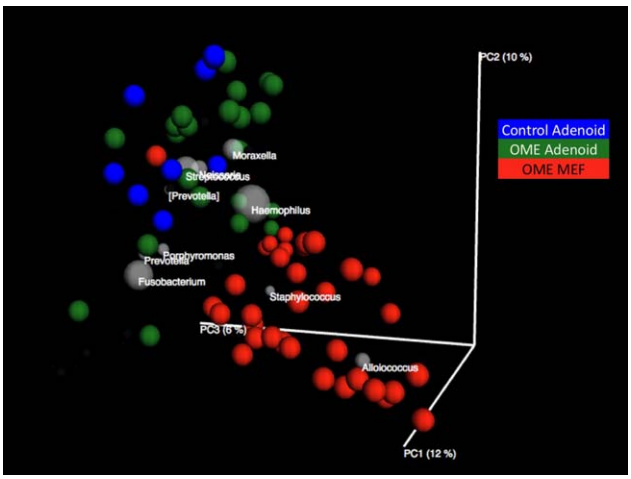

Fig. 5. Principal coordinates analysis biplot based on Bray-Curtis similarity of genus level data. Colored spheres indicate individual samples. The top 10 genera are displayed in grey spheres with volume indicating relative abundance (larger volumes indicate increased relative abundance). MEF = middle ear fluid; OME = otitis media with effusion. PC 1, 2 and 3 indicate percent of Bray–Curtis similarity explained by the PC axis.

DISCUSSION

This study is the largest published study to date utilizing culture-independent, 16S rRNA sequencing techniques to characterize the middle ear and adenoid microbiome in patients with OME. It provides further evidence that OME is not a sterile condition and importantly casts doubt on the commonly held belief that the adenoid pad serves as a bacterial reservoir for OME.

We found that the microbiome of the middle ear consisted of six main genera of bacteria: (in descending order) *Alloiococcus*, *Haemophilus*, *Staphylococcus, Corynebacteria*, *Streptococcus,* and *Moraxella*. These findings are consistent with previously published works using both traditional culture, culture-independent PCR,[4,5,16] and a 16S pyrosequencing study by Jervis-Bardy and colleagues.[3]

Analysis of our data shows distinctly different microbiota composition between middle ear effusions and the adenoid pad, with 13 of the 17 most abundant genera showing a statistically significant difference in relative abundance. The most striking of which was the difference in relative abundance demonstrated for *Alloiococcus*, with <1% in the adenoid sample versus 23% (most abundant) in the MEF. AO is commonly considered a commensal of the external canal[6,16,17] and has been reported as the most prevalent bacterial species in patients with nonpurulent OME (20%–40%).[4,5,16] Our findings are consistent with the literature, with AO having a prevalence of 69%, and representing the greatest

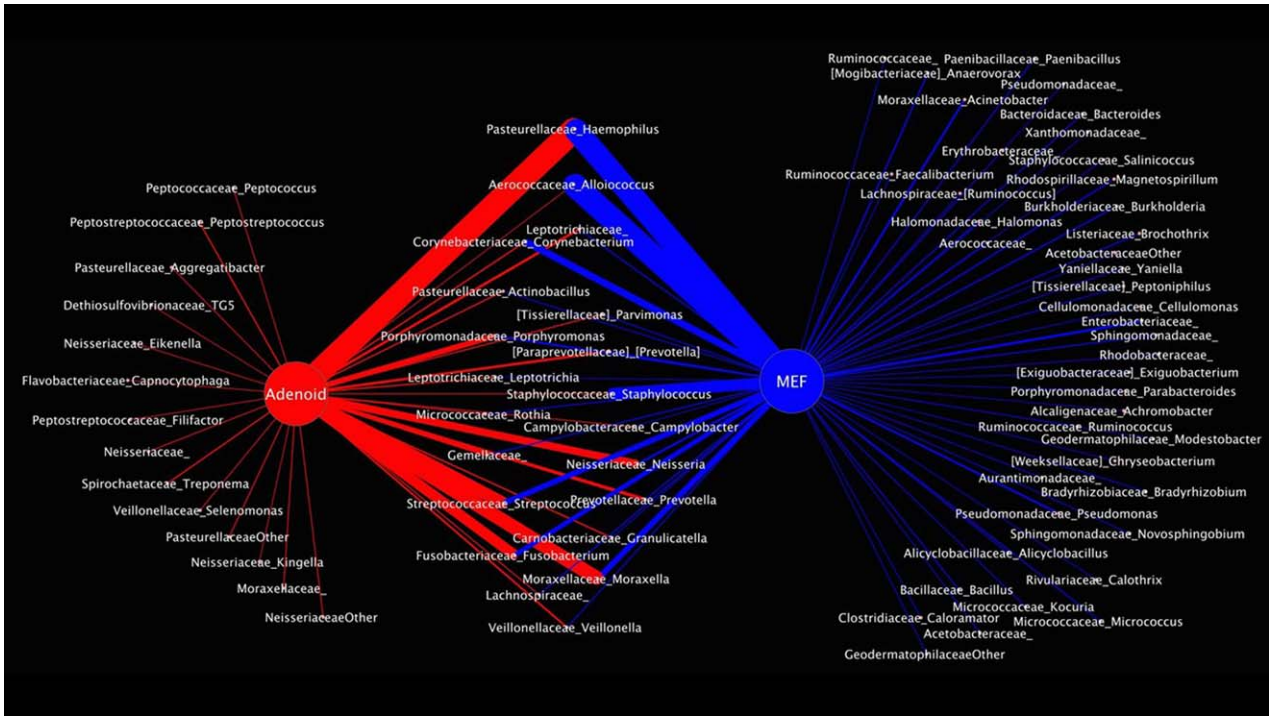

Fig. 6. Co-occurrence model showing average proportional relative abundance of genus level data by sample type. Genera with ≥1% relative abundance in at least one sample are included. MEF = middle ear fluid.

TABLE III.
Prevalence and Relative Abundance of Bacterial Genera From Adenoid Swabs of Control Patients and Patients With OME.

	Prevalence		Mean Relative Abundance		
Bacterial Genera	OME Adenoids (%)	Control Adenoids (%)	OME Adenoids (%)	Control Adenoids (%)	*P* Value
Aerobes					
Alloiococcus	78	<1	<1	Not detected	.38
Haemophilus	100	100	25	9	.13
Staphylococcus	87	100	<1	1	.13
Corynebacteria	70	100	<1	<1	.90
Streptococcus	96	100	13	19	.23
Moraxella	96	100	14	9	.51
Neisseria	100	100	7	16	.13
Pseudomonas	Not detected	Not detected	Not detected	Not detected	N/A
Anaerobes					
Fusobacteria	100	100	11	4	.22
Porphyromonas	100	100	5	3	.61
Prevotella	100	100	3	6	.13
Gemella	100	100	3	4	.44
Paraprevotella	100	100	2	3	.49
Veillonella	100	100	1	3	.15
Leptotrichia	96	100	2	1	.58

N/A = not applicable; OME = otitis media with effusion.

mean relative abundance across our cohort, particularly in patients with unilateral effusions.

In addition, *Alloiococcus*, along with the *Haemophilus* species, were the only two bacterial genera shown to be the dominant bacteria (>90% relative abundance) within the MEF, with an inverse correlation observed between the two. Although this is the first study to report possible bacterial interference between AO and

Haemophilus, numerous studies have shown *H. influenzae* to have bacterial interference with *Staphylococcus aureus* and *S. pneumoniae* within the respiratory tract.[18–20]

Interestingly, the microbiota of bilateral effusions within patients were similar, despite a significant difference with that of the adenoid tissue of the same patient. This suggests that the microenvironment of the middle ear itself may play a greater role in influencing the constitution of the microbial communities within them, rather than bacteria from the nasopharynx. This finding is supported by the wider microbiome literature, which reports distinctly different microbiomes within specific body sites,[21,22] and that the composition at each site remains relatively stable over time.[21–23] These findings imply that a core microbiota exist within body niches and that they are somewhat resistant to external factors. Although our study design precluded longitudinal analysis (only five patients have required repeat tympanostomy tube insertion to date), we believe that this is likely true of the middle ear as well.

In our patients, we found that the core bacteriology of OME involves complex polymicrobial communities rather than merely the presence of dominant bacteria. Only a minority of MEF samples displayed a dominant species, and even in these cases there were traces of other bacteria. Moreover, we observed a correlation in relative abundance between multiple genera, particularly anaerobic and facultative anaerobic bacteria. Interestingly, a similar correlation was also observed in the adenoid tissue. One possible explanation for this is the high prevalence of bacterial biofilms demonstrated in both middle ear effusions and adenoid tissue.[24] These three-dimensional structures are known to create microenvironments of low oxygen tension which may favor anaerobic growth.[25]

Based on the findings of this study, we propose a microbiome model of the pathogenesis of otitis media. We postulate that the microbiome of the healthy middle ear is likely distinctly different to that in the setting of OME and also to that of acute otitis media. We believe that adenoidal hypertrophy and eustachian tube dysfunction predispose to OME, resulting in the formation of a stable OME micriobiome (and likely biofilm), similar to that described in this article. However, subsequent changes in the local environment within the middle ear, whether via direct inflammation (for example, secondary to viral infection[26]) or host immunomodulation, creates a disequilibrium of the local microbiota, thus resulting in the emergence of a dominant species and potentially acute disease. For example, when the microenvironment favors the classic otopathogens (*H. influenzae, M. Catarrhalis, S. pneumoniae*) acute otitis media may be favored, yet when a less virulent bacteria, such as AO is dominant, then perhaps serous effusion persists. This is reflected in the fact that effusions with a high abundance of *Haemophilus* were noted as being more viscous and consistent with glue ear than effusions with high abundance of *Alloiococcus*.

Finally, the similarity of the microbiota of the MEF and external ear canal, in particular the high prevalence of *Alloiococcus, Staphylococcus,* and *Corynebacterium*, is reported in the literature.[17] This does raise the question of whether the external ear itself may act as a reservoir for the middle ear. Although bacterial translocation through an intact healthy tympanic membrane would seem unlikely, contamination through macro- or microperforations cannot be excluded. Furthermore, changes in the barrier function and permeability of the tympanic membrane during an acute infection remain unknown and therefore are the current subjects of an in vitro study in our department. The possibility of contamination during sampling may also explain our finding, although the methodology employed for this study aimed to minimize the likelihood of this.

CONCLUSION

The microbiome of the middle ear in OME is diverse with both aerobic and anaerobic bacteria represented. AO is found in high abundance and may play a major role in the pathogenesis of OME, especially unilateral effusions, and displays an antagonistic relationship with *Haemophilus*. Furthermore, dissimilarities between the local microbiota of the adenoid and the middle ear were demonstrated, suggesting that the microenvironment of the middle ear plays a greater role in the composition of the microbiota than the potential bacterial seeding from the adenoid pad to the middle ear in children with OME.

Acknowledgments

The authors thank the operating room and administration staff and at the Women's and Children's hospital for their support of this research.

BIBLIOGRAPHY

1. Minovi A, Dazert S. Diseases of the middle ear in childhood. *GMS Curr Top Otorhinolaryngol Head Neck Surg* 2014;13:Doc11.
2. Liu CM, Cosetti MK, Aziz M, et al. The otologic microbiome: a study of the bacterial microbiota in a pediatric patient with chronic serous otitis media using 16SrRNA gene-based pyrosequencing. *Arch Otolaryngol Head Neck Surg* 2011;137:664–668.
3. Jervis-Bardy J, Rogers GB, Morris PS, et al. The microbiome of otitis media with effusion in Indigenous Australian children. *Int J Pediatr Otorhinolaryngol* 2015;79:1548–1555.
4. Leskinen K, Hendolin P, Virolainen-Julkunen A, Ylikoski J, Jero J. The clinical role of Alloiococcus otitidis in otitis media with effusion. *Int J Pediatr Otorhinolaryngol* 2002;66:41–48.
5. Guvenc MG, Midilli K, Inci E, et al. Lack of Chlamydophila pneumoniae and predominance of Alloiococcus otitidis in middle ear fluids of children with otitis media with effusion. *Auris Nasus Larynx* 2010;37:269–273.
6. Tano K, Essen von R, Eriksson PO, Sjöstedt A. Alloiococcus otitidis—otitis media pathogen or normal bacterial flora? *APMIS* 2008;116:785–790.
7. Harimaya A, Takada R, Hendolin PH, Fujii N, Ylikoski J, Himi T. High incidence of Alloiococcus otitidis in children with otitis media, despite treatment with antibiotics. *J Clin Microbiol* 2006;44:946–949.
8. Zhang J, Kobert K, Flouri T, Stamatakis A. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* 2014;30:614–620.
9. Rideout JR, He Y, Navas-Molina JA, et al. Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. *PeerJ* 2014;2:e545.
10. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;26:2460–2461.
11. Pruesse E, Quast C, Knittel K, et al. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* 2007;35:7188–7196.
12. Jervis-Bardy J, Leong LEX, Marri S, et al. Deriving accurate microbiota profiles from human samples with low bacterial content through post-sequencing processing of Illumina MiSeq data. *Microbiome* 2015;3:19.
13. Clarke KR. Non-parametric multivariate analyses of changes in community structure. *Austral Ecology* 1993;18:117–143.
14. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003;13:2498–2504.

15. Wheeler DL, Church DM, Lash AE, et al. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res* 2001;29:11–16.
16. Marsh RL, Binks MJ, Beissbarth J, et al. Quantitative PCR of ear discharge from Indigenous Australian children with acute otitis media with perforation supports a role for Alloiococcus otitidis as a secondary pathogen. *BMC Ear Nose Throat Disord* 2012;12:11.
17. Frank DN, Spiegelman GB, Davis W, Wagner E, Lyons E, Pace NR. Culture-independent molecular analysis of microbial constituents of the healthy human outer ear. *J Clin Microbiol* 2003;41:295–303.
18. The bacterial interactions in the nasopharynx of children receiving adenoidectomy. *BioMedicine* 2015;5:6.
19. Pettigrew MM, Gent JF, Revai K, Patel JA, Chonmaitree T. Microbial Interactions during upper respiratory tract infections. *Emerg Infect Dis* 2008;14:1584–1591.
20. Korona-Glowniak I, Niedzielski A, Kosikowska U, Grzegorczyk A, Malm A. Nasopharyngeal vs. adenoid cultures in children undergoing adenoidectomy: prevalence of bacterial pathogens, their interactions and risk factors. *Epidemiol Infect* 2015;143:821–830.
21. Li K, Bihan M, Methe BA. Analyses of the stability and core taxonomic memberships of the human microbiome. *PLoS One* 2013;8: e63139.
22. Zhou Y, Gao H, Mihindukulasuriya KA, et al. Biogeography of the ecosystems of the healthy human body. *Genome Biol* 2013;14:R1.
23. Microbial co-occurrence relationships in the human microbiome. *PLoS Comput Biol* 2012;8:e1002606.
24. Hall-Stoodley L, Hu FZ, Gieseke A, et al. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA* 2006;296:202–211.
25. Aruni AW, Dou Y, Mishra A, Fletcher HM. The biofilm community: rebels with a cause. *Curr Oral Health Rep* 2015;2:48–56.
26. Cadwell K. The virome in host health and disease. *Immunity* 2015;42: 805–813.