Identification of the Bacterial Reservoirs for the Middle Ear Using Phylogenic Analysis

Chun Ling Chan, BMBS; David Wabnitz, MBBS, MS, FRACS; Ahmed Bassiouni, MBCh, PhD; Peter-John Wormald, MD, FRACS; Sarah Vreugde, MD, PhD; Alkis James Psaltis, MBBS(Hons), PhD, FRACS

IMPORTANCE The adenoid pad has long been considered a reservoir for bacteria in the pathogenesis of otitis media with effusion (OME). However, bacteria more reminiscent of external auditory canal (EAC) commensals are often demonstrated within middle ear aspirates.

OBJECTIVE To compare the microbiota of the EAC, the middle ear with OME, and the adenoid pad to further clarify the true source of middle ear bacteria.

DESIGN, SETTING, AND PARTICIPANTS Middle ear fluid (MEF) aspirates and EAC lavages were collected from 18 children with OME undergoing ventilation tube insertion from June 1, 2014, to August 31, 2015, at Women and Children’s Hospital, Adelaide, Australia. Adenoid pad and MEF samples were included from a previous study. Samples were analyzed using sequencing of the 16S ribosomal RNA gene. Previously collected microbiota data from the adenoid pad were collated for analysis.

MAIN OUTCOME MEASURES Mean relative abundance of top bacterial genera for the MEF, EAC, and adenoid pad samples.

RESULTS Eighteen pediatric patients with chronic OME (6 female; 12 male; mean [SD] age, 48 [36] months) were recruited prospectively, with 34 paired MEF and EAC samples. The MEF microbiota (mean relative abundance [SD]) consisted of Alloiococcus otitidis (37.5% [40.0%]), Haemophilus (14.4% [29.1%]), Moraxella (10.0% [26.4%]), Staphylococcus (8.2% [21.9%]), and Streptococcus (3.8% [13.1%]). The mean relative abundance (SD) microbiota of the EAC demonstrated a sparsity of classic otopathogens, including Haemophilus (0.3% [0.8%]), Moraxella (0.3% [0.7%]), and Streptococcus (0.2% [0.6%]), but had a high abundance of Alloiococcus (58.0% [44.1%]), Staphylococcus (20.8% [34.0%]), and Pseudomonas (3.2% [171%]). In contrast, based on previously collected data, the microbiota of the adenoid pad showed a high abundance of the classic otopathogens with a sparsity of EAC genera for Alloiococcus (0.1% vs 28.9%, respectively; $P < .001$), Haemophilus (25.2% vs 18.2%, respectively; $P = .002$), Staphylococcus (0.2% vs 10.8%, respectively; $P = .02$), Streptococcus (12.7% vs 4.2%, respectively; $P < .001$), and Pseudomonas (0 vs 2.1% respectively; $P < .001$). The microbiota of the MEF collected during 2 consecutive years were similar (Alloiococcus, 22.7% vs 37.5%; Haemophilus, 22.5% vs 14.0%; Staphylococcus, 10.9% vs 10.7%; Moraxella, 5.0% vs 9.7%; Corynebacterium, 6.2% vs 3.1%; Streptococcus, 4.8% vs 3.7%; and Pseudomonas, 1.1% vs 3.0%; $P \geq .05$).

CONCLUSIONS AND RELEVANCE The EAC and the nasopharynx could serve as reservoirs for microbiota of the middle ear. Furthermore, the microbiota of the middle ear with effusion appear to be relatively stable over time and between populations with OME.
The typical bacteria responsible for otitis media are *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Haemophilus influenzae*. These bacteria are commensals within the oropharynx and nasopharynx, and these pathogens are commonly believed to ascend from the aerodigestive tract into the middle ear via the eustachian tube.\(^1\)

In recent times, with the increasing interest in and access to culture-independent techniques (ie, polymerase chain reaction [PCR], fluorescence in situ hybridization, and most recently 16S ribosomal RNA [16SrRNA] gene sequencing), *Alloiococcus otitidis* has emerged as a bacterial species that has been reported in high abundance in specimens from the middle ear.\(^2\)\(^3\) However, *A otitidis*, although commonly believed to be a commensal of the external auditory canal (EAC), is often not found in the nasopharynx.

Chan et al\(^4\) recently characterized the microbiome of the middle ear and found that *A otitidis* was the most abundant bacteria found within middle ear effusions of children (cumulative relative abundance, 23%). Furthermore, EAC commensals such as *Pseudomonas*, *Staphylococcus*, and *Corynebacterium* species were also demonstrated in high abundance from middle ear fluid (MEF) samples. We therefore designed a follow-up study to investigate whether the EAC could be a potential reservoir for microbiota of the middle ear.

### Methods

#### Study Group

This study was conducted with ethics approval from the institutional research boards of the Women and Children’s Hospital and the University of Adelaide, Adelaide, Australia. Collection of samples was performed during the Australian winter period (June 1 to August 31) of 2015. All patients were aged 1 to 16 years, and written informed consent was obtained from the parents or guardians before enrollment. Children wait-listed for ventilation tube (grommet) insertion with or without adenoidectomy for otitis media with effusion (OME) were enrolled. Exclusion criteria consisted of effusions that had resolved by the time of surgery, upper respiratory tract infection on the day of surgery, and antibiotic or corticosteroid use in the 4 weeks preceding surgery. We collected demographic data, including age, sex, and parental smoking status, as well as clinical data, including presenting symptoms, previous tympanometry, medication history, and medical and surgical history.

#### Sample Collection

Swabs and aspirates were obtained with the patient under general anesthesia. All samples were immediately placed on ice, transferred to a freezer, and stored at −80°C.

#### EAC Aspirate

We examined all ears using an operating microscope. The EAC underwent lavage with 1 mL of sterile normal saline solution. The lavage was then aspirated into a specimen trap (Argyle; Covidien).

#### Middle Ear Effusion Collection

Middle ear effusions were sampled using a standardized protocol previously described.\(^5\) In brief, a myringotomy using sterile instruments was performed, and the middle ear effusion was aspirated into a specimen trap through the incision site (Video). The utmost care was taken to avoid direct contact with the EAC or other previously used instruments. If contact with the EAC inadvertently occurred, the equipment was exchanged for a new sterile set. After collection of the aspirate, 2 μL of sterile normal saline solution was used to flush the circuit.

#### DNA Extraction

We performed DNA extraction using an isolation kit (PowerLyzer PowerSoil; MoBio Laboratories). Samples were thawed on ice and placed directly on the beads for homogenization. Total DNA was extracted from all clinical samples and 2 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.

#### PCR Amplification of the 16SrRNA Gene and Sequencing

The Australian Genome Research Facility performed PCR amplification and sequencing. Libraries were generated by amplifying the V3 to V4 (341F-806R) hypervariable region of the 16SrRNA gene. The PCR amplicons were generated using the primers CCTAYGGGGRBGCASCAG in the forward sequence and GGACTACNNGGGTATCTAAT in the reverse sequence with a master mix kit (AmpliTaq Gold 360; Life Technologies) following local protocol. The resulting amplicons were measured by fluorometry (Picogreen assay; Thermo Fisher Scientific) and normalized. The equimolar pool then underwent quantitative PCR (KAPA; Illumina) and setup for sequencing on the paired-end chemistry system (MiSeq; Illumina). Reads from sequencing were used as raw data for bioinformatic analysis.

#### Bioinformatics Pipeline

We used PEAR software (Paired-End Read Merger; version 0.9.5)\(^6\) to pair forward and reverse reads in each sample and to quality filter the paired reads. Open-reference operational...
taxonomic unit (OTU) picking strategy in QIIME software (version 1.8) was used to cluster OTUs. Within the open-reference method, UCLUST (version 1.2.22) was used to cluster OTUs at 97% similarity. The Greengenes 16S database was used for reference-based OTU picking and for taxonomic assignment. Taxonomic assignment for all other OTUs remained at genus level during taxonomic assignment. All samples were subsampled to 950 reads (rarefaction). Diversity estimates were performed on subsampled data.

**Statistical Analysis**

We used the Shannon index of diversity for diversity. A Friedman rank sum test was used to determine whether a significant difference of diversity exists between sample types (while controlling for the repeated measure of the covariate “side in patient”).

Distance matrices for β diversity metrics (Bray-Curtis dissimilarity, unweighted UniFrac, and weighted UniFrac) were generated using QIIME software. Distances in these matrices were used to calculate mean distances within and between sample type groups. Mean within- and between-sample type distances were then compared using nonparametric Mann-Whitney tests. A permutational multivariate analysis of variance (PERMANOVA) test was also performed to test whether a statistically significant difference was found between the bacterial communities in the MEF and the canal samples, with samples from the same side (of the same patient) grouped in the analysis (as a random factor).

Species level presumptive identification was performed on OTUs classified as *A. otitidis*, using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database. Taxonomic assignment for all other OTUs remained at genus or family (if genera classification was not possible) level during taxonomic assignment.

Pearson GraphPad software (version 6; GraphPad Software Inc) was used to analyze the data, generate box plots, and calculate the statistical tests used. Mean relative abundance of top genera was calculated for the MEF and EAC groups and compared using a paired t test. We applied the Šidák correction for multiple comparisons as a follow-up to ANOVA to correct for potentially introduced type I error.

Correlation analysis between genera was performed using the Pearson product moment correlation coefficient (r), which measures the linear correlation between 2 variables.

**Results**

Eighteen patients with OME, aged 1 to 14 years (mean [SD] age, 48 [36] months) were recruited. Twelve patients (67%) were male and 6 (33%) were female. Three patients (18%) had undergone previous ventilation tube insertion. Sixteen patients had bilateral effusions and 2 had unilateral effusions (total number of MEF samples, 34). No patient was a primary smoker, although 1 patient lived with at least 1 caregiver who smoked.

**Microbiome of Middle Ear With Effusion**

The genera of bacteria that occurred at a mean (cumulative) relative abundance (SD) of at least 1% were *Alloilococcus* (37.5% [40.0%]), *Haemophilus* (14.4% [29.1%]), *Moraxella* (10.0% [26.4%]), *Staphylococcus* (8.2% [21.9%]), *Streptococcus* (3.8% [13.1%]), *Corynebacteria* (3.3% [9.1%]), and *Pseudomonas* (3.0% [16.8%]) (Table and Figure 1). Genera occurring at relative abundance of less than 1% were reported as absent because of the potential for background noise to confound these findings. Furthermore, the *Alloilococcus* genus shared an inverse correlation association with *Staphylococcus* (r = −0.433; P = .01).

**Microbiome of the EAC**

The genera of bacteria that occurred at a mean (cumulative) relative abundance (SD) greater than 1% were *Alloilococcus* (58.0% [44.1%]), *Staphylococcus* (20.8% [34.0%]), *Pseudomonas* (3.2% [17.1%]), *Corynebacteria* (2.7% [6.6%]), and *Enterobacteria* (1.4% [7.4%]). *Haemophilus* (0.3% [0.8%]), *Moraxella* (0.3% [0.7%]), and *Streptococcus* (0.2% [0.6%]) were less abundant, despite prevalence ranging from 11.4% to 17.1% (Table).

We found inverse correlation associations between the relative abundance of bacterial genera (Figure 2), including *Alloilococcus* with *Staphylococcus* (r = −0.787; P < .001), *Haemophilus* (r = −0.356; P = .04), *Moraxella* (r = −0.456; P = .007), and *Streptococcus* (r = −0.419; P = .01). *Haemophilus* shared a correlation with *Moraxella* (r = 0.794; P < .001) and...
Corynebacteria \((r = 0.632; \ P < .001)\), whereas Moraxella also had a positive correlation with Streptococcus \((r = 0.736; \ P < .001)\). Patients with previous myringotomy were found to have a high abundance of Alloiococcus (73.3%-94.1%) or Staphylococcus (68.9%) (when Aerococcus was found at a relative abundance of 5%); however, owing to the small sample size, no formal statistical analysis was possible.

**Comparison of the Microbiomes of the MEF and EAC**

The microbiomes of the MEF and EAC were dissimilar. The microbiome of the MEF was more diverse; the Simpson Diversity Index for the EAC was 1.169 compared with 1.702 for the MEF (PERMANOVA, \(P < .001\)). Analysis of \(\beta\) diversity also showed a difference between MEF and EAC microbiota based on the \(\beta\) diversity (mean of distances within samples vs mean of distances between samples, 0.654 vs 0.667; Mann-Whitney test, \(P = .004\); PERMANOVA, \(P < .001\)). However, despite overall dissimilarities between the 2 areas, we found a positive correlation among bacterial genera commonly associated with EAC commensals, including Alloiococcus \((r = 0.680; \ P < .001)\), Staphylococcus \((r = 0.868; \ P < .001)\), and Corynebacteria \((r = 0.963; \ P < .001)\). Patients with previous myringotomy were found to have a high abundance of Alloiococcus (73.3%-94.1%) or Staphylococcus (68.9%) (when Aerococcus was found at a relative abundance of 5%); however, owing to the small sample size, no formal statistical analysis was possible.

**Bilateral Effusions**

Bilateral effusions were similar in composition (1-way ANOVA, \(P < .001\)). The genera of bacteria in the left ears occurred at a mean relative abundance (SD) of 27.5% (37.3%) for Alloiococcus, 21.5% (34.3%) for Haemophilus, 11.4% (20.6%) for Moraxella, 3.1% (10.9%) for Staphylococcus, 3.6% (6.1%) for Streptococcus, 4.3% (12.3%) for Pseudomonas, and 4.6% (19.2%) for Corynebacteria. The genera of bacteria in the right ears occurred at a mean relative abundance (SD) of 26.1% (35.7%) for Alloiococcus, 20.6% (34.5%) for Haemophilus, 5.3% (11.0%) for Moraxella, 13.2% (29.0%) for Staphylococcus, 4.1% (12.4%) for Streptococcus, 6.1% (17.8%) for Pseudomonas, and 0.6% (1.5%) for Corynebacteria. We found no significant differences between

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**Figure 1. Comparison of the Microbiomes of the Adenoid Pad, Middle Ear Fluid, and External Auditory Canal**

![Graph showing comparison of microbiomes](https://example.com/graph1)

**Figure 2. Bacterial Correlations of Middle Ear Fluid (MEF) and External Auditory Canal (EAC)**

A, Findings from microbiota data of MEF from the previously published work of Chan et al.\(^5\) B, Graphic representation of the correlation between the relative abundance of bacterial genera within the MEF and EAC. Blue arrows represent positive correlation; yellow arrows, inverse correlation.
matched groups for individual genera (Šidák multiple comparisons test) (Figure 3).

**Combined With Previous Data Set**

When combined with the previously published data by Chan et al,5 we found a total of 69 MEF samples, with 24 paired, matched adenoid pad samples and 34 paired, matched EAC samples. The 2 data sets had comparable distribution of age (mean [SD], 40 [21] vs 48 [36] months; P = .39) and sex (male, 14 [61%] vs 12 [67%]; P = .84). The microbiome of the middle ears was similar when comparing the 2014 and 2015 cohorts (Alloiococcus, 22.7% vs 37.5%; Haemophilus, 22.5% vs 14.0%; Staphylococcus, 10.9% vs 10.7%; Moraxella, 5.0% vs 9.7%; Corynebacteria, 6.2% vs 3.1%; Streptococcus, 4.8% vs 3.7%; and Pseudomonas, 1.1% vs 3.0%)(1-way ANOVA, P < .001) (Figure 4); however on further analysis, we found a greater relative abundance of Alloiococcus in the second year (Šidák multiple comparisonstest, P = .046).

With the combined data set for MEF, analysis again revealed inverse correlation between Alloiococcus and Staphylococcus (r = 0.356; P = .003). In addition, a further inverse correlation was demonstrated between Alloiococcus and Haemophilus (r = −0.300; P = .01). When we compared the microbiome of the adenoid pad with that of the EAC (Mann-Whitney test), we found significant differences in relative abundance for Alloiococcus (0.1% vs 28.9%, respectively; P < .001), Haemophilus (25.2% vs 18.2%, respectively; P = .002), Staphylococcus (0.2% vs 10.8%, respectively; P = .02), Streptococcus (12.7% vs 4.2%, respectively; P < .001), and Pseudomonas (0 vs 2.1%, respectively; P < .001).

**Discussion**

This study is a follow-up to the previously published study by Chan et al,5 which was, to our knowledge, the first to describe the microbiome of the middle ear in children with OME in a nonindigenous Australian population using 16S rRNA gene sequencing. The microbiota of the middle ears from the present study and the study by Chan et al5 were similar between...
the 2 cohorts. Alloiococcus was again demonstrated to be the most abundant bacterial genera found within the middle ear, with the classic ear pathogens (Moraxella, Haemophilus, and Streptococcus) also present in abundance. The only significant difference was an increase in the mean relative abundance of Alloiococcus in the present study. The reason for this difference is unclear but may reflect a natural variation in commensal carriage of the EAC and middle ear cleft between populations. Alternatively, as discussed above, more patients had undergone previous myringotomy in the present cohort, leading to a greater opportunity for the EAC bacteria, including Alloiococcus, to translocate into the middle ear. However, the ecological niche subsequently created by the development of an effusion within the middle ear cavity is such that Alloiococcus growth is favored compared with other external canal bacteria (ie, Staphylococcus and Pseudomonas), which consequently do not exhibit a proportional rise in abundance. Despite this variation in relative abundance, we believe that bacterial genera identified from the middle ears of our cohort reflect a core microbiome of the middle ear, which remains relatively stable over time and between different populations, a characteristic that has been reported at other body sites.13,14

Our findings support those of a previous culture-independent phylogenetic study by Frank et al.8 Despite differences in sampling techniques, geographical location, and seasonal and climatic factors, Frank et al8 also demonstrated a major abundance of Alloiococcus (57%), moderate abundance of Corynebacterium (24%) and Staphylococcus (10%), but only minimal abundance of Pseudomonas (<1%).

We found that the microbiome of the middle ear in OME appears to reflect the microbiomes of the adenoid pad and the EAC (Figure 1 and Table). Haemophilus, Streptococcus and Moraxella genera were in high abundance in the adenoids and the middle ear but absent (<1%) in the EAC. Conversely, Alloiococcus, Staphylococcus, and Pseudomonas genera were found in high relative abundance in the EAC but were absent in the adenoids. We therefore propose not only that the adenoid seeds the middle ear as previously described1 but that the EAC may also serve as a bacterial reservoir for middle ear infections.

Although bacterial translocation across intact mucosal surfaces has been previously documented in gut mucosa in certain pathologic states,15,16 translocation across an intact tympanic membrane has not been demonstrated, to our knowledge. In health, the tympanic membrane has been shown to allow only oxygen and carbon dioxide to diffuse through it.17 This process is largely owing to its multilayered, multicellular structure, consisting of epidermis (external layer), a radiating layer of collagen fibers, a circular layer of collagen fibers, and a layer of simple cuboidal epithelium (middle ear mucosa). Furthermore, during acute infections, the thickness of the tympanic membrane has been found to increase further owing to edema and inflammation.18

Given these data, the presence of a tympanic perforation would seem to be needed to facilitate entry of bacteria from the EAC into the middle ear. Epidemiologic studies suggest that almost 80% of children will have an episode of acute otitis media by 3 years of age,19 with perforations reported in as many as 30% of such cases.19 Therefore, such an episode could be responsible for the translocation of EAC bacteria observed in our study. Unfortunately, a history of perforation was not obtained from our patient cohort; therefore, this cannot be commented on directly. However, as mentioned above, we observed a statistically significant increase in the mean relative abundance of Alloiococcus in our present patient cohort compared with the previous cohort.6 This finding may reflect the greater number of patients with previous ventilation tube insertion (1 of 17 [6%] in the first study vs 3 of 18 [17%] in the present study), thus allowing for a greater chance for EAC bacteria to translocate in the middle ear. In fact, examples of EAC commensals causing middle ear disease via perforation are evident in the frequent bacterial culture of Pseudomonas aeruginosa and Staphylococcus aureus in cases of chronic suppurative otitis media.20,21

However, unlike P aeruginosa and S aureus, the role of A otitidis in otitis media remains unclear. Alloiococcus otitidis is an aerobic, gram-positive cocci first documented in MEF by Faden and Dryja22 in 1989. It is typically considered a commensal of the EAC and is not commonly identified with standard techniques.4,23-25 However, the potential for pathogenicity has been documented; A otitidis has been found to invade intracellularly22 and to modulate immune responses in vitro26,27 and has been implicated in cases of endocarditis28 and endophthalmitis.29 In addition, A otitidis is the most prevalent bacterial species found in patients with nonpurulent OME (20%-40%)25,30,31 and has been reported to persist within middle ear aspirates despite antibiotic treatment.4 However, the effect and role of Alloiococcus in the pathogenesis or development of OME remains elusive. We believe that further investigation to elucidate the role of Alloiococcus in OME is warranted.

However, given the similarity between the microbiome of the EAC and middle ear, the possibility of contamination must be considered. We believe that contamination is less likely for the following reasons. First, to reduce contamination before myringotomy and aspiration, the EAC is first toileted and washed. The aspiration technique itself then avoids any external canal contact, with minimal contact with the tympanic membrane (Video). Although a theoretical risk for contamination exists because of the brief contact with the external surface of the tympanic membrane, the comparatively large volume of MEF relative to this brief contact would unlikely yield such high relative abundances seen in our study. Therefore, we believe that our results accurately represent the microbiota within the middle ear with effusion.

Conclusions
We have demonstrated that the microbiome of the middle ear with effusion is grossly stable over time and across populations. Furthermore, owing to the distribution of the microbiome characterized, we postulate that the nasopharynx and EAC contribute the microbiota within the middle ear.
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