1 Bacterial diversity of chronic middle ear lesions revealed by
2 high-throughput sequencing

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13

14 **Abstract**

15 Chronic otitis media is a common middle ear disease in otolaryngology and
16 head and neck surgery. Bacterial infection is considered the main cause of
17 disease, but relying on conventional bacterial cultures can be problematic
18 for identification of specific pathogens. Current research suggests that
19 bacteria in microbial communities can only be identified by rDNA
20 sequencing of bacteria. This prospective study utilized broad-range PCR
21 amplification of 16S rRNA genes with clone analysis to compare bacterial
22 diversity in lesions from 6 patients with chronic suppurative otitis media
23 (CSOM) and 10 patients with cholesteatoma of middle ear lesions. Bacteria
24 were analyzed at the phylum, order, family, genus, and species levels.
25 Bacterial species abundance and species diversity were greater in
26 cholesteatoma of the middle ear lesions than in CSOM lesions. At all
27 bacterial taxonomic levels, the epithelial tissue of middle ear cholesteatoma

was complex in terms of bacterial diversity, covering a large number of Gram-positive and Gram-negative bacteria, likely related to bacterial microbiome formation. In contrast, bacteriology of the CSOM lesions was relatively simple at all taxonomic levels, with all sequences characterized as belonging to Gram-negative bacteria. These results suggest that persistent infection of middle ear cholesteatoma may be a microbial flora disorder, which is related to conditional pathogenic bacteria, rather than a single bacterial infectious disease. Findings from the study may have implications in the selection of antimicrobial agents for the treatment of chronic otitis media.

INTRODUCTION

Chronic otitis media, a common disease in otorhinolaryngology, is usually caused by inflammatory lesions in the middle ear and/or mastoid cavity,

42 and is characterized by ear discharge (otorrhea) and hearing loss. Some
 43 patients experience serious complications that can be life-threatening (1, 2).
 44 Clinically, chronic otitis media usually refers to chronic suppurative otitis
 45 media (CSOM) and middle ear cholesteatoma. Bacterial infection is
 46 considered to be the main pathogenic factor of chronic otitis media (3).
 47 Bacterial culture of secretions from the middle or outer ear canal is often
 48 used to confirm the presence of pathogenic bacteria, and various
 49 pathogenic bacteria, even methicillin-resistant *Staphylococcus aureus*, have
 50 been obtained through conventional bacterial culture (4).
 51 Sensitive antimicrobial agents based on the culture results have been used
 52 to treat patients with success, but there remains some cases where the
 53 treatment is ineffective (5, 6). Subsequent studies confirmed that bacterial
 54 biofilm formation leading to resistance to antimicrobial agents may be one
 55 reason for the persistence of symptoms in some patients (7-10). Bacterial

56 culture results are affected by many factors including the inability of some
57 bacteria to grow on standard medium and restrictions on specimen
58 collection. Thus, in some cases it is difficult to successfully identify and treat
59 the causative pathogen (11).

60 In recent years, there have been numerous reports on molecular biology
61 techniques for the study of bacteria of otitis media. No bacteria were
62 isolated from middle ear secretions of adults and children by conventional
63 bacterial culture methods, but gene sequencing resulted in the isolation of
64 various bacterial sequences, including the phylum Thunb and the genus
65 Grapevine (12). Neeff et al. demonstrated the use of molecular biological
66 techniques to analyze CSOM and healthy human middle ear mucosa, which
67 can more accurately evaluate the information of microbial communities in
68 the middle ear or mastoid mucosa (13). However, there are limited reports
69 on the distribution of microbes in different types of chronic otitis media.

In the current study, the molecular technique of 16S rRNA gene amplicon sequencing was employed to study microbial communities in CSOM and cholesteatoma of middle ear (CME) diseased tissues. Differences in microbial species abundance, diversity, and at the levels of phylum, class, order, family, genus, and species were compared. These analyses provide a basis for future studies on the characterization of bacteria at the molecular level in chronic otitis media.

MATERIALS AND METHODS

Patient information

All patients in this study had chronic otitis media and received surgical treatment in the otolaryngology department of Xinjiang Uygur Autonomous Region People's Hospital from January 2017 to June 2019. Among the 16 eligible patients, six cases were chronic suppurative otitis

84 media and 10 were middle ear cholesteatoma. None of the patients used
85 antibiotic ear drops or systemic antibiotics prior to surgery, but according
86 to the requirement of preventive use of antibiotics, all patients received
87 intravenous cephalosporins 30 min ~ 2 h before surgery. Patients were not
88 associated with basic metabolic diseases such as diabetes or rheumatism,
89 and did not have fungal infections of the external auditory canal.

90 The study was approved by the Ethics Committee of the People's
91 Hospital of Xinjiang Uygur Autonomous Region (Xinjiang District Hospital
92 Ethics Committee 2015054).

93 All patients were admitted to Xinjiang Uygur Autonomous Region People's
94 Hospital for surgery. The operation mode of canal wall up [CWU] tympanic
95 forming or canal wall down [CWD] tympanic forming was selected
96 according to the disease severity of the patient. After opening the tympanic
97 sinus and mastoid cavity during the operation, the middle ear lesion tissue

was retained (after cleaning the matrix of cholesteatoma, the cholesteatom

aepithelium and granulation tissue of cholesteatoma were retained).

Following collection of pathological tissues, the accompanying blood and

floats were washed with normal saline, immediately placed into a specimen

tube, frozen in liquid nitrogen, and then stored at -80°.

Absolute quantification of 16S rRNA amplicon sequencing

Absolute quantification of 16S rRNA amplicon sequencing was performed

by Genesky Biotechnologies Inc. (Shanghai, 201315, China). Briefly, total

genomic DNA was extracted using a Fast DNA SPIN Kit for soil (MP

Biommedicals, Santa Ana, CA, USA) according to the manufacturer' s

instructions. The integrity of the genomic DNA was assessed through

agarose gel electrophoresis, and the concentration and purity of genomic

DNA were measured using a NanoDrop 2000 spectrophotometer and Qubit

3. 0 fluorometer.

GC content were artificially synthesized. Then, an appropriate proportion of spike-ins mixture with known gradient copy numbers were added to the sample DNA. The V3-V4 hypervariable regions of the 16s rRNA gene and spike-ins were amplified with the primers xxxF (5' -CCTACGGGNGGCWGCAG-3') and xxxR (5' -GACTAACHVGGGTATCTAATCC-3') and then sequenced using an Illumina NovaSeq 6000 sequencer.

Sequence data processing and analysis

Raw read sequences were processed in QIME2 [14]. The adaptor and primer sequences were trimmed using the cutadapt plugin, and the DADA2 plugin was used for quality control and to identify amplicon sequence variants (ASVs) [15]. Taxonomic assignments of representative ASVs were performed with a confidence threshold of 0.8 by a pre-trained Naive Bayes classifier that was trained on the Greengenes database (version 13.8). The spike-in

sequences were then identified, and reads were counted. A standard curve for each sample was generated based on the read counts versus spike-in copy number, and the absolute copy number of ASVs in each sample was calculated by using the read counts of the corresponding ASV. Since the spike-in sequence is not a component of the sample flora, the spike-in sequence was removed in the subsequent analysis [16].

RESULTS

Analysis of species abundance and diversity

High-throughput 16SrRNA gene sequencing technology was used to sequence samples from 16 patients with CSOM or cholesteatoma. A total of 4,672,171 raw sequence reads and 3,415,184 optimized sequence reads were obtained from the 16 patients with middle ear lesions (6 CSOM cases, 10 cholesteatoma cases). The average sequence length from the total of 1,243,460,816 bases was 364.10 bp.

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142 FIG 1 Box diagrams of α -diversity. A: Observed ; B: Chao 1 index,
 143 $P=0.093407$; C: ACE, $P=0.093407$; D: Shannon index, $P=0.3131868$;
 144 E: Simpson index, $P=0.562188$; F: Coverage, $P=0.022478$. Asterisk
 145 indicates statistical significance at $P \leq 0.05$.

146 The number of sequences obtained from each patient group were 582,102

and 1,485,436 for CSOM and cholesteatoma, respectively. Chao 1, ACE, Shannon, and Simpson indices indicated there were no differences in α -diversity between the two disease groups. However, coverage was significantly different between the two groups ($P=0.022478$), illustrating that the coverage of microbial communities in cholesteatoma samples was higher than that in the CSOM group.

Taxonomic analysis of pathological tissue flora from patients with CSOM or cholesteatoma

A total of 833 operational taxonomic units (OTU) were subjected to taxonomic classification, and 28 phyla, 50 classes, 63 orders, 128 families, 264 genera, and 232 species were identified and further analyzed.

Samples from different disease groups have certain characteristics and commonalities. Based on the OTU abundance table, the unique OTU, and common OTU in each group were screened and visualized. The total

161 number of detected OTU was 838, comprising 788 OTU detected in
 162 cholesteatoma pathological tissues, 230 in CSOM pathological tissues, and
 163 180 OTU common to both groups (FIG 2).

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166 FIG 2 Venn diagram of OTUs of CSOM and cholesteatoma lesions

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170 FIG 3 Pie charts showing phylum breakdown of bacterial communities

171 obtained from CSOM and cholesteatoma pathological tissues.

172 **Phylum**

173 Bacterial community analysis at the phylum level revealed that the

174 community flora of CSOM tissues was simpler than that of Cholesteatoma

175 samples. The phylum *Proteus* (*Proteobacteria*) was detected in both groups.

176 Statistically, detection of *Proteus* indicates this phylum is a major part of

177 CSOM rt (99.46%, P=0.000321). And *Pseudomonas aeruginosa*, *Escherichia*

178 *coli* and so on in the door of *Proteus* are also our common

179 Gram-staining-negative pathogens. The microbial community of

180 Cholesteatoma samples was more complex. Aside from the detection of

181 *Proteus* (*Proteobacteria*) (35.77%), statistically significant phyla of the

182 Cholesteatoma samples were *thikum* (*Firmicutes*) (44.21%, $P=0.001071$) and

183 *Actinomyces* (*Actinobacteria*) (16.66%, $P=0.032464$). These two phyla

184 encompass the vast majority of Gram-positive bacteria (FIG 3).

185 Class

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187 FIG 4 Class level differences in bacterial community of CSOM and
 188 cholesteatoma pathological tissues

189 Analysis of bacterial sequences at the class level showed statistical
 190 differences between the two groups (CSOM and cholesteatoma) in the
 191 classes *Gammaproteobacteria* γ -Proteus (P=0.03), *Bacilli* (P=0.01034),
 192 *Clostridia* (P=0.01034), and *Bacteroidia* (P=0.041) (FIG 4) . In general, the
 193 measured species of the cholesteatoma lesions were higher than those of
 194 the CSOM group, indicating that the abundance of microbes was greater. In
 195 the CSOM group, *Gammaproteobacteria* γ - and β - Proteus
 196 (*Betaproteobacteria*) were predominantly detected, indicating that the
 197 number of microbial species present at the class level is relatively small in
 198 this group (FIG 5).

199

200 FIG 5 Bubble diagram of class level differences in bacterial communities of

201 CSOM and cholesteatoma pathological tissues

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203 **ORDER**

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205 FIG 6 Order level differences in bacterial communities of CSOM and

206 cholesteatoma pathological tissues

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208 FIG 7 Bar plot diagram showing bacterial composition at the order level in

209 CSOM and cholesteatomas pathological tissues

210 At the order level, *Pseudomonadales* and *Burkholderiales* have an absolute

211 advantage in CSOM, with *Pseudomonadales* being statistically significant

212 ($P=0.00129$). *Pseudomonas aeruginosa* strain PAO was identified in the

213 bacterial species map at the sample sequencing level. In the cholesteatoma

214 group, the statistically significant orders were *Bacillales* ($P=0.011887$),

215 *Clostridiales* (P=0.011887), and *Bacteroidales* (P=0.002532) (FIG6).

216 Description of the bacterial communities at the order level also suggests

217 that cholesteatoma is more complex in bacterial diversity (FIG 7).

218 FAMILY

221 FIG 8 Family level differences in bacterial communities of CSOM and

222 cholesteatoma lesions

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225 FIG 9 Pie charts showing composition of bacterial communities at the family
226 level in CSOM and cholesteatoma lesions

227 Analysis of sequencing results at the family level indicated that

228 *Pseudomonadaceae* (37.74%), *Moraxellaceae* (35.13%), and *Alcaligenaceae*

229 (26.13%) were predominant in the CSOM samples. In particular, detection of

230 *Pseudomonadaceae* ($P=0.000976$) was statistically significant. Bacterial

231 diversity at the family level in the cholesteatoma group was more complex

232 than that of the CSOM group. Families with broad coverage in the

233 cholesteatoma group were *Staphylococcaceae* (33.53%), *Enterobacteriaceae*

234 (22.93%), *Brevibacteriaceae* (8.49%), *Pseudomonadaceae* (8.39%), and

235 *Corynebacteriaceae* (6.31%). The statistically significant families
 236 *Porphyromonadaceae* and *Lachnospiraceae* are mostly conditional
 237 pathogenic bacteria (FIG 8, FIG 9).

238 **Genus**

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241 FIG 9 Genus level differences in bacterial communities of CSOM and

242 cholesteatoma lesions

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247 FIG 10 Pie charts showing genus level composition of bacterial communities
248 in CSOM and cholesteatoma lesions

249 Sequencing analysis of bacterial communities of CSOM and cholesteatoma
250 lesions at the genus level revealed that 16 genera, including 'Others' and

251 'No_Rank', were detected across the two groups. However, only

252 *Pseudomonas* was detected in both groups and showed a statistically

253 significance difference in relative abundance between the two groups

254 (P=0.000601) (FIG 9). *Pseudomonas* in the CSOM group (37.74%) was

255 significantly higher than that in the middle ear cholesteatoma group
 256 (8.39%). Other genera detected in the CSOM group were *Acinetobacter*
 257 (35.13%) and *Alkaligenes* (26.02%) (FIG 10). A total of the G-negative genera
 258 were dominant. These genera include *Pseudomonas aeruginosa*,
 259 *Acinetobacter baumannii*, and fecal alkaloid bacteria, which are the most
 260 common resistant strains of nosocomial infections. In contrast, the
 261 composition of bacterial genera of the cholesteatoma group was more
 262 elaborate, comprising *Staphylococcus* (33.53%), *Providencia* (12.34%),
 263 Others (9.56%), *Proteus* (9.05%), *Brevibacterium* (8.49%), *Pseudomonas*
 264 (8.39%), *Corynebacterium* (6.31%), *Finogoldia* (2.74%), No_Rank (2.15%),
 265 *Achromobacter* (2.09%), *Clostridium* (1.53%), *Tissierella* (1.32%),
 266 *Enterococcus* (1.26 %), and *Ralstonia* (1.08%) (FIG 10). These genera are
 267 diverse and include Gram-positive and Gram-negative bacteria, cocci and
 268 bacilli, aerobic and anaerobic bacteria, and so on.

Species

FIG 11. Species level differences between bacterial communities of CSOM

and cholesteatoma lesions

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276 FIG 12 Bar plot showing species composition of bacterial communities of

277 CSOM and cholesteatoma lesions

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279 Sequencing analysis at the species level demonstrated that the composition

280 of pathogenic bacterial species in cholesteatoma lesions was more complex

281 than that of CSOM lesions. And the types of pathogenic bacteria were

282 detected much more than CSOM. The statistical results is

283 uncultured-organism ($P=0.001476$), and uncultured-bacterium
 284 ($P=0.0043290$). The detection rate of *Pseudomonas aeruginosa* in the
 285 CSOM group was much higher than that in the middle ear cholesteatoma
 286 group ($P=0.000576$) (FIG 11, FIG 12).

287 **DISCUSSION**

288 Although the clinical manifestations of each type of otitis media are
 289 different, both CSOM and middle ear cholesteatoma require surgical
 290 treatment to cure the infection. There are limited differences in the clinical
 291 use of antibiotics for anti-infection treatment of two types of otitis media
 292 similar, and selection of an appropriate antimicrobial agent is solely based
 293 on ear secretion culture and drug sensitivity test. In practical clinical work,
 294 the results of bacterial culture of ear secretions often do not meet the
 295 purpose of curing otitis media (17). Clinicians seldom treat CSOM and
 296 middle ear cholesteatoma differently and do not select individualized

297 treatments. Thus, there are limited studies on the detailed bacteriological
 298 differences between chronic otitis media and middle ear cholesteatoma.

299 In almost all molecular biological studies on the bacteriology of chronic
 300 otitis media, secretion swabs are collected from the middle ear cavity or the
 301 outer ear canal for gene sequencing. However, the collected secretions can
 302 be contaminated by bacteria in the external auditory canal or can be
 303 affected by the acquisition process. This may lead to false positive results
 304 and thus these studies do not accurately reflect the true bacteriology of
 305 chronic otitis media (12,18,19). Simultaneously, the formation of bacterial
 306 biofilm, like in many chronic inflammatory diseases, severely limits the
 307 ability to obtain positive results by conventional bacterial culture.

308 Consequently, these analyses on chronic otitis media do not reflect the true
 309 clinical situation (20,21).

310 An alternative approach, utilizing gene sequencing to analyze unusual or
 311 dominant flora in the diseased tissue, may be beneficial for the study of the
 312 pathogenesis of otitis media. In this prospective study, high-throughput
 313 gene sequencing of diseased tissues of CSOM and cholesteatoma was
 314 performed). Bacterial species abundance and species diversity both differed
 315 in the two types of otitis media. Bacterial species abundance and diversity of
 316 the cholesteatoma group was greater than that of the CSOM group. This
 317 finding is congruent with previous studies (22). Collectively data from these
 318 studies suggest that the bacterial infection situation in middle ear
 319 cholesteatoma may be different and more complex than that of CSOM.

320 The current study revealed that the bacterial community composition in the
 321 middle ear lesion tissue of cholesteatoma was significantly more complex
 322 than that of CSOM. Lesions of the cholesteatoma group contained
 323 numerous Gram-positive and Gram-negative bacteria, which may be related

324 to the patient's long-term illness, with the bacteria forming a unique
 325 microenvironment, a coexistence relationship, and biofilm. In contrast, the
 326 bacteriology of CSOM lesions is relatively simple, with bacterial
 327 communities at all taxonomic levels (phyla, classes, orders, families, genera,
 328 and species) dominated by Gram-negative bacteria. These results indirectly
 329 explain the clinical phenomenon that broad-spectrum antibiotics are
 330 sometimes effective in patients with CSOM but have no effect on patients
 331 with middle ear cholesteatoma.

332 The findings of the current study differ from previous bacteriological studies
 333 on chronic otitis media which usually only produce one or two kinds of
 334 bacteria. This is due to difficulties in cultivating a variety of bacteria using
 335 conventional bacteriological techniques, meaning such studies cannot
 336 reflect the full picture of bacterial infection in patients. However, using
 337 high-throughput gene sequencing, the detection rate of pathogenic

338 bacteria in middle ear cholesteatoma lesions was significantly higher than
 339 that in CSOM lesions. This indicates that cholesteatoma has a much higher
 340 complexity of bacterial species and it is not an infectious disease caused by
 341 a single pathogenic bacterial species. Cholesteatoma may therefore
 342 constitute a bacterial microenvironment, whereby the combination of
 343 multiple conditional pathogens leads to the continuous onset of clinical
 344 symptoms of middle ear cholesteatoma, and a series of complications
 345 eventually emerges. This finding warrants further attention in future
 346 research.

347 Based on results of the current study, it is suggested that in future clinical
 348 work, antimicrobial agents are rationally selected according to the disease
 349 types of patients in addition to the clinical microbial culture and drug
 350 sensitivity test. Patients with CSOM could use broad-spectrum antibiotics
 351 against Gram-negative bacteria, but relying solely on antibiotics would

most likely be ineffective for middle ear cholesteatoma. After surgical removal of diseased tissue, appropriate antimicrobial agents against infection could be selected in combination with actual drug sensitivity tests. Furthermore, considering the complexity of the bacterial infection would avoid drug resistance and flora disorders caused by the excessive use of broad-spectrum antimicrobial therapy.

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