Otitis media microbes: culture, PCR, and confocal laser scanning microscopy


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Abstract. Otitis media microbes: culture, PCR, and confocal laser scanning microscopy. Objectives: To assess the presence of middle ear pathogens in nasopharynx (NP), middle ear fluid (MEF), and middle ear mucosal swabs (MES) of 14 patients undergoing middle ear surgery. Methodology: Bacteria were assessed by culture and species specific PCR. Biofilm was investigated by confocal laser scanning microscopy (CLSM) of middle ear biopsies (MEBs). Results: Bacteria were absent in CLSM of MEBs in three of the four closed and healthy middle ears. Bacteria occurred in the ear with a foreign body (middle ear prosthesis), which showed localized living and dead bacteria, indicating biofilm. Bacterial growth was present in ten patient ears, but biofilm occurred in only one patient. CLSM indicated biofilm in the middle ear of two patients for whom PCR detected Haemophilus influenzae in the MEF. The three classical pathogens could frequently be found in the nasopharynx, by culture and PCR, but not from the middle ear. Alloiococcus otitidis was detected in the MEF of all five patients with open inflamed ears, though virtually absent from the nasopharynx. Pseudomonas aeruginosa was present in seven. It was the only pathogen found on several occasions in all three locations in one patient. Conclusions: This study confirms the association of H. influenzae with middle ear biofilm, and indicates a potential role of P. aeruginosa in middle ear inflammation and biofilm formation. Biofilm does not seem to cause inflammation. It is unclear whether the predominance of A. otitidis in chronically inflamed open middle ears indicates a pathogenic or contaminant role for this organism.

Introduction

The concept of biofilm as a model of microbial pathogenesis has altered the understanding and treatment of various human infections. Biofilms are aggregates of microbial cells, living within an extracellular slime matrix. The matrix protects the bacteria from host defences and antibiotic treatment. Biofilms are the preferential bacterial phenotype for indolent, long-term persistence. Bacterial cells from biofilms are frequently difficult to culture, resulting in culture-negative, PCR-positive results. In the head and neck region, biofilms have been shown to be involved in chronic otitis media, otitis media with effusion, chronic tonsillitis, cholesteatoma, and device-associated infections.1 Biofilm formation has been documented for bacterial species that are important in otolaryngologic diseases, i.e. Haemophilus influenzae, Pseudomonas aeruginosa, Staphylococcus aureus and Streptococcus pneumoniae.2

In this study, we investigated the mucosal biofilm model in the middle ear pathology of 14 patients scheduled for middle ear surgery. We took middle ear biopsies and mucosal swabs, which were microbiologically analysed by means of culture, species specific PCR, and confocal laser scanning microscopy of Live/Dead stained biopsies.

Materials and methods

Patients and sampling

This study was a prospective case series, including a total of 14 patients scheduled for middle ear surgery. Their categorization into seven groups is listed in Table 1. The open ears were from patients with eardrum perforations, or patients with chronic otitis media without cholesteatoma. There was no otorrhea at the moment of surgery, nor had the patients received any antibiotic treatment (topical or systemic) in a period of four weeks before surgery. All patients provided written consent and were under general anaesthesia during surgery.
closed ears, disinfection was routinely performed before draping the patient. In the open ears, care was taken that no disinfectant entered the outer ear canal. After elevation of the tympanic membrane, middle ear effusions (MEF) were collected using a Juhn tym-tap (Medtronic-Xomed, Jacksonville, Fl). When no effusion was present, the middle ear was rinsed with saline and aspirated, with the aspirated rinse serving as the MEF sample.

Using an operating microscope (Carl Zeiss Inc, Thornwood, NY) and cupped forceps, middle ear mucosal biopsies (MEB) of a few square millimetres were taken at the level of the hypotympanum. The protocol was approved by the ethics committee of the University Hospital of Ghent and was in accordance with the ethical standards stipulated in the 1964 Declaration of Helsinki for research involving human subjects.

Middle ear and nasopharyngeal samples (obtained transnasally) were taken with a flexible sterile calcium alginate swab (Medical Wire & Equipment, Wiltshire, UK).

**Culture**

Upon collection, the samples were immediately transported to the microbiology laboratory.

The nasopharyngeal swab and the middle ear mucosal swab were placed in separate Eppendorf tubes containing 400 µl of physiological water and homogenized by swirling for 30 seconds. The effusion fluid was collected in an Eppendorf tube, and if the volume was lower than 400 µl, physiological water was added up to a total volume of 400 µl.

Fifty µl of each sample were cultured on two different media: i) Tryptic soy agar + 5% sheep blood, and ii) Gonococcal Culture (GC) II agar with Isovitalex enrichment (Becton Dickinson, Sparks, MD), in both aerobic and anaerobic growth conditions. Anaerobic conditions were obtained by using airtight jars and the Gaspack anaerobic envelope system (Becton Dickinson). One representative of each colony type was isolated, and DNA was extracted by alkaline lysis.³

**Identification of the cultured bacteria**

The bacterial isolates were identified by tRNA-intergenic length polymorphism analysis (tDNA-PCR), followed by fragment analysis in ABI310 capillary electrophoresis.⁴ Those bacteria that could not be identified by tDNA-PCR were analysed by sequencing of the 16S rRNA gene, followed by comparison with a selection of quality-controlled sequences in Genbank, using the SmartGene IDNS™ bacteria software module (SmartGene, Zug, Switzerland).

**Species-specific PCRs for identification**

The homogenized samples were also used for species-specific PCRs. Genomic DNA extraction of the samples (QiAamp DNA mini Kit, Hilden, Germany) was followed by species-specific PCRs for four species, which have been described to occur frequently and/or play a pathogenic role in middle ear infections, i.e. *Alloïococcus otitidis*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae*, as described by Hendelin et al.³ The fragment lengths amplified for each species were 264, 525, 237, and 484 bp, respectively. Because the fragment lengths of *A. otitidis* and *M. catarrhalis* migrate close to each other in agarose gel electrophoresis, and because in our hands the multiplex PCR format yielded false negative results when artificial mixtures of DNA-extracts of these organisms were used, we opted to apply two duplex PCRs instead of one quadruplex PCR. One PCR amplified *A. otitidis* and *H. influenzae* (fragment lengths of 237 and 525 bp, respectively). The other PCR amplified *M. catarrhalis* and *S. pneumoniae* (264 and 484 bp, respectively). In addition, species-specific PCRs were carried out for *Staphylococcus aureus*,⁶ *Pseudomonas aeruginosa*,⁷ and *Streptococcus pyogenes*.⁸

**Confocal laser scanning microscopy of the middle ear biopsies**

The MEBs were placed immediately on ice, and stained by means of the Live/Dead BacLight Bacterial Viability Kit (Invitrogen, Carlsbad, CA). The staining was carried out with SYTO9, which enters both living and dead bacteria (green), and propidium iodide, which only enters cells with damaged cell walls (red).⁹ The MEB was put in an eppendorf tube of 1.7 ml and submerged in 30 µl of the Live/Dead stain working solution. The mixture was incubated for 15 minutes in the dark at room temperature. The staining solution was removed by pipette, and the biopsy was washed twice with 100 µl phosphate buffer. The biopsy was put on a glass slide with Vectashield containing DAPI (1.5 µg/ml) (Vector Laboratories, Burlingame, CA), which stains
nucleic acids blue. CLSM was carried out on a Radiance 2100 blue CLSM system mounted on an inverted confocal microscope (Bio-Rad, Hercules, CA), controlled by the Lasersharp 2000 software (Bio-Rad). Analysis of the images was done by ImageJ software (http://rsb.info.nih.gov/ij).

Results

Table 1 shows the categorisation of the 14 patients into seven groups, based on the surgeon’s clinical interpretation of the conditions of their middle ears. Seven patients, all scheduled for myringoplasty, presented with open ears, of which five were considered inflamed (OI), one with normal middle ear mucosa (ON), and one with a middle ear with mucus, but without signs of inflammation (OM). Another seven patients, mostly scheduled for reconstructive surgery after previous surgery, presented with clinically healthy closed ears, of which only one middle ear was considered as inflamed (CI), three had normal mucosa (CN, healthy controls), one contained a foreign body in the middle ear (CFB), and two had mucous secretions (CM). The three CN subjects were taken as the healthy control group.

A selection of confocal laser scanning microscopy (CLSM) images of middle ear biopsies (MEB) is presented in Figure 1. MEBs were stained using universal DAPI nucleic acid stain (blue) and bacterial Live/Dead (green/red) staining. CLSM of the MEBs showed the absence of bacteria in the three healthy controls (Figure 1A). Bacterial growth, generalized (Figure 1B) or localized (Figure 1C), was present in all ears of the patient group. Bacterial growth was also present in the only patient with normal middle ear mucosa (ON1), who was also the only one in the patient group for whom biofilm could be observed. Also, for the sample that resembled a clinically healthy ear but presented with a foreign body (CFB), CLSM showed the localized presence of living as well as dead bacteria (Figure 1D), interpreted as the presence of biofilm.

In PCR of MEF, MES, and NP, the three classical pathogens were amplified from four patients, and were even simultaneously present in three of them. None of those pathogens were found in the MES. *S. pneumoniae* was not detected in MEF and MES. *H. influenzae* was detected only twice, in the MEF of two of the five subjects with normal mucosa, who were also the only two subjects (ON1 and CFB) for whom biofilm could be documented. *M. catarrhalis* was detected only once in MEF, in association with *H. influenzae*.

In contrast, *A. otitidis* was detected in the MEF of all five OI patients, in the MES of two of these, and further, in the MEF of a patient with closed ear and glue in the middle ear. *A. otitidis* was present only in the NP of subject CFB, with a closed ear, normal mucosa, and the presence of a foreign body. The three classical ORL pathogens were present in the NP of three subjects (OI4, OI5, and CM1) for whom *A. otitidis* could be detected in the middle ear.

Species-specific PCR of other pathogens demonstrated a high prevalence of *Pseudomonas aeruginosa*, which was found in seven of the MEF samples, i.e. 4/7 open ears and 3/7 closed ears. *P. aeruginosa* was the only species that could be (and was usually) encountered in all sites that were sampled. *P. aeruginosa* was also associated with *H. influenzae* and biofilm in both subjects where biofilm was observed. *P. aeruginosa* and *A. otitidis* were the most frequently found species in MEF samples, with *A. otitidis* more clearly associated with open ears. *Staphylococcus aureus* was detected only twice in MEF, both from open ear samples. *Streptococcus pyogenes* was found in only one nasopharyngal sample (OI4), in which four of the other six species were also present.

Aerobic and anaerobic cultures on two enriched media were carried out with MEF of 12 subjects and MES of 10 subjects. A total of 190 different colonies were picked from all samples. As expected, many different species were cultured from the nasopharynx, but no straightforward correspondence with culture results from the middle ear could be established. Culture results for MEF and MES are presented in Table 2. The MEF of six out of 11 subjects tested and the MES of three out of 12 MES yielded no growth. Four subjects had both MEF and MES free of bacteria, according to culture. *P. aeruginosa* was cultured twice from patients with normal mucosa (one with an open ear, one closed). *Turicella otitidis* was cultured once from MEF and once from MES, both from patients with inflamed ears. *Actinomyces turicensis* was cultured from MEF and MES for the only patient with a closed but inflamed ear (CI1). All other isolates from both the patient and control groups, including closed ears, belonged to different *Staphylococcus* species.
### Table 1

Patient data, patient categorization, and the results of the species-specific PCR

<table>
<thead>
<tr>
<th>Category</th>
<th>Patient</th>
<th>Age (yr)</th>
<th>PCR MEF</th>
<th>PCR MES</th>
<th>PCR NP</th>
<th>CLSM of MEP</th>
<th>Clinical interpretation</th>
<th>Reason for surgery</th>
</tr>
</thead>
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<tr>
<td>OI</td>
<td>OI1</td>
<td>13</td>
<td>AO, PA</td>
<td>NT</td>
<td>NT</td>
<td>G-L</td>
<td>glue, inflammation of the mucosa</td>
<td>myringoplasty</td>
</tr>
<tr>
<td>OI</td>
<td>OI2</td>
<td>14</td>
<td>AO</td>
<td>N⁰</td>
<td>NT</td>
<td>G-O</td>
<td>inflammation of the mucosa</td>
<td>myringoplasty</td>
</tr>
<tr>
<td>OI</td>
<td>OI3</td>
<td>24</td>
<td>AO, PA</td>
<td>PA</td>
<td>PA</td>
<td>G-O-L</td>
<td>mild inflammation of the mucosa, badly aerated middle ear</td>
<td>myringoplasty</td>
</tr>
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<td>OI</td>
<td>OI4</td>
<td>7</td>
<td>AO</td>
<td>AO</td>
<td>HI, MC, SP, SA, SPY</td>
<td>G-L</td>
<td>glue, inflammation of the mucosa</td>
<td>myringoplasty</td>
</tr>
<tr>
<td>OI</td>
<td>OI5</td>
<td>6</td>
<td>AO, SA</td>
<td>AO, SA</td>
<td>HI, MC, SP, PA</td>
<td>G-L</td>
<td>glue, inflammation of the mucosa</td>
<td>myringoplasty</td>
</tr>
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<td>OM</td>
<td>OM1</td>
<td>8</td>
<td>PA</td>
<td>PA</td>
<td>HI, SP, PA</td>
<td>G-O⁻</td>
<td>glue</td>
<td>myringoplasty</td>
</tr>
<tr>
<td>ON</td>
<td>ON1</td>
<td>9</td>
<td>HI, MC, PA, SA</td>
<td>NT</td>
<td>NT</td>
<td>G/R-L</td>
<td>normal mucosa</td>
<td>myringoplasty</td>
</tr>
<tr>
<td>CI</td>
<td>CI1</td>
<td>55</td>
<td>N</td>
<td>N</td>
<td>NT</td>
<td>G-L</td>
<td>inflammation of the mucosa</td>
<td>mastoiditis</td>
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<tr>
<td>CM</td>
<td>CM1</td>
<td>14</td>
<td>AO</td>
<td>N</td>
<td>HI, MC, SP</td>
<td>G-L</td>
<td>glue</td>
<td>second look + reconstruction</td>
</tr>
<tr>
<td>CM</td>
<td>CM2</td>
<td>5</td>
<td>PA</td>
<td>PA</td>
<td>NT</td>
<td>G-L</td>
<td>glue</td>
<td>second look</td>
</tr>
<tr>
<td>CN</td>
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<td>N</td>
<td>NT</td>
<td>NT</td>
<td>B</td>
<td>healthy ear</td>
<td>second look + reconstruction</td>
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<td>18</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>B</td>
<td>healthy ear</td>
<td>second look</td>
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<tr>
<td>CN</td>
<td>CN3</td>
<td>13</td>
<td>PA</td>
<td>NT</td>
<td>NT</td>
<td>B</td>
<td>healthy ear</td>
<td>second look</td>
</tr>
<tr>
<td>CFB</td>
<td>CFB</td>
<td>51</td>
<td>HI, PA</td>
<td>PA</td>
<td>AO, SA, PA</td>
<td>G/R-L</td>
<td>normal mucosa, presence of foreign body</td>
<td>middle ear exploration (contaminated prosthesis)</td>
</tr>
</tbody>
</table>

Legend:
- a) C: closed ear; O: open ear, i.e. any perforation of the tympanic membrane; I: inflamed, i.e. presence of discharge and inflammation of the mucosa; M: mucoid, i.e. presence of mucoid secretions; N: neutral, no discharge; FB: foreign body
- b) MEF: middle ear fluid; MES: middle ear mucosal swab; NP: nasopharyngeal swab
- c) N: negative PCR; NT: not tested due to either no sample received or no DNA-extraction carried out; AO: Alloiococcus otitidis; HI: Haemophilus influenzae; MC: Moraxella catarrhalis; PA: Pseudomonas aeruginosa; SA: Staphylococcus aureus; SP: Streptococcus pneumoniae; SPY: Streptococcus pyogenes
- d) CLSM: confocal laser scanning microscopy; MEP: middle ear biopsy
- e) B: blue (DAPI stain, i.e. no bacterial cells); G: green (living bacterial cells); R: red (dead bacterial cells); G/R: both green and red (living and dead) cells; L: localized; O: overall
Microbial analysis of otitis media

No streptococci could be cultured. In general, few bacteria and none of the four pathogens could be cultured from middle ears or from open ears.

Discussion

The mucosal biofilm paradigm of middle ear disease is a model that may explain conflicting observations. For some types of middle ear disease, antibiotic treatment is not efficient and no bacteria can be cultured, although bacterial DNA and RNA can be detected in the middle ear fluid.7

In this study, we attempted to demonstrate the presence of biofilm on the middle ear mucosa by CLSM of Live/Dead stained middle ear biopsies, taken from 14 patients undergoing middle ear surgery for various reasons. We also evaluated the presence of bacteria by tDNA-PCR based identification of all the colony types that could be cultured from nasopharynx (NP), middle ear fluid (MEF), and middle ear mucosal swabs (MES). Species-specific PCR was carried out on samples from NP, MEF and MES for the four species previously reported to be frequently related to middle ear pathology, i.e. *Alloccoccus otitidis*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae*, and for *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pyogenes*, which also have been related to otitis media.10

CLSM confirmed the presence of bacteria in all 10 patients and the absence of bacteria in the three subjects of the control group. By taking the presence of dead bacterial cells as the indication of biofilm growth,11 biofilm could be shown only twice. Interestingly, the only two samples presenting biofilm were from the only two patients with normal mucosa, one of whom had a foreign body in the ear. Also, these were the only two subjects with *Haemophilus influenzae* in the middle ear, confirming the association between this organism and biofilm in the middle ear.12,13 Both subjects also had *Pseudomonas aeruginosa* in the middle ear. However, *P. aeruginosa* was found in five other middle ears, and also frequently in the nasopharynx, making it less specifically associated with biofilm.

Furthermore, PCR indicated the absence of the three classical pathogens from all six inflamed middle ears (five open, one closed). This result was in contrast to a strong association of *A. otitidis* with inflamed open ears, i.e. present in all five open inflamed middle ears. In addition, no correlation could be found between PCR results from the nasopharynx and those from the middle ear. The three classical pathogens were frequently found in association with each other in the nasopharynx (i.e. in four out of the seven subjects for whom PCR on NP was carried.
A. otitidis was detected only once in the nasopharynx, moreover, from a patient with normal middle ear mucosa. The combined results on A. otitidis in this study, i.e. prevalence in effusion fluids of six out of nine (100% of those with open inflamed ears) and nasopharyngeal prevalence of one out of 14, correspond to published results, which are summarized in Table 3. Culture of MEF and MES indicated the absence of all four pathogens. However, it should be taken into consideration that A. otitidis is very difficult to culture. P. aeruginosa was cultured from two of the seven middle ears in which its presence had been established by PCR. S. aureus was cultured twice as well, but not from the single middle ear from which its presence had been established with PCR. As has been reported previously, the culture of NP yielded a diverse microflora with no correlation to bacteria cultured from the middle ear.

**Conclusion**

The use of species-specific PCR enabled us to demonstrate a high incidence of A. otitidis in chronically inflamed middle ears. A. otitidis was predominantly present in samples collected from middle ears with open eardrums. Further research should elucidate whether this organism has a pathogenic role or is merely an outer ear contaminant. The established route of middle ear infection is via the Eustachian Tube, which demands nasopharyngeal colonization prior to ear infection, while A. otitidis appears to be absent from the nasopharynx.

Confocal laser scanning microscopy demonstrated biofilm in only two middle ear biopsies. Both samples were negative for A. otitidis species-specific PCR, but were the only two middle ear biopsies that were not taken from middle ear mucosa with open eardrums.

### Table 2

Culture results for middle ear effusion fluid and middle ear mucosal swabs, after identification with tDNA-PCR

<table>
<thead>
<tr>
<th>Patient</th>
<th>No growth</th>
<th>Not tested</th>
<th>Achromobacter xylosoxidans</th>
<th>Acinetobacter sp.</th>
<th>Actinomyces turgidus</th>
<th>Corynebacterium species</th>
<th>Pseudomonas aeruginosa</th>
<th>Staphylococcus capitis</th>
<th>Staphylococcus epidermidis</th>
<th>Staphylococcus hominis</th>
<th>Staphylococcus warneri</th>
<th>Staphylococcus sp.</th>
<th>Turicella otitidis</th>
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<tbody>
<tr>
<td>O11</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>S</td>
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<tr>
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<td>F</td>
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<td></td>
<td></td>
<td></td>
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<td>S</td>
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<td></td>
<td>S</td>
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<tr>
<td>O13</td>
<td>F</td>
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<td></td>
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<td></td>
<td></td>
<td>S</td>
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<td>S</td>
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<tr>
<td>O14</td>
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</tbody>
</table>

**Legend:**

a) See legend of Table 1 for patient abbreviations
b) F: present in MEF; S: present in MES; FS: present in both MEF and MES.
samples positive for *H. influenzae*, adding further evidence for the role of *H. influenzae* in middle ear biofilm formation.

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References


