Mastoid Biofilm in Chronic Otitis Media

Henri Lampikoski, Antti A. Aarnisalo, Jussi Jero, and Teemu J. Kinnari

Department of Otorhinolaryngology–Head and Neck Surgery, Helsinki University Central Hospital, Helsinki, Finland

Hypothesis: We designed a study to determine the role of mastoid mucosal biofilm in chronic otitis media (COM).

Background: Biofilm formation has been found in several chronic airway infections. COM is associated with chronic, recalcitrant infection of the mastoid mucosa, and surgery often is required.

Methods: COM patients were divided into 2 groups: one with chronic suppurative otitis media (CSOM) and one with cholesteatoma presence. All COM patients had mastoid involvement in a preoperative computed tomographic scan. The control group consisted of patients undergoing cochlear implantation, with no previous history of chronic otitis media. Mastoid mucosa samples were harvested during mastoidectomy. The samples were studied with multiplex-polymerase chain reaction and with CSLM using BacLight Live/Dead stain. Routine bacterial culture was performed in selected cases.

Results: A total of 29 COM patients underwent mastoidectomy. Mastoid mucosal biofilm formation could be found in 19 (66%) of these patients. In the control group, there were 11 cases of cochlear implantation, and 1 patient (9%) presented mastoid mucosal biofilm. In the cholesteatoma group, there were 17 patients, of which, 14 (82%) presented biofilm, whereas in the CSOM group, 5 (42%) of 12 patients presented biofilm. The correlation between COM and biofilm was statistically significant (Fisher’s exact test, \[ p = 0.003 \]), as was the correlation between cholesteatoma and biofilm, in comparison with the CSOM group (Fisher’s exact test, \[ p = 0.046 \]).

Conclusion: Mastoid mucosal biofilm could be seen in patients with COM with or without cholesteatoma. The role of mastoid biofilm in the development of cholesteatoma should be studied further.

Key Words: Biofilm—Cholesteatoma—Chronic otitis media—Chronic suppurative otitis media—Confocal—Confocal scanning laser microscopy—Mastoid—Mastoiditis—Otitis—Polymerase chain reaction.


Biofilms are 3-dimensional polymicrobial colonies that grow attached to both nonbiological and biological surfaces, including human tissue. These polymicrobial colonies protect themselves from environmental conditions and the defenses of the host organism, using mechanisms such as the exopolysaccharide matrix (1). Conventional antibiotic treatments often prove insufficient in the eradication of biofilm infection (2). The presence of biofilm infection has been demonstrated in several chronic and recalcitrant infections, including airway diseases like cystic fibrosis, chronic rhinosinusitis, and chronic otitis media (COM) (3–5). Furthermore, biofilm growth has been linked with tenacious forms of infection with potential for devastating inflammatory response (6).

There are previous studies that use confocal scanning laser microscopy (CSLM) and scanning electron microscopy techniques to demonstrate a connection between biofilm presence and chronic suppurative otitis media (CSOM) without cholesteatoma (7), as well as studies based on scanning electron microscopy imaging that show evidence of biofilm presence in CSOM with or without cholesteatoma (8,9). The difference between imaging methods in biofilm diagnostics has been studied, and CSLM has been found to be the most objective technique for biofilm detection (10).

In the present work, our intention was to study and compare the presence of mastoid mucosal biofilm in COM with or without cholesteatoma in a prospective, controlled setting, using CSLM and multiplex polymerase chain reaction (PCR).

MATERIALS AND METHODS

Patients

The study protocol was approved by the ethics committee of Helsinki University Central Hospital. Before each procedure patients signed an informed consent.

A prospective, clinically controlled study was performed on 40 patients. These included 19 female and 21 male patients. Patient samples were collected from August 2009 to June 2010.
There were 29 patients who underwent mastoidectomy for the treatment of COM. The control group consisted of 11 patients with no previous history of ear infection, undergoing cochlear implantation for the treatment of sensorineural hearing loss. We further divided the 29 COM cases into 2 groups: one comprising 17 patients with middle-ear or mastoid cholesteatoma presence and another consisting of 12 patients with no cholesteatoma presence but with CSOM and mastoid involvement. In preoperative computed tomographic imaging of the 17 cholesteatoma patients, 2 cholesteatomas presented only epitympanic involvement, and 15 cholesteatomas extended to the mastoid cavity. There were no congenital cholesteatomas among these patients. In the CSOM group, all 12 patients showed mastoid opacification in a preoperative computed tomographic scan. The mean age of patients was 34.3 years for the cholesteatoma group, 37.9 years for the CSOM group, and 27.3 years for the control group. Two representative mastoid mucosa samples were obtained from each patient during the mastoidectomy operation. Immediately after collection, the mastoid mucosa samples were stored in Eppendorf tubes and snap frozen in liquid nitrogen, until the date of further analysis. One sample was studied with CSLM, and the other sample was sent for PCR analysis. An intraoperative routine bacterial culture was performed on selected patients when deemed necessary by the surgeon.

**CSLM Technique**

In preparation for the CSLM, the mucosa samples were thoroughly washed 3 times in mQ water to rinse away any planktonic bacteria, then stained for 10 minutes in dark conditions with BacLight Live/Dead stain (Molecular Probes, Leiden, The Netherlands), and finally rinsed again in three washes with mQ water. The samples were viewed with a Leica TCS SP2 (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) confocal scanning laser microscope, using water immersion objectives with ×63 magnification. The BacLight Live/Dead stain provides generic nucleic acid labeling, which stains living cells green (Syto 9) and dead or damaged cells red (propidium iodide). The detection of biofilm structures was based on morphologic analysis of the samples, noting features that are characteristic to biofilms, such as microcolony formation and presence of exopolysaccharide matrix. Bacteria were recognized by their size, shape, and green fluorescent stain. Image analysis was performed by 2 independent observers (H. L. and T. J. K.). Both observers were blinded to the disease state of the patient (Fig. 1).

**Bacterial Culture**

Various biochemical tests for bacterial identification were undertaken according to Clinical and Laboratory Standards Institute guidelines using Mueller-Hinton II agar base (catalogue number 212257; Becton, Dickinson and Company, Sparks, MD, USA) and antibiotic discs (Oxoid, Basingstoke, UK), at 35°C.

**PCR Technique**

**DNA Extraction**

Samples were pretreated by adding 10 to 30 µl of 100 mM DTT (Fluka Chemie GmbH, Buchs, Switzerland) and incubating for 30 minutes at 37°C with 300 rpm agitation. Thereafter, 20 µl of protease K (Roche, Mannheim, Germany) was added to the samples and incubated for 1 hour 50 minutes at 60°C with 300 rpm agitation. To enhance cell lysis, 20 µl of the lysis buffer (Arrow VIRAL NA kit, Nordiag, Norway) was mixed with the samples and incubated for 10 minutes at 60°C with 300 rpm agitation. After the lysis step, the initial sample volume was brought up to a volume of 200 µl with 1× PBS. DNA extraction was conducted using the Arrow VIRAL NA kit, the NorDiag Arrow device, and the Viral 010 program according to the instructions of the manufacturer (NorDiag). Elution volume was 100 µl. One negative extraction control was included in the test series.

**Analysis With PCR and Microarray-Based Prove-it Assay**

DNA samples were analyzed with the modified Prove-it sepsis assay in conjunction with the StripArray Reader and the Prove-it advisor analysis software (Mobidiag, Helsinki, Finland) (11,12). The PCR reactions were conducted according to the instructions of the Prove-it sepsis assay using the application for bone and joint infections. The amplified PCR products were hybridized onto the microarray, and the hybridization protocol was adapted from the instructions of the Prove-it sepsis StripArray with slight modifications. To evaluate the reliability and success of the analysis, the negative DNA extraction and negative PCR controls were required to be negative, and the positive PCR control (DNA from methicillin-resistant *Staphylococcus aureus*) was required to be positive for both *S. aureus* and the mecA marker for the acceptance of the results of a particular test series.

**DNA Sequencing**

DNA sequencing was conducted to the PCR products amplified in the Prove-it assay but which were negative in the microarray analysis. The PCR products of 300 bp were run and extracted from the 2% agarose gel using the QiAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Sequencing was performed using cycle sequencing with Big Dye Terminator kit (version 3.1) supplied by Applied Biosystems (ABI, CA, USA), and the reactions were run on ABI 3130xl capillary sequencer according to the manufacturer’s instructions. Sequences were
TABLE 1. Confocal scanning laser microscopy results regarding biofilm presence

<table>
<thead>
<tr>
<th></th>
<th>Chronic otitis media (n = 29)</th>
<th>Controls (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chronic supplicative otitis media (n = 12)</td>
<td>Cholesteatoma (n = 17)</td>
</tr>
<tr>
<td>Biofilm positive</td>
<td>5 (42%)</td>
<td>14 (82%)</td>
</tr>
<tr>
<td>Biofilm negative</td>
<td>7 (58%)</td>
<td>3 (18%)</td>
</tr>
</tbody>
</table>

We analyzed the microbiologic profile of all cases, where biofilm could be seen with CSLM. According to these results, multiplex PCR and bacterial culture are not reliable methods for the detection of biofilm presence.

TABLE 2. Microbiologic profiles according to PCR and bacterial culture

<table>
<thead>
<tr>
<th>Biofilm positive cases (n = 20)</th>
<th>Positive PCR or bacterial culture</th>
<th>Negative PCR and bacterial culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1. Klebsiella oxytoca and Escherichia coli both in culture and PCR</td>
<td>n = 14</td>
</tr>
<tr>
<td></td>
<td>2. Haemophilus influenzae in culture and coagulase negative staphylococci in PCR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. Staphylococcus aureus in culture and Staphylococcus epidermidis in PCR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4. Staphylococcus aureus in culture and coagulase negative staphylococci in PCR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5. Pseudomonas aeruginosa in culture</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6. Pseudomonas aeruginosa in culture</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biofilm negative cases (n = 20)</th>
<th>Positive PCR or bacterial culture</th>
<th>n = 18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1. Staphylococcus aureus in culture</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. Candida parapsilosis in PCR (control case)</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

Our study shows that there is a correlation between chronic supplicative otitis media (including both chronic supplicative otitis media and cholesteatoma disease) and mastoid mucosal biofilm infection. There is a higher incidence of biofilm in cholesteatoma disease than in chronic supplicative otitis media, the difference being statistically significant.

The pathogenesis of acquired cholesteatoma disease has been studied extensively in the past, and the mechanisms involved are still a matter of ongoing research. One of the main etiologic theories for acquired cholesteatoma has been described as pneumatization failure or inflammatory conditions of the middle ear and mastoid cavity, leading to reduced middle-ear pressure, retraction pocket formation, epidermal migration failure, and eventually, cholesteatoma formation, along with associated complications, such as bone resorption of the ossicular chain and otic capsule. The study of the role of diseased mucosa in the development of retraction pockets and cholesteatoma formation has been gaining prominence as of late (15). Bacterial biofilm infection has been linked with severe inflammatory changes of the host mucosa, as well as ventilation failure of the middle ear (16). *Pseudomonas aeruginosa*, a common biofilm forming ear pathogen, has been linked with osteoclastic remodeling (17). In our study, there is a higher incidence of biofilm presence in ears with cholesteatoma than in ears with chronic supplicative otitis media. One could hypothesize that the cholesteatoma tissue functions as a beneficial substrate for biofilms to settle on, but the possibility of biofilm infection causing favorable conditions for cholesteatoma pathogenesis also should be considered in further research.

There was a disparity between the results of CSLM and both microbiologic methods (PCR and routine bacterial culture). These results are explained in Table 2. A microbiologic profile could be determined using multiplex PCR or routine bacterial culture in 6 of 20 cases where biofilm could be seen with CSLM. According to these results, multiplex PCR and bacterial culture are not reliable methods for the detection of biofilm presence.
We found biofilm presence in the mastoid mucosa of 1 healthy control patient undergoing cochlear implantation. Although not statistically significant, this finding should be considered in light of its implications for cochlear implantation. There is a previous report of an explanted cochlear implant with biofilm presence (18), as well as research showing that the incidence of pneumococcal meningitis in cochlear implant recipients is greater than that in age-matched general population (19).

There is an ongoing research effort in the field of biofilm treatment, and some pharmacologic options seem to hold a promise. Exopolysaccharide-dissolving agents in combination with antibiotics have been studied (20), as have quorum sensing inhibiting agents (21,22) and long-term antibiotic treatment (23).

In the light of current knowledge, surgical revision and aeration of both chronic suppurative otitis media ears and ears affected with cholesteatoma holds its place as the treatment of choice. The biofilm findings of our study support the current treatment modalities.

In conclusion, here is a statistically significant correlation between mastoid mucosal biofilm infection and COM. Furthermore, the incidence of biofilm in COM is even higher when cholesteatoma growth is present. The association between mastoid mucosal biofilm and chronic suppurative otitis media with or without cholesteatoma has not been studied before in a prospective, controlled trial. Further research regarding the role of biofilm infection in the pathogenesis of cholesteatoma should be encouraged.

REFERENCES