

ORIGINAL ARTICLE

Demonstration of Bacterial Biofilms in Chronic Otitis Media

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OBJECTIVE: To establish the presence of biofilms in surgical tissue specimens from patients with chronic otitis media.

MATERIAL AND METHODS: 22 patients with chronic otitis media scheduled for tympanomastoid surgery were enrolled in this study between September 2007 and January 2008. Biopsies of the middle ear mucosa and cultures were taken at the time of surgery. Tissues were cultured using conventional methods for *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. Bacteria identification was performed using the Becton Dickinson automatic identification system. Slime forming ability was tested on congo red agar for culture positive bacteria. The presence of *icaA* and *icaD* DNA were detected by polymerase chain reaction using forward and reverse primers for *icaA* and *icaD* for *staphylococcus aureus*.

RESULTS: 6 of 22 patients' tissue specimens were culture-positive(72.7%). 5 *Staphylococcus aureus* and 11 *Pseudomonas aeruginosa* were identified on 16 specimens. Bacterial biofilms were present on 9 of 16(56.2%) culture-positive specimens. 2 of 5(40%) *Staphylococcus aureus* and 7 of 11(63.6%) *Pseudomonas aeruginosa* produced bacterial biofilms.

CONCLUSION: *Pseudomonas aeruginosa* was the most commonly bacteria in chronic otitis media. Biofilm forming ability was higher in *Pseudomonas aeruginosa* compared with other bacteria. The presence of biofilms on the mucosa of patients with chronic otitis media offers a possible cause of antimicrobial therapy failure.

Biofilms are increasingly recognised as playing a role in diseases of otolaryngologic interest. Biofilms are organised, heterogeneous bacterial communities. Biofilm bacteria are embedded in a matrix rich in polysaccharides, nucleic acids, and proteins known as the extracellular polymeric substances^[1]. This matrix guarantees better survival and protection from macrophage action, antibiotics, temperature and pH fluctuations. Bacterial biofilms are 10-1000 times or more resistant to antibiotic treatment when compared with genetically identical planktonic bacteria^[2].

Bacterial biofilms play an important role in chronic otitis media, chronic tonsillitis, cholesteatoma, and device-related infections. Bacterial biofilms identified in various medical devices used in otorhinolaryngology, including tympanostomy tubes, voice prostheses, and cochlear implants^[3,4]. The upper airways seem to be at high risk for this type of colonisation. Furthermore, it has been demonstrated that several bacterial species are able to develop a biofilm, including the most frequent organisms responsible for otorhinolaryngologic disorders such as *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Staphylococcus aureus*^[5].

COM infections may recur even though they are treated with sensitive antibiotics. These observations have led us to hypothesize that bacteria from biofilms within the chronically infected middle ear can resist eradication by antibiotics and host defenses.

The aim of this study was to establish the presence of biofilms in surgical tissue specimens from patients with chronic otitis media.

MATERIAL AND METHODS

We examined 22 samples of middle ear tissues obtained during tympanomastoid surgery from 19 adults (mean age 35 years) and three children (mean age ten years) between September 2007 and January 2008. This study was approved by the ethics committee of the hospital and patients gave their written informed consent before participation. All 22

patients were suffering from chronic otitis media infections documented by clinical findings and the results of computed tomography (CT) scans. The infections had proved to be refractory to repeated cycles of antibiotic regimens with demonstrated in vitro potency, and surgical treatment was thus being planned. Biopsies of the middle ear mucosa and cultures were taken at the time of surgery. Tissues were cultured using conventional methods.

Characterization of bacterial strains: After collection, middle ear specimens were immediately transported to the clinical microbiology laboratory for Gram staining and for bacterial culture. Samples were plated on blood agar, chocolate agar, EMB agar and incubated in aerobic conditions at 37 C for 48 hours. Bacteria identification was performed using the Becton Dickinson automatic(Phoenix 100) identification system.

Phenotypic characterization of slime-producing bacteria: Qualitative detection of biofilm formation by all slime-producing strains was studied by culturing the strains on Congo red agar plates (CRA; Sigma Chemical Company, St Louis, MO, USA). Inoculated CRA plates, made by mixing 0.8 g Congo red with 36 g saccharose (Sigma) in 1 L of brain heart infusion agar, were incubated for 24 h at 37 C under aerobic conditions and followed overnight at room temperature. Slime-positive variants appear as reddish-black colonies with a rough, dry and crystalline consistency on CRA, whereas slime negative strains develop pinkish-red, smooth colonies with a darkening at the centre. Biofilm formation was confirmed for positive and negative controls. Two *S. epidermidis* reference strains were used, the well-known slime-producing strain ATCC 35984 (RP62A) and the non-slime-producing strain ATCC 12228. *S. aureus* LSPQ 2520 (Positive), *P. aeruginosa* LSPQ 3332 (Positive), *P. aeruginosa* ATCC 10145 (Negative).

Detection of *icaA* and *icaD* loci

The presence of *icaA* and *icaD* DNA were detected by polymerase chain reaction(PCR) using forward and reverse primers for *icaA* and *icaD* for *Staphylococcus aureus*. For *icaA*, the forward primer (corresponding to

nucleotides 1337-1356) had the following sequence: 50-TCT CTT GCA GGA GCA ATC AA-30; and the reverse primer (corresponding to nucleotides 1505-1524) had the following sequence: 50-TCA GGC ACT AAC ATC CAG CA-30. The primer sequences for *icaD* (187-bp amplicon size) were: forward (nucleotides 1963-1982), 50-ATG GTC AAG CCC AGA CAG AG-30; and reverse (nucleotides 2138-2160), 50-CGT GTT TTC AAC ATT TAA TGC AA-30. Intercellular adhesion genes (*icaADB*) are defined as biofilm mediating operon.

RESULTS

16 of 22 patients' tissue specimens were culture-positive (72.7%). 5 *Staphylococcus aureus* and 11 *Pseudomonas aeruginosa* were identified on 16 specimens. Bacterial biofilms were present on 9 of 16 (56.2%) culture-positive specimens. 2 of 5 (40%) *Staphylococcus aureus* and 7 of 11 (63.6%) *Pseudomonas aeruginosa* produced bacterial biofilms (Table-1). The PCR technique was applied to the 5 staphylococcal strains to detect *icaA* and *icaD*. All strains that were positive for *icaA* were also positive for *icaD*. Among the 4 strains positive for the *ica* operon, 2 (50%) were biofilm positive on CRA, 2 were biofilm negative.

DISCUSSION

Microbial biofilms seem to play important roles in a large number of human infections. Biofilms can directly colonise mucosal tissues, producing chronic or recurrent infections that are resistant to all types of antibiotic treatment. The upper airways seem to be at high risk for this type of colonisation.

Biofilm formation is a dynamic process that begins with the casual attachment of one or more bacteria to an inorganic or organic surface. The attachment of bacteria is facilitated, in inorganic materials (prosthesis or medical devices), by the presence of an irregular surface, and, inorganic surfaces, by cilia or flagella, or organic polymeric liquids such as blood, saliva or respiratory secretions. After the attachment, the phenotypic change is guided by an interbacterial communicating system called "quorum sensing". Quorum sensing is crucial in determining the density of the bacterial population, and it increases locally as more bacteria attach [6]. Regulation of this type coordinates bacterial behavior at the population level. Chronic and recurrent upper airway infections may be related to the complex structural and biochemical (quorum sensing) organisation of the biofilm which interferes with the activity of antibiotics (including those with proven in vitro efficacy), thus promoting the establishment of a chronic infection eradicable only by surgical treatment.

Current methods for biofilm detection include scanning electron microscopy, transmission electron microscopy, confocal laser microscopy, and detection of the presence of genes identified with biofilm-forming capacity [1,7]. However, biofilm detection by confocal or electron microscopy or genetic methods is expensive and requires expensive, specialized equipment that is not routinely available. In addition, although these methods can help identify the presence of a biofilm, they are unable to identify the agents involved. We evaluated the capacity of a simple, in vitro method to detect biofilm-forming capacity in commonly identified species of bacteria (*S. aureus*, *P. aeruginosa*) recovered from individuals with COM. Slime-forming ability was tested on congo red agar plates in our study. Using this method, biofilm-

Table-1: Relationship between bacteria and biofilms.

Tissue samples n:22	<i>Pseudomonas Aeruginosa</i>	<i>Staphylococcus Aureus</i>
Culture positive n:16 (72.7%)	n:11(68.7%)	n:5(31.2%)
Production of biofilm n:9 (56.2%)	n: 7(63.6%)	n:2(40%)

forming capacity could be established in 16 of 22 of the specimens in our study. Bendouah et al. also used inexpensive in vitro test using crystal violet to assess biofilm production bacteria recovered from individuals with chronic rhinosinusitis. Biofilm-forming ability was established in 22 of 31 patients in this study^[8]. Galli et al. reported that bacterial biofilms were found in well over half of the tissue specimens collected from patients undergoing surgery for the eradication of chronic upper respiratory tract infections^[9]. However, bacterial biofilm was found in 17(44%) of the 38 chronic rhinosinusitis patients using confocal laser microscopy in a previous study. The authors concluded that pathogenesis of chronic rhinosinusitis is multifactorial and needs further investigations^[10].

Pseudomonas aeruginosa is the organism most commonly isolated from aural cholesteatoma, representing 25% to 35% of total isolates^[11]. In addition, *P. aeruginosa* is widely studied because of its propensity to form biofilms under a variety of conditions and in diseases where biofilm formation is suspected^[12]. *P. aeruginosa* was the most commonly isolated bacterium in our study. 7 of 11 culture positive *Pseudomonas* produced bacterial biofilms (%63.6). Wang et al. have demonstrated that *P. aeruginosa* is a common cholesteatoma organism and variant strains of otopathogenic *P. aeruginosa* are capable of forming biofilms on cholesteatomas, which explains persistent infection that can be eradicated only by surgery^[13]. Chole and Faddis reported that there was strong anatomic evidence for the presence of bacterial biofilms in experimental and human cholesteatomas. They concluded that the existence of bacterial biofilms within cholesteatomas may explain the clinical characteristics of infected cholesteatomas^[14].

PCR analysis could be used to detect microorganisms and biofilm forming ability genes. We used PCR analysis to investigate intercellular adhesion genes(ica) operon for *Staphylococcus aureus*. *S. aureus* can produce slime carries the ica operon responsible for slime production. All strains that were positive for icaA were also positive for icaD in our study. We found that

among the 4 strains positive for the ica operon, 2 (50%) were biofilm positive on CRA, 2 were biofilm negative. Arciola et al. used PCR method to detect icaA and icaD genes and found that 61% of *S. aureus* were icaA and icaD positive and slime forming^[15]. Furthermore, Chaieb et al. demonstrated that 50% *S. epidermidis* isolates were biofilm positive on CRA plates. Among the 23 strains positive for the ica operon, 15 were biofilm positive on CRA, eight were biofilm negative^[16]. PCR method was also used in patients with secretory otitis media. 24 of 24 effusions were PCR-positive for at least 1 otitis media pathogen, and 6 (22%) of 27 effusions were culture-positive for any pathogen in this study^[17]. Moreover, PCR-based assay system detected the presence of bacterial mRNA in a significant percentage (31%) of culturally sterile middle ear effusions, thus establishing the presence of viable, metabolically active, intact organisms in some culture-negative OME^[18].

CONCLUSION

Pseudomonas aeruginosa was the most commonly isolated bacteria in our study. Biofilm forming ability was higher in *Pseudomonas aeruginosa* compared with *Staphylococcus aureus*. PCR technique should be added to the conventional methods to detect biofilm forming ability genes. Among the 4 strains of *Staphylococcus aureus* positive for the ica operon, 2 (50%) were biofilm positive on CRA, 2 were biofilm negative. These data suggest that the phenotypic change may be influenced by the environmental conditions.

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