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Identification of adenoid biofilms with middle ear pathogens in otitis-prone children utilizing SEM and FISH

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ABSTRACT

Objectives: Biofilms have been implicated in the development of several chronic infections. We sought to demonstrate middle ear pathogens in adenoid biofilms using scanning electron microscopy (SEM) and fluorescent in situ hybridization (FISH) with confocal laser scanning microscopy (CLSM).
Methods: Comparative micro-anatomic investigation of adenoid mucosa using SEM and FISH with confocal scanning laser microscopic (CLSM) imaging from patients with recurrent acute otitis media (RAOM).
Results: All otitis-prone children demonstrated biofilm surface area presence greater than 85% by SEM. FISH accompanied by CLSM imaging also demonstrated patchy biofilms All biofilms contained middle ear pathogens and were frequent in polymicrobial distributions: 4 of 6, 4 of 6 and 3 of 6 samples contained *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis*, respectively.
Conclusions: Dense adenoid biofilms may act as a reservoir for reinfection of the tubotympanum. Aspiration of planktonic middle ear pathogens existing in resistant adenoid biofilms during a viral upper respiratory tract infection may be an important event in the development of RAOM.

1. Introduction

Acute otitis media (AOM) is one of the most common pediatric illnesses seen by primary care physicians with an estimated 16 million total office visits in 2000, resulting in roughly 13 million prescriptions written in 2000 for AOM [1,2]. Including treatment costs, lost work and school days, and its contributions to the rising levels of antibiotic resistance, AOM places a significant burden on the US economy with the 1990 combined direct and indirect costs estimated at nearly $3–4 billion [3,4]. Recurrent otitis media is also known to have adverse effects on speech and hearing [5–7]. The high morbidity compounded by increasing prevalence rates of recurrent AOM makes determining the underlying mechanism of AOM of vital importance to the advancement of treatment and prevention options.

Recurrent acute otitis media (RAOM) is defined as three or more distinct, well-documented episodes of AOM in 6 months or four or more episodes in 12 months. RAOM is most often treated with antimicrobial prophylaxis, however with increasing levels of antimicrobial resistance as well as improved outcomes with the addition of adenoidectomy, studies have supported treatment of otitis-prone children refractory to tympanostomy tubes with adenoidectomy. Adenoidectomy has resulted in improved resolution and decreased recurrence of AOM [8,9]. The concept of biofilm establishment in the adenoid bed with subsequent removal through adenoidectomy has helped to explain the effectiveness of this procedure.

The occurrence of a biofilm was originally demonstrated in relation to chronic infections [10,11]. Biofilms are organized communities of adherent micro-organisms that are encased in a complex extracellular polymeric substance (EPS) matrix. The bacteria adopt a metabolically dormant state in the biofilm configuration allowing them to persist longer and rendering them more resistant to antimicrobials. The existence of biofilms on the surface of adenoid tissue has been established and numerous studies have shown a correlation between the presence of biofilms on the adenoids and the occurrence of chronic upper respiratory tract disease [12–14]. This was further supported by studies that showed decreased disease recurrence with the removal of adenoid tissue [8,9]. This evidence has been the foundation for the theory

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that biofilms play a central role in the underlying recurrence of upper airway infections, including otitis media.

Studies have shown the presence of biofilms on middle ear mucous membranes [15] and adenoids [16] of otitis-prone children using scanning electron microscopy and confocal scanning laser microscopy (CLSM). Culture and PCR data have also identified the most common pathogens from the adenoids of otitis-prone children, namely *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* [16,17]. However, while these studies have examined the connection between middle ear biofilms and in situ bacteriology, the connection between adenoid biofilm in situ bacteriology and middle ear bacteriology has not been specifically examined.

In this study, we examined specific bacteria contained within the adenoid biofilms utilizing fluorescent in situ hybridization (FISH) with probes specific for *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *Staphylococcus aureus*. Biofilm ultrastructure was demonstrated by scanning electron microscopy (SEM) and further co-localized with middle ear pathogens utilizing FISH and confocal laser scanning microscopy (CLSM).

2. Methods

2.1. Sample collection

This study was approved by the Wayne State University Institutional Review Board. Selection criteria consisted of patients with RAOM, defined by chart documentation of three or more episodes of AOM in 6 months or four or more episodes in 12 months with demonstrated resistance to antibiotic treatment, who underwent adenoidectomy at an academic tertiary care hospital. Adenoid mucosal samples were collected and divided in half. One half was placed in 2.5% glutaraldehyde in 0.1 M Sorensen's phosphate buffer and stored at −20 °C for SEM imaging and the other half was placed into 0.1 M Sorensen's phosphate buffer and shipped on ice to the Center for Genomic Sciences for FISH and CSLM imaging. There were five male patients and one female patient, ranging in age from 10 months to 9 years old.

2.2. SEM preparation

All samples were prepared for (SEM) employing the following methodology: tissue samples were fixed for 3 h in 2.5% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.4). Distilled H_2O was used for washing. Four washes were done for 15 min each. The samples were then treated with 1% osmium tetroxide for 30 min. The tissue was dehydrated with the following ethanol concentrations for 15 min each: 30%, 50%, 70%, 90%, 100%, and 100%. The tissue was then washed with HMDS (hexamethyldisilvan, Electron Microscopy Sciences) four times for 15 min. Several drops of HMDS were placed on the samples and were left to dry for 48 h under a hood. Samples were then mounted and gold sputter coated in final preparation for imaging.

2.3. SEM imaging and analysis

Imaging was performed at the University of Michigan – Ann Arbor. Imaging was performed by two of the authors (M.H. and L.S.) with guidance from imaging facility staff. Specimens were imaged at 1000× with a procedure used previously by our lab [18]. Biofilm architecture consistent with the literature, showing dense accumulations of bacteria within an amorphous matrix [19,20], was noted and analyzed using the Carnoy image analysis software as previously described [18] to quantify the percentage of adenoidal surface covered by biofilm.

2.4. Microscopic examination

CLSM imaging was performed using a Leica DM RXE microscope attached to a TCS SP2 AOBS confocal system using a high-resolution long working distance 63× water immersion objective (Leica Microsystems, Exton, PA) as noted by Hall-Stoodley et al. [15]. Use of the long distance water immersion objective for observing biofilms on clinical specimens has three advantages. First, the specimen can be observed while fully hydrated, which ensures that that there are no structural dehydration artifacts to the tissue or biofilm and also loosely adherent and free-floating planktonic cells are washed away leaving only firmly attached or invasive bacterial cells and biofilms. Secondly, the specimen can be observed in "plain view" (as opposed to thin section) so that the surface distribution of bacteria can be better assessed. Thirdly, the long working distance minimizes the possibility of scraping the specimen with the objective.

2.5. Evaluation of AdM specimens with pathogen specific 16S rRNA fluorescence in situ hybridization (FISH)

Adenoid mucosa specimens were mounted in a 35 mm plastic Petri (Thermo Fisher Scientific, Hampton, NH) by gently placing into a few drops of cooled 5% molten agar or Lubriseal Stopcock grease (Thomas Scientific, Swedesboro, NJ) with sterile tweezers, avoiding touching the intended viewing area. The specimen was fixed by flooding with fresh 4% paraformaldehyde in phosphate buffered saline (PBS) followed by washes with PBS and PBS–ethanol (1:1), with subsequent 3-min incubations in 80% and 100% ethanol. Specimens were incubated with 10 mg/mL of lysozyme (Sigma–Aldrich, St Louis, MO) in 0.1-M Tris (hydroxymethyl) aminomethane hydrochloride and 0.05-M Na_2 EDTA at 37 °C and washed with ultrapure water. Samples were then ready for FISH evaluation.

The fluorescence in situ hybridization protocol utilizing 16S rRNA probes for *H. influenzae*, *S. pneumoniae*, *M. catarrhalis*, *S. aureus*, and a universal eubacterial 16S rRNA probe [15] was adopted from Nistico et al. [21]. A maximum of three FISH probes were performed on any single specimen to minimize cross-interference of the fluorescent dyes. FISH was performed as previously described [23–25] using the following 16S ribosomal probe sequences labeled with either Cy3, Cy5, or 6-FAM fluorescent dyes (Integrated DNA Technologies Inc, Coralville, IA): EUB338, 5′-GCTGCCTCCCGTAGGAGT-3′ (16S[338–355]) Domain Bacteria [22]; Spn 5′-GTGATGCAAGTGCACCTT-3′ (16S[195–212]) *S. pneumoniae* [23]; Hinf 5′-CCGCACTTTCATCTCCG-3′ (16S[185–202]) *H. influenzae* [24]; and Mcat 5′-CCGCCACUAA-GUAUCAGA-3′ (16S[88–105]) *M. catarrhalis*. The probe specificity was established by hybridizing the probes with a pure culture of the respective target organism and a non-target organism, which was then washed with graduated concentrations of formamide (0–50%, in 5% increments). The probe was then mixed with hybridization buffer and applied to the tissue samples. The tissue samples were allowed to hybridize for 90–120 min at 46 °C, and then washed with pre-warmed hybridization buffer twice and with MilliQ water once before drying at room temperature. After hybridization the samples were immersed in PBS and visualized using CSLM with a 63× long working distance objective.

2.6. Image analysis and interpretation

Specimens were evaluated by LHS, LN, and PS for bacterial clusters showing morphology consistent with *S. pneumoniae* and *H. influenzae* based on in vivo and in vitro biofilms [25,26]. Images suggestive of biofilm ultrastructure were further evaluated using the CLSM electronic high-resolution zoom function, and specimens were scored biofilm-positive when high-resolution images