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Direct Detection of Bacterial Biofilms on the Middle-Ear Mucosa of Children With Chronic Otitis Media

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Abstract

Context—Chronic otitis media (OM) is a common pediatric infectious disease. Previous studies demonstrating that metabolically active bacteria exist in culture-negative pediatric middle-ear effusions and that experimental infection with *Haemophilus influenzae* in the chinchilla model of otitis media results in the formation of adherent mucosal biofilms suggest that chronic OM may result from a mucosal biofilm infection.

Objective—To test the hypothesis that chronic OM in humans is biofilm-related.

Design, Setting, and Patients—Middle-ear mucosa (MEM) biopsy specimens were obtained from 26 children (mean age, 2.5 [range, 0.5–14] years) undergoing tympanostomy tube placement for treatment of otitis media with effusion (OME) and recurrent OM and were analyzed using microbiological culture, polymerase chain reaction (PCR)-based diagnostics, direct microscopic examination, fluorescence in situ hybridization, and immunostaining. Uninfected (control) MEM specimens were obtained from 3 children and 5 adults undergoing cochlear implantation. Patients were enrolled between February 2004 and April 2005 from a single US tertiary referral otolaryngology practice.

Main Outcome Measures—Confocal laser scanning microscopic (CLSM) images were obtained from MEM biopsy specimens and were evaluated for biofilm morphology using generic stains and species-specific probes for *H influenzae, Streptococcus pneumoniae,* and *Moraxella catarrhalis.* Effusions, when present, were evaluated by PCR and culture for evidence of pathogen-specific nucleic acid sequences and bacterial growth, respectively.

Results—Of the 26 children undergoing tympanostomy tube placement, 13 (50%) had OME, 20 (77%) had recurrent OM, and 7 (27%) had both diagnoses; 27 of 52 (52%) of the ears had effusions, 24 of 24 effusions were PCR-positive for at least 1 OM pathogen, and 6 (22%) of 27 effusions were culture-positive for any pathogen. Mucosal biofilms were visualized by CLSM on 46 (92%) of 50 MEM specimens from children with OME and recurrent OM using generic and pathogen-specific probes. Biofilms were not observed on 8 control MEM specimens obtained from the patients undergoing cochlear implantation.

Conclusion—Direct detection of biofilms on MEM biopsy specimens from children with OME and recurrent OM supports the hypothesis that these chronic middle-ear disorders are biofilm-related.

OTITIS MEDIA (OM) IS THE most common illness for which children visit a physician, receive antibiotics, or undergo surgery in the United States. Chronic OM includes both OM with effusion (OME) and recurrent OM in which clinical evidence of OM and the middle-ear effusion (MEE) resolves between episodes. Otitis media with effusion can result in conductive hearing loss, which has been linked to the delayed development of speech and socialization skills.[1] *Streptococcus pneumoniae, Haemophilus influenzae*, and *Moraxella catarrhalis* are isolated from approximately 25% of children with OME, but polymerase chain reaction (PCR)-based methods have demonstrated sequence-specific DNA and RNA for these pathogens in nearly 80% of cases.[2–5]

Evidence that OME is associated with persistent bacterial infection in the absence of culture, combined with its recalcitrance to antibiotic treatment, led to the development of the biofilm hypothesis.[4,6] Biofilms consist of aggregated bacteria, usually adherent to a surface, surrounded by an extracellular matrix, and have been implicated in several chronic bacterial infections.[7–10]

Previous laboratory studies with low-passage clinical isolates of *H influenzae* have demonstrated that biofilms form on the middle-ear mucosa (MEM) of the chinchilla[6]; however, no studies have directly examined human MEM for biofilms. Our objective in this study was to perform imaging of MEM biopsy specimens from children with OME and recurrent OM to test the hypotheses that these conditions are biofilm-related.

METHODS

Patient Population

Institutional review board approval was obtained from the Medical College of Wisconsin. Patients were invited to participate at the time of consultation for either tympanostomy tube (TT) placement (study group) or cochlear implantation (control group). Written informed consent was obtained from the parents or guardians of the children in the study and control groups, as well as from adults in the control group. Consecutive patients fulfilling the study criteria were invited to participate between February 2004 and April 2005 from a single tertiary referral pediatric otolaryngology practice (J.E.K.). The participation rate was approximately 50% in the TT group.

To be considered for inclusion, study patients had to be between 6 months and 15 years of age and meet clinical criteria for TT insertion for either OME or recurrent OM. Otitis media with effusion was defined as the persistence of an MEE for longer than 3 months with minimal constitutional symptoms. Recurrent OM was defined as 3 or more acute OM presentations within a 6-month period in which clinical evidence of OM and MEE resolved between episodes. Exclusion criteria included immunological abnormality, either intrinsic or pharmacological; anatomical or physiological defect of the ear; syndrome associated with OM (eg, Down syndrome, cleft palate); sensorineural hearing loss; and chronic mastoiditis, cholesteatoma, or other OM complications except for conductive hearing loss.

Two groups of control biopsy specimens were obtained from patients in the same otolaryngology practice undergoing cochlear implantation: a pediatric group (n = 3), in which the age range was the same as for the experimental group, and an adult group (n = 5). For inclusion in either control group, patients had to have a negligible history of OM defined as 1 or fewer episodes per year and no middle-ear pathology. Middle-ear mucosa specimens were obtained at the time of cochlear implantation and prepared in the same manner as the specimens from the study group. Antibiotic usage was not used as an exclusion criterion in any case.

Specimen Acquisition and Tissue Preparation

General mask anesthesia was used during the surgical procedure. Using an operating microscope (Carl Zeiss Inc, Thornwood, NY), a myringotomy incision was made in the anterior-inferior tympanic membrane. Middle-ear effusion was collected, if present, using a sterile tympanostomy trap for culture and PCR, A cup forceps was inserted through the myringotomy incision using high-power magnification, and MEM specimens ($<.1 \text{ mm}^2$) were obtained from the middle-ear promontory proximal to the eustachian tube.

The specimens were placed in Hank balanced salt solution (HBSS) (Invitrogen, Carlsbad, Calif) and shipped overnight on ice to the Center for Genomic Sciences. Because unfixed specimens were evaluated immediately after shipment it was not possible to randomize samples. Unfixed MEM specimens were rinsed and incubated with either BacLight LIVE/ DEAD nucleic acid probes (Invitrogen) or with antipneumococcal antibody (PnAb) diluted in HBSS, then rinsed to remove excess probe and imaged in an enclosed coverslip chamber containing HBSS. Specimens for 16S rRNA fluorescence in situ hybridization (FISH) were fixed overnight and stored at −20°C prior to hybridization.

Aerobic Cultures—After collection, MEE specimens were immediately transported to the clinical microbiology laboratory for Gram staining and for bacterial growth on blood agar, chocolate agar, MacConkey agar, and colistin-naladixic acid, with incubation in 5% CO_2.

PCR-Based Bacterial Detection—DNA was isolated from MEEs using TRIzol (Invitrogen). Twenty-microliter PCRs were performed containing 1.0 unit of Platinum Taq DNA polymerase (Invitrogen); 5 mM each of the 4 deoxyribonucleotide triphosphates (Invitrogen); 0.1 μM of each primer; 1.5 mM $MgCl_2$; and 2 μL of DNA. A no-template negative control was used for each reaction. The following primers were used: *H influenzae* (forward) 5′-GGAGTGGGGTTGTACCAGAAGTAGAT-3′ [124-base pair [bp] amplicon] and *S pneumoniae* (forward) 5′-AGTCGGTGAGGTAACCGTAAG-3′ [105-bp amplicon], both with a universal reverse 5′-AGGAGGTGATCCAACCGCA-3′ primer; and *M catarrhalis* (forward) 5′-TTGGCTTGTGCTAAAATATC-3′ [140-bp amplicon] with an *M catarrhalis* (reverse) 5′-GTCATCGCTATCATTCACCT-3′ primer. The specificity of these primers has been previously established.[11]

Thirty-six-cycle amplifications were performed using a GeneAmp PCR System 2400 thermal cycler (Applied Biosystems, Foster City, Calif) with 30 seconds of denaturation at 95°C, 30 seconds of annealing at 55°C, and 30 seconds of elongation at 72°C. PCR products were restriction digested, electrophoretically separated, and visualized using GelStar Nucleic Acid Stain with a Kodak image analysis system (Eastman Kodak, Rochester, NY).

Microscopic Examination

Confocal laser scanning microscopic (CLSM) imaging was performed using a Leica DM RXE microscope attached to a TCS SP2 AOBS confocal system using high-resolution objectives (Leica Microsystems, Exton, Pa).

Evaluation of MEM Specimens With Pathogen-Specific Probes

Immunostaining—Specimens were incubated with 40 to 50 μg/mL of rabbit anti-*S pneumoniae* antibody (Biodesign, Saco, Me) specific for *S pneumoniae* serotypes 3, 4, 6, 7, 9, 14, 18, 19, and 23, washed to remove excess antibody, blocked with 10% fetal bovine serum (Hyclone, Logan, Utah) in HBSS, and incubated with 150 μg/mL of AffiniPure F(ab)$_2$ fragment Texas Red-conjugated donkey antirabbit IgG (H$^+$L chain) antibody (Jackson ImmunoResearch, West Grove, Pa). Specimens were rinsed with 10% fetal bovine serum/ HBSS and evaluated using the CLSM transmission mode to define the mucosal epithelium substratum and the fluorescent mode to detect the Texas Red-conjugated antibody. In vitro specificity studies demonstrated that this antibody reacted with all of the pneumococcal strains tested, and neither primary nor secondary antibodies cross-reacted with any of 31 clinical isolates of *H influenzae, M catarrhalis, Pseudomonas aeruginosa, Staphylococcus aureus,* or *Klebsiella pneumoniae* (data not shown).

16S rRNA FISH—Middle-ear mucosa specimens were fixed with fresh 4% paraformaldehyde in phosphate-buffered saline (PBS) followed by washes with PBS and PBS-ethanol (1:1), with subsequent 3-minute incubations in 80% and 100% ethanol. For FISH, 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. The lysozyme step was omitted for some specimens to optimize conditions for gram-negative bacteria.

To obtain bacterial species-specific information for the specimens from children with recurrent OM, which lacked effusions for PCR analyses, FISH protocols were developed using 16S rRNA probes for *H inftuenzae, S pneumoniae,* and *M catarrhalis,* as well as a universal eubacterial 16S rRNA probe. These assays were also applied to OME specimens. A maximum of 3 FISH probes could be performed on any single specimen due to limitations of the

fluorescent dyes. Therefore, FISH provides evidence that a specific pathogen is present or absent when a specific probe is used.

Fluorescence in situ hybridization was performed as previously described [12–14] using the following 16S ribosomal probe sequences: EUB338, 5′-GCTGCCTCCCGTAGGAGT-3′ (16S [338–355]) Domain Bacteria[12]; Spn 5′-GTGATGCAAGTGCACCTT-3′ (16S [195–212]) *S pneumoniae*[13]; Hinf 5′-CCGCACTTTCATCTCCG-3′ (16S [185–202]) *H influenzae*[14]; and Mcat 5′-CCGCCACUAAGUAUCAGA-3′ (16S [88–105]) *M catarrhalis.* Conditions for FISH were developed and rigorously tested in vitro to ensure specificity for each species probe, and the generic character of the eubacterial probe (positive and negative controls[12,15]) was tested using panels of bacterial strains. Probes were labeled with Cy3, Cy5, or 6-FAM (Integrated DNA Technologies Inc, Coralville, Iowa). Hybridized samples were evaluated using CLSM.

Specificity of 16S rRNA Probes—Clinical isolates of *S pneumoniae, H influenzae, M catarrhalis, P aeruginosa, S aureus,* and *K pneumoniae* were obtained, cultured, and serotyped as described[16,17] from the Children's Hospital of Pittsburgh and the Medical College of Wisconsin for testing of the 16S probes. All *H influenzae* isolates hybridized with the *H influenzae* probe; no *H influenzae* isolates hybridized with any probe for the other target species. *Streptococcus pneumoniae* and *M catarrhalis* probes were similarly specific (data not shown). The eubacterial probe hybridized with all bacterial isolates, whereas a nonsense-sequence probe[15] gave negative results for all bacteria tested. Mixed pneumococcal/nontypeable *H influenzae* cultures examined with the *S pneumoniae, H influenzae,* and eubacterial 16S probes demonstrated appropriate species-specific as well as eubacterial probe hybridization (data not shown).

Image Analysis and Interpretation

The *H influenzae*[6] and *S pneumoniae* (data not shown) chinchilla OM biofilm models were used as guides for assessing pediatric MEM specimens. Specimens were evaluated by 2 independent observers (L.H.-S., L.N.) (3 observers for specimens evaluated with FISH [L.H.-S., L.N., P.S.]) for bacterial clusters showing morphology consistent with *S pneumoniae* and *H influenzae* based on in vivo and in vitro biofilms[6,18]; no instances of disagreement were recorded. 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 demonstrated bacterial morphology. In specimens from the first 16 children in the study group (children 5–20), biofilms were identified solely by morphology using standard visual clues including size (approximately 1–2 μm), shape (cocci or coccobacilli), and biofilm ultrastructure. When species-specific probes (FISH and PnAb) were used, the same criteria were used in addition to fluorescence with the appropriate signal. The epithelial surface of the specimen was evident by staining with nonspecific dyes or by transmission imaging. Control specimens were exhaustively evaluated by 2 independent observers (L.H.-S., L.N.) for evidence of bacteria and biofilms using identical protocols and a comprehensive x-y raster scanning approach to ensure that no minimal pockets of biofilm were missed.

RESULTS

Patient Characteristics

Of 52 MEM specimens obtained from 26 children undergoing TT placement, 50 were evaluated by CLSM for evidence of bacterial biofilms. Of these 26 children, 13 (50%) had a diagnosis of OME, 20 (77%) had a diagnosis of recurrent OM, and 7 (27%) had both diagnoses. Of the 26 children with OM, 13 (50%) were girls, and the mean age was 2.5 (range, 0.514) years.

Uninfected control specimens were obtained from 3 pediatric patients (mean age, 5.8 [range, 0.9–14.8] years). All 3 pediatric control patients were boys. In addition, MEM specimens from 5 adult patients receiving cochlear implantation (4 women) were evaluated (mean age, 49.9 [range, 22.9–69.9] years).

Among the 26 children undergoing TT placement, 27 (52%) of their 52 ears had effusions, and 24 of these 27 effusions were assayed by PCR for *H influenzae, S pneumoniae,* and *M catarrhalis*; the 3 remaining effusions yielded insufficient effusion for both culture and PCR or produced no testable DNA. Only 5 (19%) of the 27 effusions were culture-positive for any of the 3 major OM pathogens (*H influenzae* [2], *S pneumoniae* [2], *M catarrhalis* [l]), with 1 additional effusion testing positive for *Klebsiella* species. However, all 24 effusions assessed by PCR were positive for at least 1 OM pathogen (*H influenzae* [17], *S pneumoniae* [12], *M catarrhalis* [6]). Of the 24 effusions, 4 (17%) were PCR-positive for all 3 bacteria, and 3 (13%) were PCR-positive for both *H influenzae* and *S pneumoniae* (Table 1).

Evidence of Biofilms

Middle-ear mucosa specimens from all patients were examined by CLSM for evidence of bacterial biofilms using techniques that included generic nucleic acid labeling of cells with the BacLight kit, FISH, and pneumococcal immunostaining. Because of the small specimen size (< 1 mm^2), only a subset of these analyses could be performed on each specimen. Of 30 OM specimens examined using the BacLight kit, 28 were positive for bacterial biofilms based on morphological criteria. In these images, green fluorescence indicates live bacteria and healthy host cells, and red stains the nuclei of dead cells (Figure 1). Yellow and orange fluorescence indicates the presence of both stains within a particular host cell, suggesting cell membrane damage. A specimen from a patient with an effusion that was PCR-positive for *S pneumoniae* shows clusters of cocci that appear to be adherent to the MEM (see z-axes images) that are surrounded by an amorphous matrix that stains lightly with the Syto 9 (green) nucleic acid–binding dye (Figure 1B). Similarly, a specimen with an effusion that was PCR-positive for *H influenzae* shows bacteria in matrix-enclosed bacterial clusters (Figure 1C). Overall, biofilms were present on 46 (92%) of 50 OM specimens and were not limited to those cases in which an effusion was present but were also present on specimens of children with recurrent OM (Table 1).

To obtain bacterial species–specific information for the specimens from children with recurrent OM, which lacked effusions, 16S RNA FISH was developed for the major OM pathogens (Figure 2). Fluorescence in situ hybridization for *H influenzae* was positive for 6 of 7 MEM specimens (86%), including 2 of 3 recurrent OM specimens. These specimens showed a range of biofilm morphologies, from micro-colonies to large clusters of bacteria. Of 4 specimens from OME cases that were FISH-positive for *H influenzae*, all 4 also had effusions that were PCR-positive for *H influenzae*.

Streptococcus pneumoniae–specific FISH demonstrated bacterial morphology in clusters from 10 of 10 specimens examined. Six of these 10 were also hybridized with the eubacterial probe, which colocalized with the *S pneumoniae* 16S in all cases. The 4 remaining FISH-positive pneumococcal specimens were hybridized with the *H influenzae* 16S probe; all demonstrated hybridization (Figure 2A). Of 5 children with MEM specimens that were FISH-positive for *S pneumoniae,* 3 also had MEEs that were PCR-positive for *S pneumoniae*. Of 2 specimens examined using FISH for *M catarrhalis,* 1 was positive. Overall, FISH and PCR gave comparable percentages of positive results for *H influenzae*, *S pneumoniae*, and *M catarrhalis*.

Pneumococcal-specific immunostaining with PnAb was developed as a confirmatory assay for children with recurrent OM and FISH-positive MEM specimens that lacked a cognate effusion (ie, an effusion from the same child, same ear). When the 2 pneumococcal-specific in situ visualization methods were used sequentially, 5 of 5 specimens demonstrated *S pneumoniae* by both methods (Table 1). A recurrent OM specimen that lacked a cognate effusion was PnAb-positive and also FISH-positive using pneumococcal-specific and eubacterial 16S probes (Figure 3B). Immunostaining also provided confirmation of *S pneumoniae* biofilms in patients with pneumococcal-specific PCR-positive effusions (Figure 3C).

Overall, biofilm structures were identified by at least 2 methods in 18 (90%) of 20 specimens assayed using in situ pathogen-specific probes. This compares with 28 of 30 specimens (93%) that were biofilm-positive using nonspecific probes. Of ears assayed with 2 or more specific modalities, 12 of 12 were PCR effusion–positive, 14 of 15 (93%) were FISH-positive, and 16 of 17 (94%) were PnAb-positive. Five of 6 (83%) of recurrent OM specimens lacking effusions were positive for OM pathogens using 1 or more specific in situ methods (FISH or immunostaining).

Control Specimen Findings

No uninfected control specimens from the 3 pediatric or 5 adult patients undergoing cochlear implantation demonstrated bacterial morphology or evidence of biofilm ultrastructure using the *H influenzae*, *S pneumoniae,* or eubacterial FISH probes (Figure 3A). For all control specimens, the entire mucosal surface of the specimen was examined thoroughly to rule out any occult biofilms. Negative results were also obtained for 2 control specimens immunostained with PnAb (Table 2).

COMMENT

Increasing evidence suggests that microbial biofilms play a role in chronic human infections.[7,9,10,19,20] Criteria proposed for characterizing biofilm infections include direct examination of an infected tissue revealing pathogenic bacteria in clusters within a matrix attached to a surface, infections localized to a particular anatomical site, and evidence of recalcitrance to antibiotic treatment despite antibiotic sensitivity demonstrated by planktonic forms.[20] In this investigation, we observed biofilms characterized by bacteria in matrix-enclosed adherent clusters in 46 of 50 evaluable specimens (92%) from children with chronic localized middle-ear disease undergoing TT placement who had not responded to multiple courses of antibiotics, thus fulfilling all 3 biofilm diagnostic criteria.

CLSM imaging revealed clusters of bacteria on the MEM of patients with both OME and recurrent OM. Since specimens from those with recurrent OM lacked accompanying effusions for PCR and culture, in situ pathogen-specific identification methods were developed to further characterize these biofilms. CSLM imaging, using both antibody and FISH probes specific for pneumococcus, also identified coccal bacteria within matrices on the pediatric MEM specimens that corresponded morphologically with pneumococcal biofilms imaged in vitro[18] and on chinchilla bullar epithelium (data not shown), supporting the hypothesis that pneumococcus forms biofilms during chronic infections.

All of the specimens that demonstrated pneumococcus were rated as positive by at least 2 independent diagnostic modalities, suggesting that the prevalence of *S pneumoniae* is high in chronic OM. This percentage is higher than the number of PCR-positive pneumococcal specimens found in this and other studies[4]; however, because PCR is performed only when an effusion is present, this method does not assess recurrent OM. Middle-ear mucosa specimens in the current study also revealed a high prevalence of biofilms that were FISH 16S–positive

for *H influenzae.* The *H influenzae* observed in these biofilms assumed a coccobacillary form, similar to those forms observed in experimental *H influenzae* biofilms[6] and distinct from the pure bacillary form seen during planktonic growth.

Fluorescence in situ hybridization has been demonstrated to be a specific and sensitive tool for the assessment of bacteria in clinical samples and has proven useful for providing spatial and morphological data unobtainable by PCR[14]; however, there are technical issues associated with FISH-based imaging. First, specimen preparation requires stringent wash steps, which undoubtedly remove a significant fraction of any biofilm. Second, when using small biopsy specimens that cannot be subdivided, there is a maximum of 2 species that can be evaluated per specimen when also using the eubacterial probe, because only 3 fluorescent dyes are available for FISH-based analyses. Notwithstanding these difficulties, in all but 1 case for which positive FISH results were obtained, species-specific corroborating data were obtained using other methods.

In this study, the in situ assessment of MEM biopsy specimens using both generic and species-specific bacterial probes is supportive of a biofilm etiology for chronic OM. The vast majority of MEM specimens obtained from children undergoing TT placement evidenced matrix-enclosed adherent bacteria, whereas control specimens from patients undergoing cochlear implantation had no evidence of bacteria. Moreover, these data suggest that the bacterial species involved in these mucosal biofilms are the same pathogens commonly associated with OM. However, because of the small amount of tissue and the inability to simultaneously perform positive and negative 16S controls on each specimen, we cannot entirely exclude the possibility of nonspecific interactions with the probes used in these analyses. We attempted to address this limitation by using a larger number of tests in the aggregate than could be performed on each specimen. Moreover, in vitro evaluations of all of the 16S and antibody probes used in this investigation identified no cross-reactivity when tested against a battery of clinical strains representing the common human mucosal pathogens. A second limitation of this investigation was the small number of age-matched controls who were available for participation in the study; to partially compensate for this difficulty, we expanded the age range for obtaining control specimens.

Collectively, the data presented in this study support the hypothesis that biofilms may play a role in the pathogenesis and chronicity of OME and also provide evidence that at least some mucosal specimens from patients with recurrent OM harbor bacterial biofilms. This finding is surprising in light of the body of literature that suggests that consecutive episodes of acute OM are associated with unique bacterial strains.[21] Our findings are more consistent with recurrent OM as a chronic disease with episodic acute exacerbations. Leibovitz et al[22] have demonstrated that approximately 30% of cases of recurrent OM result from relapses attributable to the original organism. The true relationship between bacterial persistence demonstrated on biopsy and the clinical observation of a disease-free period clearly requires further investigation.

Importantly, the findings from our study do not exclude other potential pathogenic factors associated with OME, such as an antecedent viral upper respiratory infection, eustachian tube dysfunction with impaired gas exchange, a genetically predisposed host, persistent inflammatory mediators, or exacerbation by gastroesophageal reflux. However, these findings do argue against the notion that OME is the result of a non-bacterial inflammatory process and also indicate that equating culture negativity and absence of bacteria is incorrect.

The finding that biofilms are present in almost all cases of OME in this study may help to explain the lack of antibiotic efficacy for this disorder, given that biofilm bacteria are more

antibiotic resistant than single cells in suspension.[23–28] This resistance may stem from the fact that oxygen and nutrient limitation within biofilms induces metabolic quiescence, which in turn reduces antibiotic effectiveness.[2,23] Other evidence suggests that biofilm bacteria may have genetic mechanisms, selected for in the biofilm, that provide antimicrobial protection.[29–31] In addition, the biofilm provides a physical barrier that enhances pathogen resistance to host defenses such as opsonization, lysis by complement, and phagocytosis.[7]

CONCLUSIONS

Direct detection of biofilms on MEM biopsy specimens from children with OME and recurrent OM supports the hypothesis that these chronic middle-ear disorders may be biofilm-related and may help to explain multiple patho-physiological aspects of chronic OM. Much remains to be learned about the mechanisms by which bacteria in these biofilms persist and resist both the host immune response and antibiotic treatment.

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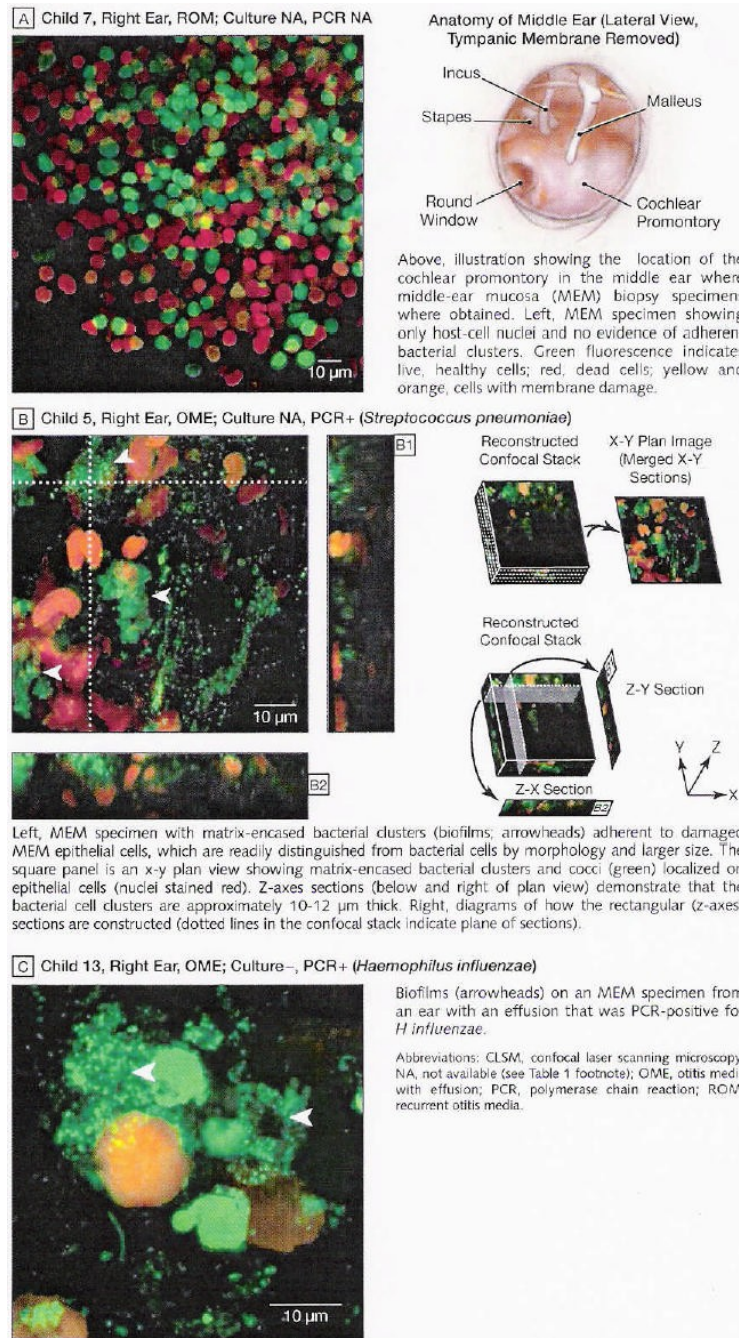
Figure 1.
CLSM Imaging of MEM Biopsy Specimens From Children With Chronic Otitis Media Stained With BacLight LIVE/DEAD Nucleic Acid Probes

A Child 30, Right Ear, ROM; Culture NA, PCR NA, FISH+ *(Haemophilus influenzae, Streptococcus pneumoniae)*

CLSM images showing coinfection by *H influenzae* and *S pneumoniae* on a middle-ear mucosa (MEM) biopsy specimen visualized by fluorescence in situ hybridization (FISH) with 16S rRNA probes specific for *H influenzae* (left) labeled with Cy5 (red), for *S pneumoniae* (middle) labeled with Cy3 (green), and overlay of both (right). Each species colonizes discrete regions not inhabited by the other.

B Child 27, Left Ear, OME; Culture−, PCR+ *(Moraxella catarrhalis)*, FISH+ *(M catarrhalis, Eubacteria)*

CLSM images showing bacterial morphology in cell clusters of an MEM specimen visualized by FISH with, left, FAM-labeled *M catarrhalis* 16S rRNA probe (green); middle, Cy5-labeled eubacterial 16S probe (red); and right, overlay of both (yellow). This specimen was also PCR-positive for *M catarrhalis*.

Figure 2.
Fish of Bacterial Biofilms on MEM Biopsy Specimens From Children With Chronic Otitis Media
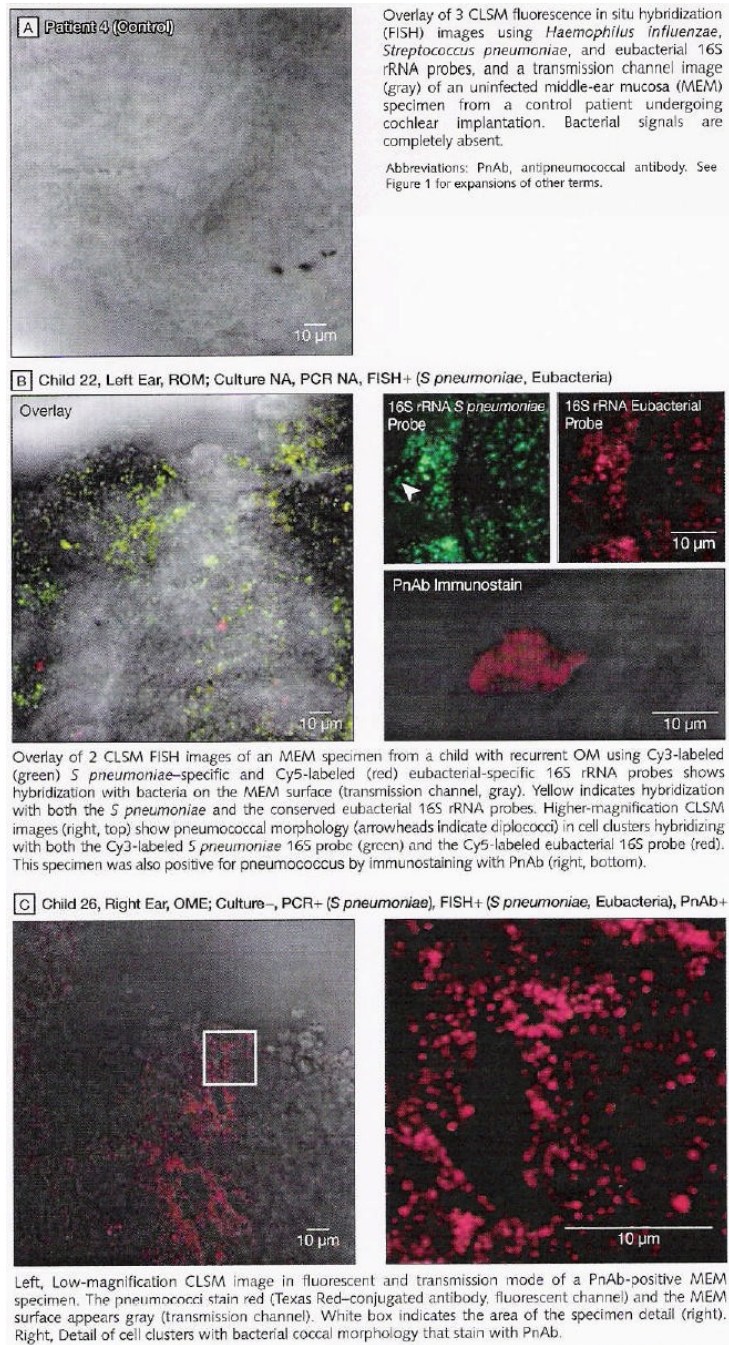
Figure 3.
Biofilms on MEM Biopsy Specimens Visualized by FISH and Immunostaining With PnAb

Table 1

Results for Pediatric Middle-Ear Mucosa Biopsy Specimens Assayed With Multiple Molecular Methods for Otitis Media Pathogens—Study Group (Children Undergoing Tympanostomy Tube Placement)*

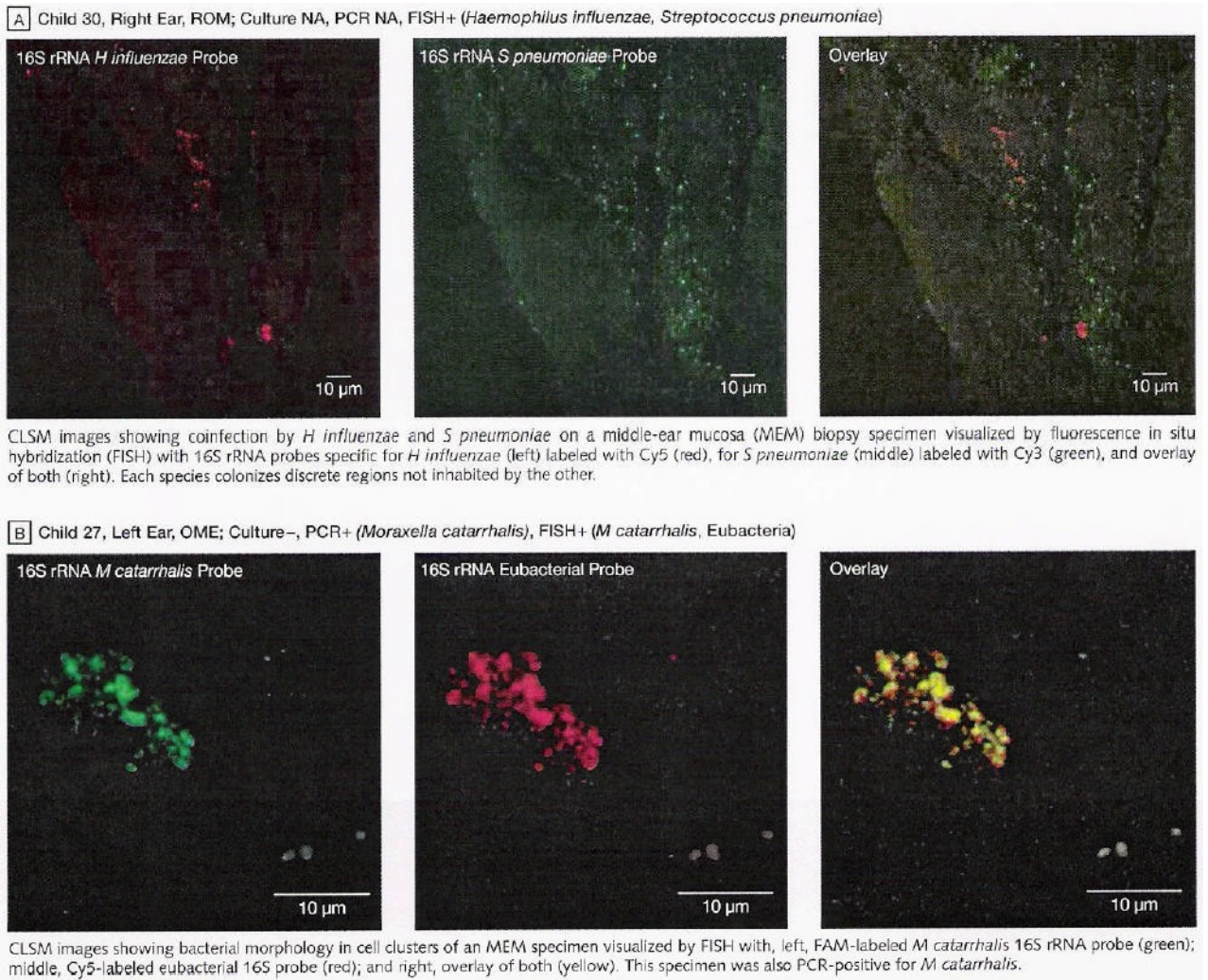| Child No.	Sex (Age, y)	Ear	Diagnosis	Culture	PCR HI	PCR SP	PCR MC	FISH HI	FISH SP	FISH MC	FISH EUB	BacLight Probe	PnAb	Biofilm
5	F (13.8)	L	ROM	NA	NA	NA	NA					+	NA	+
		R	OME	NA	-	NA	-					+	NA	+
6	F (1.3)	L	ROM	NA	NA	NA	NA					NA	NA	NA
		R	ROM	NA	NA	+	-					+	NA	+
7	F (4.5)	L	OME	-	+	-	-					+	NA	+
		R	ROM	NA	NA	NA	NA					NA	NA	NA
8	M (1.8)	L	ROM	NA	NA	NA	NA					+	NA	+
		R	OME	-	+	-	-					+	NA	+
9	F (0.8)	L	ROM	NA	+	NA	NA					+	NA	+
		R	ROM	NA	NA	NA	NA					+	NA	+
10	M (0.8)	L	OME	+HI	+	-	-					+	NA	+
		R	OME	-	+	-	-					+	NA	+
11	M (0.8)	L	OME	NA	NA	NA	NA					+	NA	+
		R	ROM	NA	NA	NA	NA					+	NA	+
12	F (0.9)	L	ROM	NA	NA	NA	NA					+	NA	+
		R	ROM	NA	NA	NA	NA					+	NA	+
13	M (2.0)	L	ROM	NA	+	-	-					+	NA	-
		R	OME	-	+	-	-					+	NA	+
14	F (0.8)	L	OME	+MC	+	-	+					+	NA	+
		R	OME	-	-	-	-					+	NA	+
15	F (1.3)	L	ROM	NA	NA	NA	NA					+	NA	+
		R	ROM	NA	NA	NA	NA					+	NA	+
16	M (0.8)	L	OME	-	+	-	-					+	NA	+
		R	ROM	NA	NA	NA	NA					+	NA	+
17	M (1.1)	L	ROM	NA	NA	NA	NA					+	NA	+
		R	ROM	NA	NA	NA	NA					+	NA	+
18	M (2.8)	L	ROM	NA	+	+	+					+	NA	+
		R	ROM	NA	-	+	-					+	NA	+
19	M (0.9)	L	OME	NA	NA	NA	NA					+	NA	+
		R	OME	NA	+	+	+					+	NA	+
20	F (4.5)	R	ROM	NA	NA	NA	NA					+	NA	+
		R	ROM	NA	NA	NA	NA					+	NA	+
21	M (0.9)	L	OME	-	+	+	-	NA	+	NA	+	NA	+	+
		R	OME	-	+	+	-	NA	+	NA	+	NA	+	+
22	F (2.5)	L	ROM	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	+
		R	ROM	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	+
23	M (0.5)	L	OME	+SP	NA	NA	NA	NA	NA	NA	NA	NA	+	+
		R	OME	+SP	NA	NA	NA	NA	NA	NA	NA	NA	+	+
24	F (0.8)	L	OME	-	-	-	-	+	+	NA	+	NA	+	+
		R	OME	+KP	+	+	+	NA	NA	NA	NA	NA	+	+
25	M (1.2)	L	OME	-	+	+	+	NA	+	NA	+	NA	+	+
		R	OME	-	+	+	-	NA	+	NA	+	NA	+	+
26	F (1.0)	L	ROM	NA	-	NA	NA	NA	NA	NA	+	NA	+	+
		R	OME	-	-	+	-	NA	+	+	+	NA	+	+
27	F (7.8)	L	OME	-	-	-	+	NA	NA	+	+	NAD	NA	+†
		R	OME	-	-	-	+	NA	NA	NA	+	NA	NA	+†
28	M (7.8)	L	ROM	NA	NA	NA	NA	+	NA	NA	+	NA	-	+
		R	OME	-	-	-	-	+	NA	NA	NA	NA	NA	+
29	M (2.5)	L	OME	-	+	-	-	+	+	NA	NA	NA	+	+

Child No.	Sex (Age, y)	Ear	Diagnosis	Culture	PCR			FISH 16S RNA Probe[†]				BacLight Probe	PnAb	Biofilm
					HI	SP	MC	HI	SP	MC	EUB			
30	F (1.8)	R	OME	+HI	+	–	–	+	+	NA	NA	NA	+	+
		L	ROM	NA	NA	NA	NA	+	+	NA	NA	NA	+	+
		R	ROM	NA	NA	NA	NA	+	+	NA	NA	NA	+	+

Abbreviations: EUB, eubacterial probe; FISH, fluorescence in situ hybridization; HI, *Haemophilus influenzae* (organism and probe); KP, *Klebsiella pneumoniae*; MC, *Moraxella catarrhalis* (organism and probe); NA, not available (see below); OME, otitis media with effusion; PCR, polymerase chain reaction; PnAb, antipneumococcal antibody; ROM, recurrent otitis media; SP, *Streptococcus pneumoniae* (organism and probe).

*
Cells marked NA indicate that assay was not performed due to sample limitations. PCR and culture were only possible if an effusion was present and therefore were never performed on ROM samples. For microscopic examination, the small size of the sample (<1 mm) did not allow subsectioning and, therefore, limited the examination to performance of a subset of visualization techniques for any given sample.

[†]
FISH assays began with patient 22.

[‡]
Denotes 16S signal in the absence of conclusive biofilm morphology.

Table 2

Results for Middle-Ear Mucosa Biopsy Specimens Assayed With Multiple Molecular Methods for Otitis Media Pathogens—Control Group (Patients Undergoing Cochlear Implantation)[*]

Patient No.	Sex (Age, y)	Culture	PCR			FISH 16S Probes				BacLightProbes	PnAb	Biofilm
			HI	SP	MC	HI	SP	MC	EUB			
4[†]	M (0.9)	NA	NA	NA	NA	-	-	NA	-	NA	NA	-
7[‡]	F (adult)	NA	NA	NA	NA	-	NA	NA	-	NA	-	-
8[‡]	F (adult)	NA	NA	NA	NA	-	NA	NA	-	NA	-	-
9[†]	M (1.8)	NA	NA	NA	NA	-	-	NA	-	NA	NA	-
10[‡]	F (adult)	NA	NA	NA	NA	-	-	NA	-	NA	NA	-
11[‡]	M (adult)	NA	NA	NA	NA	-	-	NA	-	NA	NA	-
12[‡]	F (adult)	NA	NA	NA	NA	-	-	NA	-	NA	NA	-
13[†]	M (14.8)	NA	NA	NA	NA	-	-	NA	-	NA	NA	-

Abbreviations: EUB, eubacterial probe; FISH, fluorescence in situ hybridization; HI, *Haemophilus influenzae* probe; MC, *Moraxella catarrhalis* probe; NA, not available (see below); PCR, polymerase chain reaction; PnAb, antipneumococcal antibody; SP, *Streptococcus pneumoniae* probe.

[*] Cells marked NA indicate that assay was not performed due to sample limitations. For microscopic examination, the small size of the sample (< 1 mm) did not allow subsectioning and, therefore, limited the examination to performance of a subset of visualization techniques for any given sample.

[†] Pediatric specimens that met inclusion criteria of negligible history of otitis media with effusion or recurrent otitis media (≤1 episodes of otitis media per 12 months of life and no middle-ear pathology). Biopsy specimens for patients 1–3 and 5–6 (pediatric) were not obtained due to failure to meet inclusion criteria.

[‡] An additional 5 specimens from adults (mean age, 49.9 years; 20% men) receiving cochlear implants were analyzed for biofilm ultrastructure: none of these 5 specimens showed any sign of biofilm or otitis media pathogens.