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What is This?
Nasopharyngeal Biofilm-Producing Otopathogens in Children with Nonsevere Recurrent Acute Otitis Media

Sara Torretta, MD¹, Paola Marchisio, MD², Lorenzo Drago, MD³, Elena Baggi, MD², Elena De Vecchi, MD³, Werner Garavello, MD⁴, Erica Nazzari, MD², Lorenzo Pignataro, MD¹, and Susanna Esposito, MD²

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Abstract

Objective. Bacterial biofilms have been detected in biopsies of the adenoid and middle ear mucosa of otitis-prone children and children with chronic middle otitis media. However, the invasiveness of biopsy makes it unsuitable for routine clinical practice, especially in pediatrics. This study aimed to investigate nasopharyngeal biofilm-producing otopathogens (BPOs) of nasopharyngeal swabs (NPS) in children with a history of nonsevere recurrent acute otitis media (RAOM) and healthy controls.

Study Design. A cross-sectional study with planned data collection.

Setting. University of Milan.

Subjects and Methods. Transoral NPS were taken from infants and children aged 10 months to 11 years with nonsevere RAOM or healthy controls without adenoid hypertrophy. Nasopharyngeal colonization by otopathogens was assessed by means of microbiological cultures and standard bacterial identification, as well as nasopharyngeal BPOs by means of spectrophotometric analysis.

Results. The study involved 113 children (56.6% males; median age 40 months; range, 10-132 months): 58 with a history of nonsevere RAOM (51.3%) and 55 controls (48.7%). Otopathogens were significantly more frequently detected in the RAOM group (24/58, 41.4%) than in controls (8/55, 14.5%; \( P = .003 \)); the main pathogens were respectively Haemophilus influenzae (12/24, 50.0%) and Streptococcus pyogenes (3/8, 37.5%). Nasopharyngeal BPOs were more frequently isolated in the RAOM group (17/58, 29.3%) than in controls (6/55, 10.9%; \( P = .02 \)). H influenzae (12/17, 70.6%) was confirmed as the main pathogen in the RAOM group.

Conclusion. The presence of nasopharyngeal BPOs is an important factor favoring RAOM; it is therefore useful investigating biofilms even in children with nonsevere recurrences of AOM without adenoid hypertrophy.

Keywords
biofilm, otopathogens, recurrent acute otitis media, nasopharynx

Received October 15, 2011; revised December 9, 2011; accepted January 17, 2012.
The invasiveness of the bioptic detection of biofilms makes it unsuitable in routine clinical practice, especially in children. We have previously found that the diagnostic accuracy of nasopharyngeal swabs (NPS) in detecting biofilm-producing bacteria in a cohort of children with chronic adenoiditis is generally poorer than that of adenoidal biopsies. However, given the fairly good sensitivity and positive predictive value of NPS and the unsubstantial difference in pathogen identification, the use of such an economic and simple means of detecting biofilm-producing otopathogens (BPOs) may be useful, especially in positive cases. In addition, it has been shown that children with RAOM are more frequently colonized by nasopharyngeal otopathogens than controls and that the bacterial colonization patterns vary with the type and severity of otitis media.

The aim of this study was to investigate the feasibility of using spectrophotometric analysis of NPS to quantify biofilm formation in children with a history of nonsevere RAOM and healthy controls. Spectrophotometric assessment was chosen as a simpler and cost-effective quantitative method compared with expensive and extensive sample preparation needing electron microscopic techniques.

**Methods**

**Study Design and Setting**

This cross-sectional study with planned data collection, which was approved by the Ethics Committee of the University of Milan, was carried out between October 2009 and October 2010 in the Department of Maternal and Pediatric Sciences and the Department of Specialist Surgical Sciences of Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico (recruitment, specimen collection, and statistical analysis) and at the Clinical Microbiology Laboratory of the University of Milan’s Department of Preclinical Science (DISP) LITA (microbiological assessment). Written informed consent was obtained from the children’s parents or legal guardians, and the older children were also asked for their assent.

**Study Population**

The study involved children aged 10 months to 11 years with a history of nonsevere RAOM (defined as 3 episodes in the preceding 6 months, with the most recent episode occurring at least 4 weeks before and no more than 4 episodes in the preceding 12 months). The episodes of acute otitis media (AOM) were documented by medical records and had to include any combination of fever, earache, irritability, and hyperemia or opacity accompanied by bulging of the tympanic membrane or otorrhea. At least 2 episodes had to be supported by otoscopy and tympanometric findings. At the time of sampling, the children had to be free of AOM and middle ear effusion. The exclusion criteria were acute febrile illness; upper respiratory tract infection or antibiotic therapy in the previous 14 days; concomitant systemic diseases; craniofacial, neuromuscular, immunological, syndromic, or defined genetic abnormalities; and previous ear surgery or adenoidectomy. Age-matched children without any evidence or history of AOM were used as controls; these children were outpatients or inpatients undergoing minor surgery.

**Study Procedures**

At the enrollment visit, a complete clinical history was taken, and the children underwent a detailed clinical examination, with particular attention given to the ears, nose, and throat using pneumatic otoscopy (Model 20200; Welch Allyn, Skaneateles Falls, New York) and tympanometry (Amplaid 770; Amplifon, Plymouth, Minnesota). Obstructive hypertrophic adenoids (Cassano grade 3 or more) were detected by means of nasal fiber-optic endoscopy. The following variables were recorded: sex, age, breastfeeding, day care attendance, the presence of older siblings, passive smoking exposure, allergy (documented by a positive skin prick test within the previous 12 months), the use of a pacifier, the time since the last AOM episode and antibiotic consumption, any history of spontaneous eardrum perforation with otorrhea, the season of assessment, and the previous administration of conjugate Haemophilus influenzae type b (Hib) and/or pneumococcal vaccine.

When AOM was diagnosed, amoxicillin (80 mg/kg/d) plus clavulanic acid (ratio 7:1) was given for 10 days, and NPS collection was postponed until its complete clinical resolution. No other treatments were allowed during the follow-up period.

**Specimen Collection**

All of the transoral NPS were taken by the same specially trained examiner (PM) using an extra-thin flexible wire swab (Mini-Culturette; Becton Dickinson, Cockeysville, Maryland) with a 30° bent tip, which was inserted through the mouth and positioned 1 to 1.5 inches into the nasopharynx (taking care not to touch the uvula or tongue) and left in place for 4 to 6 seconds.

**Bacterial Growth and Identification**

A nasopharyngeal culture was obtained to determine colonization with Streptococcus pneumoniae, H influenzae, Moraxella catarrhalis, or Streptococcus pyogenes. All of the specimens were inoculated into Stuart transport medium tubes (Copan Venturi Transystem, Brescia, Italy) and processed within 2 hours by the clinical microbiology laboratory. The swabs were transferred to tubes containing 1 mL of brain/heart infusion broth (Difco, Detroit, Michigan) with the addition of 5% of sheep’s blood and vortexed for 30 seconds. The samples were appropriately diluted in the same medium, and 100 μL of each dilution was inoculated into horse blood, chocolate, and Columbia CNA agar plates. All of the plates were incubated in a 10% enriched carbon dioxide atmosphere for 18 to 24 hours at 37°C, and their morphological and gram stain characteristics were microscopically observed. S pneumoniae, S pyogenes, H influenza, and M catarrhalis were isolated and identified using the standard laboratory procedures of latex agglutination (Oxoid, Basingstoke, UK) and API System (bioMérieux, Marcy L’Étoile, France).
Evaluation of Biofilm Formation

Biofilm formation was evaluated spectrophotometrically using the method by Christensen et al. S pneumoniae, S pyogenes, and M catarrhalis were inoculated in 10 mL of brain-heart infusion (BHI) containing 5% sheep’s blood; H influenzae was inoculated in 10 mL of Haemophilus test medium (HTM) and incubated for 18 hours at 37°C in 10% CO2. Aliquots of 20 µL of broth culture were then pipetted into 3 wells in 96-well plates (BD Falcon, Franklin Lakes, New York) containing 180 µL triptone broth solution (TSB) with the addition of 5% sheep’s blood for S pneumoniae, S pyogenes, and M catarrhalis or HTM for H influenzae. After incubation for 18 hours as above, the nonadherent bacteria were removed, and the wells were rinsed 3 times with 200 µL of sterile physiological solution and stained with 200 µL of crystal violet; the excess stain was removed 15 minutes later, and each well was rinsed 3 times with distilled water.

The amount of the obtained biofilm was determined by means of spectrophotometry (wavelength 595 nm) using a microplate reader (Biorad, model 680; Segrate, Milan, Italy).

On the basis of the difference between the optical density (OD) of the biofilm bacteria and that of the negative control (ODb), the bacteria were graded as nonproducers (OD ≤ ODb), weak producers (ODb < OD ≤ 2 × ODb), moderate producers (2 × ODb < OD ≤ 4 × ODb), or strong producers (4 × ODb < OD).

Statistical Analysis

The prevalence and distribution pattern of nasopharyngeal otopathogens and nasopharyngeal BPOs were assessed. Multivariate logistic regression analysis was used to determine whether the presence of nasopharyngeal BPOs was associated with the study group, sex, age, breastfeeding, day care attendance, presence of older siblings, passive smoking exposure, allergy (documented by a positive skin prick test within the previous 12 months), regular use of a pacifier, upper respiratory tract infections in children (ie, day care attendance, the use of pacifier, the presence of older siblings, passive smoking exposure, allergy, previous vaccine, antibiotics, and season) and the variables related to the severity and season of assessment, or the previous administration of Hib and/or conjugate pneumococcal vaccine. The model initially included all of the covariates, after which backward elimination with a threshold of P = .05 was used to select the covariates for the final model. The goodness of fit of the regression models was measured by the coefficient of determination r². The differences in the prevalence of specific otopathogen biofilms (ie, specific bacterial strains and the ability of the bacterium to produce biofilm) between the children with RAOM and the controls were tested by means of the Fisher exact probability test. The data were analyzed using STATA 10.0 software (StataCorp, College Station, Texas).

Results

Study Population

The study involved 113 children (56.6% boys; median age 40 months; range, 10-132 months). As about 75% of children were younger than 5 years and as a preliminary analysis failed to find any significant difference between children ≤5 and >5 years, we decided to perform the final analysis on the expanded age range, including 58 children (51.3%) with a history of nonsevere RAOM and 55 controls (48.7%).

About one-third of the children had experienced previous spontaneous otorrhea; none had obstructive hypertrophic adenoids. Table 1 summarizes their main demographic and clinical characteristics.

Microbiological Findings

Table 2 shows all of the main microbiological results. Otopathogens were significantly more frequently detected in the children with a history of RAOM (24/58, 41.4%) than in the controls (8/55, 14.5%; P = .003); the main pathogens were respectively H influenzae (12/24, 50.0%) and S pyogenes (3/8, 37.5%). The nasopharyngeal carriage rates did not depend on a history of spontaneous otorrhea. The distribution of otopathogens was not significantly different in the 2 study groups. Nasopharyngeal BPOs were more frequently isolated in the RAOM group (17/58, 29.3%) than in the controls (6/55, 10.9%; P = .02), and H influenzae (12/17, 70.6%) was confirmed as the main pathogen in the RAOM group. There was no between-group difference in the type of otopathogens, except for H influenzae (P = .005), or their ability to produce biofilms. None of the otopathogens was a strong biofilm producer, but 3 (M catarrhalis [n = 2]; S pneumoniae [n = 1]) collected from the RAOM group and 2 (S pneumoniae) from the control group were moderate biofilm producers. The prevalence of biofilm-producing otopathogens did not depend on a history of spontaneous otorrhea.

Epidemiological Variables

Being a child with a history of RAOM was the main predictor of the presence of nasopharyngeal BPOs (odds ratio [OR] = 4.69, SE = 2.681, P = .007). The r² was 17.2%, suggesting a weak fit between the model and the data.

None of the other epidemiological variables was associated with the presence of nasopharyngeal BPOs. In particular, the variables known to modify the natural history of upper respiratory tract infections in children (ie, day care attendance, the use of pacifier, the presence of older siblings, passive smoking exposure, allergy, previous vaccine, and season) and the variables related to the severity and temporal sequence of the disease (ie, previous spontaneous otorrhea, the time since the last AOM episode, and the last antibiotic course) were not statistically related to the presence of nasopharyngeal BPOs.

Discussion

Our data suggest further evidence about the relationship between nasopharyngeal bacterial biofilms and recurrent middle ear diseases in children. In addition, this is the first study using a nonaggressive sampling procedure to evaluate the presence of nasopharyngeal biofilms in pediatric outpatients.
### Table 1. Demographic and Clinical Characteristics of the Study Groups

<table>
<thead>
<tr>
<th></th>
<th>RAOM (n = 58)</th>
<th>Controls (n = 55)</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boys</td>
<td>35 (60.4)</td>
<td>29 (52.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Median age, mo (range)</td>
<td>39 (15-115)</td>
<td>40 (10-132)</td>
<td>NS</td>
</tr>
<tr>
<td>Breastfeeding</td>
<td>44 (75.9)</td>
<td>46 (83.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Full-time daycare attendancec</td>
<td>39 (67.3)</td>
<td>39 (70.9)</td>
<td>NS</td>
</tr>
<tr>
<td>At least 1 older sibling</td>
<td>23 (39.6)</td>
<td>28 (50.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Exposure to passive smoking</td>
<td>18 (31.0)</td>
<td>15 (27.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Allergy</td>
<td>11 (19.0)</td>
<td>10 (18.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Regular use of pacifier</td>
<td>19 (32.7)</td>
<td>6 (10.9)</td>
<td>.022</td>
</tr>
<tr>
<td>Time since previous episode of AOM, mo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9 (15.7)</td>
<td>7 (12.7)</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>24 (41.4)</td>
<td>26 (47.3)</td>
<td>NS</td>
</tr>
<tr>
<td>≥3</td>
<td>25 (43.1)</td>
<td>22 (40.0)</td>
<td>NS</td>
</tr>
<tr>
<td>At least 2 previous episodes of spontaneous otorrhea</td>
<td>21 (36.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous <em>H. influenzae</em> type b vaccine</td>
<td>58 (100)</td>
<td>55 (100)</td>
<td>NS</td>
</tr>
<tr>
<td>Previous pneumococcal vaccine</td>
<td>30 (51.7)</td>
<td>21 (38.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Obstructive hypertrophic adenoids</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Season</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>September-November</td>
<td>9 (15.5)</td>
<td>8 (14.6)</td>
<td>NS</td>
</tr>
<tr>
<td>December-February</td>
<td>26 (44.8)</td>
<td>22 (40.0)</td>
<td>NS</td>
</tr>
<tr>
<td>March-May</td>
<td>11 (19.0)</td>
<td>13 (23.6)</td>
<td>NS</td>
</tr>
<tr>
<td>June-August</td>
<td>12 (20.7)</td>
<td>12 (21.8)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values presented as No. (%) unless otherwise stated. Abbreviations: AOM, acute otitis media; NS, not significant; RAOM, recurrent acute otitis media; —, not applicable.

*Fisher exact test (unless otherwise stated).

bAnalysis of variance (ANOVA).

cFive days/wk, 6 to 8 h/d.

### Table 2. Nasopharyngeal Microbiological Characteristics of the Study Groups

<table>
<thead>
<tr>
<th></th>
<th>RAOM (n = 58)</th>
<th>Controls (n = 55)</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carriers of otopathogens</td>
<td>24 (41.4)</td>
<td>8 (14.5)</td>
<td>.003</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em></td>
<td>7 (29.2)</td>
<td>2 (25.0)</td>
<td>NS</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>3 (12.5)</td>
<td>2 (25.0)</td>
<td>NS</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>2 (8.3)</td>
<td>3 (37.5)</td>
<td>.085</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>12 (50.0)</td>
<td>1 (12.50)</td>
<td>NS</td>
</tr>
<tr>
<td>Carriers of BPOsb</td>
<td>17 (29.3)</td>
<td>6 (10.9)</td>
<td>.019</td>
</tr>
<tr>
<td><em>M catarrhalis</em></td>
<td>1 (5.9)</td>
<td>2 (33.4)</td>
<td>NS</td>
</tr>
<tr>
<td><em>S pneumoniae</em></td>
<td>3 (17.6)</td>
<td>2 (33.3)</td>
<td>NS</td>
</tr>
<tr>
<td><em>S pyogenes</em></td>
<td>1 (5.9)</td>
<td>2 (33.3)</td>
<td>NS</td>
</tr>
<tr>
<td><em>H influenzae</em></td>
<td>12 (70.6)</td>
<td>0 (0.0)</td>
<td>.005</td>
</tr>
<tr>
<td>Biofilm production grading</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>14 (82.4)</td>
<td>4 (66.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Moderate</td>
<td>3 (17.6)</td>
<td>2 (33.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Strong</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: BPO, biofilm-producing otopathogens; NS, not significant; RAOM, recurrent acute otitis media.

*Fisher exact test.

bThree patients in the RAOM group carried 2 different BPOs.
affected by nonsevere RAOM and not undergoing surgery. Moreover, no direct comparison of children with RAOM with or without otorrhea has ever been made before.

The finding that nasopharyngeal colonization by otopathogens is significantly more prevalent in children with a history of RAOM than in controls is in line with those of previous studies showing increased carriage of *H influenzae* in otitis-prone children and corroborates the findings of our previous study showing a higher rate of otopathogens carriage in children with RAOM than in controls. The greater prevalence of nasopharyngeal BPOs in children with a history of RAOM than in controls is in line with the findings of previous animal and human studies. It is also in line with those of Zuliani et al., who showed that biofilms were significantly more prevalent in the adenoids of children with RAOM, although their children had a history of severe RAOM (4 episodes in 6 months or 6 in 12 months) that made them eligible for surgery.

The proportion of children harboring nasopharyngeal BPOs in our population was lower than observed in other studies, probably because of the different sampling techniques (ie, nasopharyngeal swab vs mucosal biopsy) and the different microbiological methods (ie, spectrophotometry vs scanning electron microscopy). In particular, the poor negative predictive value of NPS in detecting biofilm-producing bacteria, which is probably due to the well-known resistance of children without middle ear infections (10.9%) has been made before. There are a number of possible explanations associated with this finding, including that biofilm presence is not associated with the tympanic membrane perforation complication of AOM. However, further studies should be conducted as to the association of biofilms and severe AOM, including studies examining the biofilm presence at the time of the complication.

The finding that nasopharyngeal BPOs in a limited number of children without middle ear infections (10.9%) has been recently described by other authors and may be related to the colonization of the spare adenoidal tissue by opportunistic biofilm-producing bacteria that do not trigger any infectious exacerbation.

This study has some limitations. No attempt was made to ascertain the serotypes or the resistance of *S pneumoniae* isolates as this was considered beyond the scope of the study. However, these data would not have added relevance to our results, as no association has been found between the ability to form biofilm and serotypes, antibiotic resistance, or clinical presentation. Moreover, no attempt was made to distinguish Hib from non-typeable *H influenzae*. However, it is conceivable that the role of Hib would have been negligible as all of our patients had received anti-Hib vaccine, which has been recommended for all 3-month-old infants in Italy since 2002.

We conclude that the presence of nasopharyngeal BPOs should be considered and investigated even in children with nonsevere RAOM who do not need adenoid surgery. Clinicians should be encouraged to avoid antibiotics that are ineffective and may further select resistant organisms. In addition, while awaiting clinically suitable and efficacious treatment for nasopharyngeal biofilms, it is desirable to test new disposable devices aimed at reaching anatomic recesses where biofilm can nestle, such as the nasopharynx and peri-tubercar area.

Finally, the detection of nasopharyngeal BPOs in children during the interval between episodes of AOM suggests that bacteria are not removed from the nasopharynx after an acute episode. This raises some doubts about the adequacy of the concept of AOM “recurrence” rather than AOM.
chronicity and suggests the need for a new classification of acute, recurrent, and chronic infections.

Author Contributions

Sara Torretta, data analysis and interpretation, drafting and final approval; Paola Marchisio, conception and design, data analysis and interpretation, drafting and final approval; Lorenzo Drago, acquisition of data, drafting and final approval; Elena Baggi, acquisition of data, drafting and final approval; Erica Nazzari, acquisition of data, drafting and final approval; Werner Garavello, data interpretation, revising the article, final approval; Sara Torretta, acquisition of data, drafting and final approval; Lorenzo Pignataro, data interpretation, revising the article, final approval; Susanna Esposito, data interpretation, revising the article, final approval.

Disclosures

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