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REVIEW ARTICLE

Bacterial biofilm in adenoids of children with chronic otitis media. Part II: a case–control study of nasopharyngeal microbiota, virulence, and resistance of biofilms in adenoids

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ABSTRACT

Background: We previously described that adenoid tissue in children with chronic otitis media (COM) contained more mucosal biofilms than adenoid tissue removed for hypertrophy.

Aims/objectives: The aim of the second part was to characterize nasopharyngeal microbiota and explore virulence of the most common middle ear pathogens.

Material and methods: Bacteriological analysis was performed following a culture-based approach on the samples recovered from 30 patients of COM group (15 biofilm-positive and 15 biofilm-negative) and from 30 patients of a control group (15 biofilm-positive and 15 biofilm-negative). Virulence factors of *Streptococcus pneumoniae, Streptococcus pyogenes*, and *Haemophilus influenzae* were investigated.

Results: The most frequent species were Firmicutes followed by *Proteobacteria* and *Actinobacteria*. The presence of biofilm was statistically associated with an increase of the number of bacterial species and Firmicutes phylum regardless of the condition (case/control). No virulence factors associated with invasive isolates were found for the most common middle ear pathogens.

Conclusions and significance: This case–control study demonstrated that the presence of COM plus biofilm was associated with a given microbiota which contained more Firmicutes. Our study allows a better understanding of physiopathological mechanisms involved in chronic otitis media and paves the way for further investigations.

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Biofilms; adenoids; otitis media; microbiota; virulence; antimicrobial susceptibility

Introduction

Adenoids play a central role in most bacterial infections of the upper and lower airways. For otitis media, the origin of initial infection and recurrence has been allocated to adenoid tissues. Indeed, adenoidectomy is one of the most common surgery in the world [1]. Its goal is to remove an infection source or a breathing obstacle. Its effectiveness in reducing the prevalence of otitis media with effusion (OME) and recurrent otitis media (ROM) has been proved in many studies [2,3]. Rosenfeld and Bluestone have shown that this efficiency is more due to the elimination of a bacterial load than a reduction of the Eustachian tube obstruction [4].

Bacterial biofilms are defined as communities of bacteria embedded in a self-produced glycocalyx matrix. They form by attachment of planktonic or free-living bacteria to a surface, followed by the development of bacterial microcolonies with the production of the extracellular hydrated polymeric matrix. These attached bacteria mature into a differentiated biofilm that persists on surfaces [5]. Their adherence to the surface and their resistance to antibiotics and host immune system are major clinical features of bacterial biofilms. Little is known about the link between pathogenic bacteria and biofilm in chronic otitis media (COM) genesis.

Many bacteriological studies are based on nasopharyngeal swabs and not on adenoid tissues. Therefore, it can undervalue the nasopharyngeal microbiota. Fekete-Szabo et al. reported screening of nasopharyngeal microbiota using nasopharyngeal swabs and adenoid tissues of 20 children [6]. Their findings showed that the culture results of

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nasopharyngeal swabs and the inner part of the adenoid tissue are in close correlation. Predominant aerobic isolates in all two groups were S. *pneumoniae, H. influenzae,* and *M. catarrhalis*. Nistico et al. compared nasopharyngeal microbiota in 35 children with COM and control and reported more *H. influenzae*, *S. aureus* and *S. pneumoniae* in COM group [7].

We recently compared in a prospective study the prevalence of biofilms in adenoids of children with chronic otitis media (COM) versus a control group without any COM having adenoids removed because of hypertrophy [8]. In COM group, adenoid tissue in children with COM contained more mucosal biofilms than adenoid tissue removed for hypertrophy. The aim of the second part of this study was to characterize nasopharyngeal microbiota and explore virulence and resistance of the most common middle ear pathogens (*S. pyogenes, S. pneumoniae, H. influenzae,* and *S. aureus*).

Methods

Study design

We previously conducted a prospective monocentric unrandomized case-control study comparing the prevalence of biofilms between two groups of children matched for age and gender [8]. Cases (Group 1, $n = 52$) were children who underwent adenoidectomy for COM. Controls (Group 2, $n = 51$) were children who underwent adenoidectomy for obstructive adenoids without COM. Before performing adenoidectomy, a swab of the adenoid tissue was made in the two groups. Each specimen was immediately aliquoted into snap-frozen in cold isopentane on dry ice and stored at −80 °C. A part of specimens was dedicated to bacterial biofilm study, using confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) and another one to the microbiota analysis. Bacterial biofilm prevalence in adenoids was 63.5% (33/52) in group 1 and 47.1% (24/51) in group 2 [8].

In the current study, we performed bacteriological analysis on the samples recovered from 30 patients of group 1 (15 biofilm-positive and 15 biofilm-negative), and from 30 patients of group 2 (15 biofilm-positive and 15 biofilm-negative).

Bacteriological analysis for nasopharyngeal swab and adenoid specimen

Bacteriological analysis was performed on adenoid specimens and nasopharyngeal swabs for all four groups. Adenoid specimens were placed in sterile Nalgene™ vials containing 10 mL sterile water and 5 mL of sterile glass beads (1.5-mm diameter) and crushed by the Retsch MM301 Mixer Mill for 3.5 min at 30 Hz, as previously described. Crushed tissues and swabs were cultured at 37 °C on blood agar plates with or without antibiotic (colistin-nalidixic acid or neomycin-vancomycin agar plates) (BioMérieux, Marcy-L'Etoile, France) under aerobic and anaerobic atmosphere, polyvitex chocolate agar plate (under 5% CO2) (BioMérieux, Marcy-L'Etoile, France) during 5 days. Agar plates were examined daily and all growing colonies were replated under the same conditions for further identification analysis.

Bacterial identification using MALDI-TOF-MS and 16S rRNA amplification

Identification of colonies was performed using by Matrix-Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry (MALDI-TOF-MS). Briefly, replated colonies were harvested in 20 µL of sterile water, and cocrystallized with 1 µL of matrix solution DHB (2,5-dihydroxybenzoic acid, 80 mg/mL; 30% acetonitrile; 0.1% trifluoroacetic acid) in duplicate. Samples were processed in the MALDI-TOF-MS spectrometer (Microflex Bruker Daltonics/BioTyper™ version 2.0). Identification isolates at the species level were fixed at ≥ 1.9. When a score was lower and all proposals converged on the same genus, identification at the genus level was retained. In the other cases, colonies were subjected to 16S rRNA sequencing. All presumptive *Streptococcus pneumoniae* results obtained by MALDI-TOF-MS analysis were confirmed by optochin susceptibility testing. When necessary an additional test targeting the 16S rDNA by PCR/sequencing was performed on colonies by using A2/S15 primers for identification at the species level as previously described [9]. The sequences of the corresponding amplicons were submitted to the NCBI (http://www.ncbi.nlm.nih.gov) and BIBI database (http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi).

Bacterial classification, resistance, and clonality

All bacteria were identified at the species level and reclassified into different phyla (*Fusobacteria*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Firmicutes*). For all *S. pneumoniae, S. pyogenes, H. influenzae and S. aureus* isolates, antimicrobial susceptibility was tested and interpreted using disk diffusion method with Mueller–Hinton agar plates (BioMérieux, Marcy-L'Etoile, France) according to CA-SFM (Antibiogram Committee of the French Society of Microbiology) guidelines. For *S. pneumoniae* isolates, the MICs for penicillin G, amoxicillin, and cefotaxime were determined. Serotyping of *S. pneumoniae* isolates was performed by the National Reference Center for pneumococci (Dr. Emmanuelle Varon, Centre Hospitalier Intercommunal de Créteil, Créteil, France). For *S. pyogenes,* determination of clonality (*emm* type) and detection of *speA, speB, speC, smeZ* and *sic* virulence factor genes were performed in the laboratory of Bacteriology of Robert Debré Hospital (Dr. Philippe Bidet, Hôpital Robert Debré, APHP, France) as previously described [10]. For *H. influenzae*, virulence factor genes (*hmw1A, hmw2A, hmwC, hia* and *hifbBC*), especially those targeting capsule (*bexA* and *bexB* genes) were investigated as previously described [11].

Statistical analysis

Two-way ANOVA tests were used to assess differences and interactions between groups. P values of <.05 were considered significant. Analyses were performed using the SAS statistical analysis system 9.4 (SAS Institute, Cary, NC, USA).

Results

Bacteriological analysis was performed on the samples recovered from 30 patients of group 1 (15 biofilm-positive and 15 biofilm-negative), and from 30 patients of group 2 (15 biofilm-positive and 15 biofilm-negative). These analyses were performed for both adenoid specimens and nasopharyngeal swabs for each patient.

Comparison of the average number of bacterial species in the nasopharyngeal swab vs. adenoid specimen

In the case group, the average number of bacterial species was 2.8 in nasopharyngeal swabs and 9.3 in adenoid tissue ($p < .05$). In the control group, the average number of bacterial species was 3.8 in nasopharyngeal swabs and 9.1 in adenoid tissue ($p < .05$).

Distribution of different phyla found in the adenoid specimens

The most frequent species were bacteria usually included in the oral flora. In all 4 groups, Firmicutes (*Streptococcus* spp., *S. pneumoniae, S. pyogenes, Staphylococcus* sp., *S. aureus*, *Gemella* spp., *Parvimonas* spp., *Veillonella* spp.), Proteobacteria (*Neisseria* spp., *Haemophilus* spp., *Moraxella* spp., *Rothia* sp), Actinobacteria (*Corynebacterium* spp. and *Actinomycetes* spp.), Bacteroidetes (*Prevotella* spp.) and Fusobacteria (*Fusobacterium* spp.) were evidenced. The most frequent phyla were Firmicutes (36.7%-46.4%), followed by Proteobacteria (31.3%-37.6%) and Actinobacteria (10–14.2%) (Figure 1). The mean number of bacterial species found in culture was significantly higher in adenoids with biofilm than in those without biofilm (9.1 *vs* 7.4, $p < .05$), particularly for Firmicutes (4.2 vs 2.8, $p < .05$), regardless of the condition (case/control) (Table 1).

Study of pathogen bacteria found in the adenoid specimens

Distribution of pathogenic species (*S. pyogenes, S. pneumoniae, S. aureus,* and *H. influenzae*) in regards to the presence or absence of bacterial biofilm in the case and control groups is shown in the Table 2.

A total of 10 isolates of *S. pyogenes* were found; 6 of them were clonally-related to the *emm12* type, the others were *emm3*, *emm4* and *emm89*. In all cases, the genotype *emm12* was found and was associated with biofilm (4 in cases and 2 in controls). All isolates harbored *speB* and *smeZ* virulence factor genes and *Ssa*, *speC*, *smeZ1* and *speA* genes were found in 3, 4, 1 and 1 isolates, respectively.

A total of 12 isolates of *S. pneumoniae* were found and analyzed. Serotypes identified were 3, 6 C, 11 A, 15 A, 15B, 19 A, 19 F, mostly covered by *Pneumo23* or *Prevenar* vaccines. Five of them were Penicillin G-nonsusceptible *S. pneumoniae.*

For *H. influenzae* isolates, only one was found to be *H. influenzae* encapsulated type b in the control group without biofilm. All other *H. influenzae* isolates were unencapsulated (nontypaeable) and no virulence factor genes usually associated with invasive infections (i.e. *hmw 1 A*, *hmw2A* and *hifBC*) were detected in the collection.

Discussion

In 2017, the information of approximately 772 prokaryotic species, 70% of which are culturable, are published in the expanded Human Oral Microbiome Database (eHOMD). Six broad phyla, Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria, Bacteroidetes, and Spirochaetes are evidenced and constitute 96% of total oral bacteria [12]. In our prospective monocentric case–control study, we explored the culturable microbiota in adenoids of children with chronic otitis media (cases), compared with obstructive adenoids without chronic otitis media (controls), associated or not with biofilm.

Our study highlighted three important points regarding chronic otitis. First, considering the pathologies affecting the oropharyngeal sphere, numerous studies explored the bacteria involved in the infection of the pharynx using nasopharyngeal swabs. For all groups, our study reveals that the pharyngeal swab is not informative enough, because it reflects only a small fraction of the bacteria found in the adenoids ($p < .05$). Indeed, three times more bacteria have been found in the crush of adenoid tissue compared to the nasopharynx swab. These results are in line with many studies [13] except with that of Fekete-Szabo et al. who reported a close correlation between cultures of nasopharyngeal swabs and that of the inner part of the adenoid tissue [6].

Second, the normal oropharyngeal microbiota has recently been described with aerobic and anaerobic bacteria species included in 6 phyla. However, the normality of the oral microbiome varies with many factors as ages, ethnicities, or underlying disease [14]. For all of these reasons, our study was based on robust group control, comparable to the case group in age and gender. In our study, the oral microbiota contains a majority of proteobacteria and Firmicutes in all groups. Interestingly, the mean number of bacterial species found in culture was significantly higher in adenoids with biofilm than in those without biofilm, particularly for Firmicutes. In our original study, the biofilm prevalence in adenoids was significantly higher in the case group (63.5%, 33/52) than in the control group (47.1%, 24/51) [8]. Altogether, the higher prevalence of biofilm in children with COM suggests a modification of microbiota for these patients. This perturbation of microbiota, also called dysbiosis, is important to emphasize. Indeed, dysbiosis is more

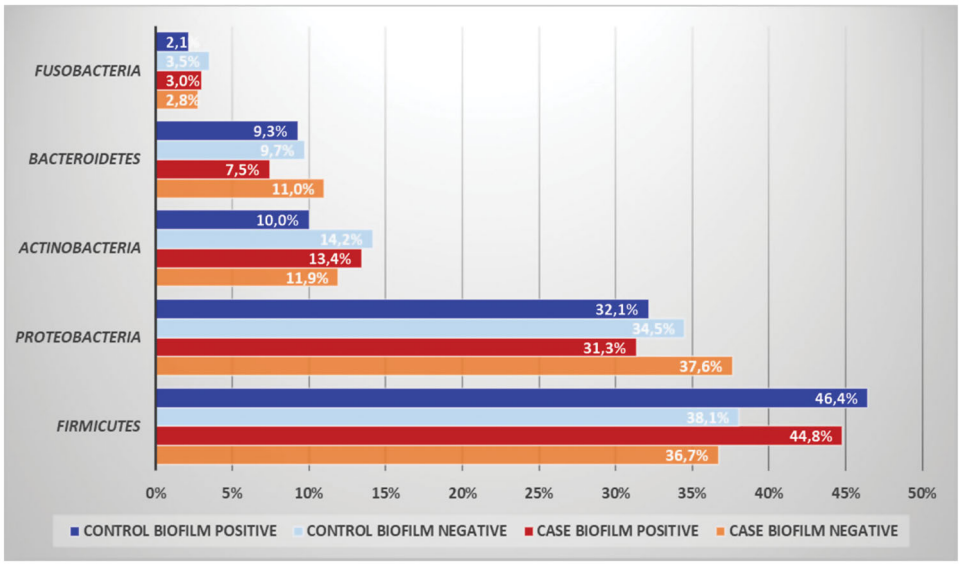

Figure 1. Phylum comparison in adenoid specimens with or without biofilm. **Firmicutes** were Streptococcus spp. (including S. pneumoniae and S. pyogenes), Staphylococcus spp. (including S. aureus), Gemella spp., Parvimonas spp., Veillonella spp.; **Proteobacteria** were Neisseria spp., Haemophilus spp., Moraxella spp., Rothia spp., **Actinobacteria** were Corynebacterium spp. and Actinomycetes spp.; **Bacteroidetes** were Prevotella spp; **Fusobacteria** were Fusobacterium spp.

Table 1. Mean numbers of bacterial species in regards to the presence or absence of bacterial biofilm in the case and control groups.

	Biofilm positive			Biofilm negative		
	Case ($n=15$)	Control ($n=15$)	Total ($n=30$)	Case ($n=15$)	Control ($n=15$)	Total ($n=30$)
Mean number of bacterial species per sample +/- SD	8.9 ± 2.8	9.3 ± 2.3	9.1 ± 2.5*	7.3 ± 1.6	7.5 ± 2.0	7.4 ± 1.8*
Mean number of Firmicutes species per sample +/- SD	4.0 ± 1.4	4.3 ± 1.8	4.2 ± 1.6^{+}	2.7 ± 1.1	2.9 ± 1.6	2.8 ± 1.3^{+}

Symbols * and $^{+}$ indicate differences between groups using two-way ANOVA (p values <.05).

Table 2. Distribution of pathogenic species in regards to the presence or absence of bacterial biofilm in the case and control groups.

	Biofilm positive			Biofilm negative		
	Case $n=15$ n(%)	Control $n=15$ n(%)	Total $n=30$ n(%)	Case $n=15$ n(%)	Control $n=15$ n(%)	Total $n=30$ n(%)
S. pyogenes	5 (33.3)	3(20.0)	8 (26.7)	1 (6.7)	1 (6.7)	2 (6.7)
S. pneumoniae	2 (13.3)	3 (20.0)	5 (16.7)	2 (13.3)	1 (6.7)	3 (10.0)
S. aureus	0 (0)	2 (20.0)	2 (6.7)	3 (20.0)	6 (40.0)	9 (30.0)
H. influenzae	3 (20.0)	6 (40.0)	9(30.0)	3 (20.0)	5 (33.3)	8 (26.7)

and more described as being associated with many chronic diseases, such as inflammatory bowel diseases.

Our findings could be compared to the result obtained by Minami *et al.*, who found a dramatic modification of microbiota in adults or children presenting a COM with active inflammation and wet ear comparing to those with dry ear [15]. In that case, the authors also highlighted a specific microbiota with an increase of Firmicutes but unfortunately, no experimentation was done to explore the presence or absence of biofilm [15]. Further clinical studies will be needed to investigate this point. In addition, concerning the control group with biofilm, our observation raises the questions that this modification of microbiota could be related to the first step of the future development of an inflammatory disease chronic disease.

Third, in acute media otitis, the main bacteria usually found are *S. pyogenes, S. pneumoniae,* and *H. influenzae*. Vermee et al., reported a high proportion of biofilm production by *H. influenzae* and *S. pneumoniae* strains isolated from the nasopharynx of children with acute otitis media, which reinforces the results of studies suggesting the importance of biofilm in the pathogenesis of acute otitis media [16]. Conversely, our observation highlights that these pathogens are not predominant in the COM case group as expected at the beginning of this work. In addition, these pathogenic bacteria were investigated and they do not produce virulence factors.

For *S. pyogenes*, we found a preponderant serotype *emm*12 (6/10, 60%), previously described with high prevalence [17]. *SpeB* and *smeZ* genes, encoding for virulence factors, were present in all isolates. These factors were not reported in a study based on 4000 strains performed in Europe on invasive isolates [17]. Virulence factors in *H. influenzae* isolates were also different than those found in invasive infections strains. *HmwC* and *hia* genes were identified respectively in 41.2% (7/17) and 23.5% (4/17) in our study. Ecevit *et al.* reported none of these factors in capsulated isolates and a prevalence of 48.0% for *hmwC* and 33.0% for *hia* in nontypeable *H. influenzae* isolates [11]. In our study, only one isolate presented a gene of the capsule (5%), which is consistent with the literature [18]. The low virulence of *S. pyogenes* and *H. influenzae* isolates found in our study paves the way for further studies. The perspectives could be to compare pathogenic and nonpathogenic bacteria to their ability to produce biofilm.

One of the limits of our study is based on the culture approach used to investigate bacterial microbiota. Indeed, the next-generation sequencing approach allows a better description of microbiota, particularly for strict anaerobic bacteria [19] that we probably missed using cultures.

However, such molecular-based approaches have limits too: 1/a majority of unnamed oral taxa are referenced by clone numbers or 16S rRNA GenBank accession numbers, often without taxonomic anchors, and 2/accurate investigation of isolated bacteria is not possible. Hence, some authors encourage researchers to improve culture methods for the phenotypic description of microbiota [20]. Following this culture-based approach, we were able to deep the descriptions of potential pathogens of upper respiratory tract and of middle ear.

Altogether, our results raise several issues:

1/A pathophysiological issue: we previously found a higher prevalence of bacterial biofilm in patients with chronic otitis media in the first part of our study [8] and suggested that the presence of a biofilm can maintain a low-noise infection and contribute to the chronicity of the infection. In this current microbiological part of the study, we emphasize the association between the presence of biofilm and dysbiosis, independently of other factors. Our study paves the way for further studies to better understand the pathophysiological mechanisms involved in chronic otitis media.

2/A clinical issue: The usual treatment of chronic otitis media is adenoidectomy. In our study, chronic otitis media is associated with the presence of biofilm and dysbiosis, but not with virulent strains of microorganisms usually found in acute otitis media. These results suggest that the use of antibiotics active against biofilm-embedded bacteria should be studied for the treatment of recurrent acute otitis media, which promotes the emergence of chronic otitis media.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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