INDEX COPERNICUS INTERNATIONAL

Prospective observational study of adenoidal biofilms in a paediatric population and their clinical implications

Joao Subtil[1], Maria Paula Bajanca-Lavado[2], Joao Carlos Rodrigues[3], Aida Duarte[4], Lucia Reis[3], Isabel Nogueira[5], Luisa Jordao[6]

[1]Servico de otorrinolaringologia (ORL), Hospital de Beatriz Angelo (HBA), Loures, Portugal
[2]Departamento de Doencas Infeciosas (DDI), Laboratorio Nacional de Referencia de Haemophilus influenzae, Instituto Nacional de Saude Dr. Ricardo Jorge (INSA), Lisbon, Portugal
[3]Departamento de Doencas Infeciosas (DDI), Unidade Laboratorial Integrada de Microbiologia, INSA, Lisbon, Portugal
[4]Departamento de Microbiologia e Imunologia, Faculdade de Farmacia da Universidade de Lisboa, Lisbon, Portugal
[5]Departamento de Engenharia Quimica, Instituto Superior Tecnico, Lisbon, Portugal
[6]Departamento de Saude Ambiental, Unidade de Investigacao e Desenvolvimento, INSA, Lisbon, Portugal

Article history: Received: 21.08.2018 Accepted: 26.09.2018 Published: 01.10.2018

ABSTRACT:

Introduction: Adenoids are composed of nasopharyngeal lymphoid tissue with a relevant role in host defence against infections of the upper respiratory tract. Nevertheless, adenoids are also a reservoir of microorganisms that can cause infections of the upper respiratory tract and otitis, particularly in children.

Objective: To evaluate and compare the association between biofilm assembly on adenoids and the incidence of recurrent infections in a paediatric population submitted to adenoidectomy for either infectious or non-infectious indications.

Methods: Scanning electron microscopy was used to assess biofilms on adenoid surface; biofilm assembly *in vitro* was monitored by crystal violet assay; antibiotic susceptibility was assessed following EUCAST guidelines; *H. influenzae* capsular typing was performed by PCR.

Results: Biofilms were present in 27.4% of adenoid samples and no statistical difference was found between infectious and non-infectious groups. *In vitro*, the most clinically relevant bacteria, *H.influenzae, S.aureus, S.pyogenes, S.pneumoniae* and *M.catarrhalis*, were mostly moderate biofilm assemblers (71.7%). As much as 55.3% of these bacteria were intermediate/resistant to at least one of the tested antibiotics. No association was found between the ability to assemble biofilms *in vitro* and the presence of biofilms on adenoids or antibiotic resistance. All *H.influenzae* were characterized as non-typeable.

Conclusion: The presence of biofilms on adenoid surface was independent from clinical sample background. Bacterial ability to assemble biofilms *in vitro* cannot be used to predict biofilm assembly *in vivo*. The lack of correlation between biofilm formation and antibiotic resistance questions the effects of biofilms on the pathogenesis of infectious diseases.

KEYWORDS: Adenoids, biofilms, scanning electron microscopy, *Haemophilus*, child

INTRODUCTION

Acute and chronic infections of the upper respiratory tract are very common, and constitute one of the most frequent causes of antibiotic prescription in children and adults [1]. Sometimes surgery is necessary, and in children, adenoidectomy is often used to aid in the control of recurrent or chronic infections [2,3]. Removal is effective irrespective of adenoid volume [4]. It is thus recognized that adenoids harbour pathogenic bacteria [5-7], from which the colonization of adjacent organs emerges. The benefit of this surgery derives from removing a natural pathogen reservoir from the upper respiratory tract. Bacterial colonization is an increasing concern, due to the emergence of infections by antibiotic-resistant microorganisms [8]. However, this colonization has claimed growing interest among ecological studies that seek to assess its universal presence and even its benefits [9]. We understand better and better the relations between colonizing microbiome, and our own organism. By forming biofilms, the bacteria reduce their metabolism and increase the interactions between individuals [10]. In nature, this form of existence protects the individual, and thus we observe biofilms in virtually all media. Likewise, we understand that under the right conditions, virtually all bacteria form biofilms, and in those formations, bacteria of the same species share defence mechanisms, and this sharing may even cross species barriers [10]. That is how in our adenoid mucosa it *exists a* vast microbiota, with which we live in symbiosis, and which, in certain circumstances, gets organized in biofilms. This colonization in biofilms is very resistant to our natural defences (specific and non-specific), and to conventional treatments, namely antibiotics [10]. They are made of a matrix of glycoproteins, which

 DOI: 10.5604/01.3001.0012.5278

houses the bacteria out of reach for these defences. At the same time, its porosity allows to maintain the distribution of nutrients to the bacterial population, and the proximity of the bacteria and the matrix itself allows for interaction with quorum sensing and even sharing of genetic heritage, which favours the emergence of resistances, one of the major concerns of the global medical and scientific (and even political) community [10]. Finally, as individuals in biofilms present reduced metabolism, their sensitivity to many conventional treatments also decreases [10].

The surface of adenoids is a site where biofilm formation is favoured, as it is irregular, well ventilated, covered with mucus, and out of reach of mechanical cleaning mechanisms that could remove the biofilm [11]. Thus, adenoidectomy provides physical removal of the surface that houses the biofilm, and the scaring that then occurs leaves a smooth surface.

Possibly, these mechanisms explain the surgery's success, even if partial, in the control of infectious pathology of the airways. With the present study, we want to document the status of our population regarding the presence of biofilms in the nasopharynx, and to evaluate if this presence correlates with the diagnosis of chronic or recurrent inflammatory or infectious conditions of the upper airways.

MATERIALS AND METHODS

Study design and Ethics: A cross-sectional study was designed to evaluate the influence of adenoid colonization on ear and upper respiratory chronic infections in a paediatric population, treated at a tertiary hospital in Lisbon. The study (reference: 0089/2014_RMRV) was approved by the hospital Medical Ethics Board in accordance with the World Medical Association Declaration of Helsinki. Sixty-two participants were consecutively enrolled, and the inclusion criteria were adenoidectomy (done by curettage under direct visual control), age between 2 and 10 years, no antibiotics during the last month preceiding surgery and absence of immunodeficiency history or craniofacial malformation. All the children were immunized in accordance with the National Vaccination Program (including *Haemophilus Influenza* B and 13 serotypes of *Pneumococcus*). An informed consent was obtained from the children's' tutors to allow the collection of 3 different samples (nasal swab, superficial adenoid swab and adenoid core biopsy) for cultivable microbiota12 and biofilm study on adenoid surface. The samples were divided in two groups: infectious diagnosis group (recurrent acute otitis media, sinusitis or adenoiditis) and non-infectious group (obstructive sleep apnoea or otitis media with effusion – OME, without any relevant upper respiratory tract infection history). This allowed a comparison between the group where biofilms would be causing relevant respiratory infections, and another group where, if present, they were not causing relevant respiratory infections.

Tissue collection and scanning electron microscopy (SEM): In each of the 62 children enrolled, one adenoid sample was processed for cultivable microbiota analysis and the other for scanning electron microscopy (SEM) analysis.

Bacteria: Bacteria were isolated and identified, being stored at -80ºC in 20% glycerol TSB until needed. Fresh overnight cultures were prepared on Chocolate Polyvitex agar (PVX) for *H. influenzae* or blood-agar (BioMerieux, Marcy l'Étoile, France) for all the other bacteria, for the biofilm assembly assay and DNA extraction.

Biofilm assembly: Three independent assays were conducted, performed in triplicate, using 96-well flat-bottomed plates (Nunc, New York, NY, USA) as described previously, with small modifications [13]. Bacteria were ranked as weak, moderate or strong biofilm assemblers according to Stepanovic and colleagues' criteria [14].

Antimicrobial susceptibility test: The antimicrobial activity for *S. aureus, S. pyogenes, M. catarrhalis* and *S. pneumoniae* was tested using either the disk diffusion method or microdilution broth method (Vitek 2 system) as recommended by EUCAST [15]. At least one antibiotic was used from each of the following groups of penicillin (benzylpenicillin, ampicillin, amoxicillin), penicillin combined with a beta-lactamase inhibitor (amoxicillin/clavulanic acid), cephalosporin (2nd generation: cefuroxime, cefoxitin; 3rd generation: ceftriaxone, cefotaxime, ceftazidime) and clindamycin. Results were interpreted according to EUCAST guidelines [15].

H. influenzae capsular typing: The capsular status was determined by PCR amplification of bexA gene (responsible for capsule transport) and capsular type was characterized by amplification of capsule-specific genes (for serotypes a-f) using primers and conditions described previously [16].

Statistical analysis: Unadjusted association between surgery indication (infectious/non-infections) and presence of biofilms on adenoid surface was evaluated by Chi-square test. A P value of <0.05 was considered statistically significant. For biofilm assembly *in vitro*, the results of at least three independent experiments were expressed as the means ± standard deviation (SD) and statistical significance assessed by the Student's t-test (two-tailed). A P value of <0.05 was considered statistically significant.

RESULTS

Sixty-two adenoid samples from a paediatric population, after adenoidectomy, indicated by infectious (n=19) or non-infectious (n=43) pathology, were collected and then analysed by SEM for the presence of biofilms. Representative images of adenoid surfaces negative (Fig. 1A) and positive (Fig. 1B) for biofilms are shown in Figure 1.

The percentage of adenoids positive for biofilms *ex vivo* (on adenoid surface) was similar in both groups: 26.3% (5/19) in the infectious and 27.9% (12/43) in the non-infectious group. No statistical difference was found for biofilm presence *ex vivo* between infectious and non-infectious samples as assessed by Chi2 test (P=0.63). For this reason, we decided to analyse the sample as a whole, instead of distinguishing between infectious and non-infectious, allowing for a more robust statistical analysis.

To evaluate if bacterial ability to assemble biofilms *in vitro* could

INDEX COPERNICUS INTERNATIONAL

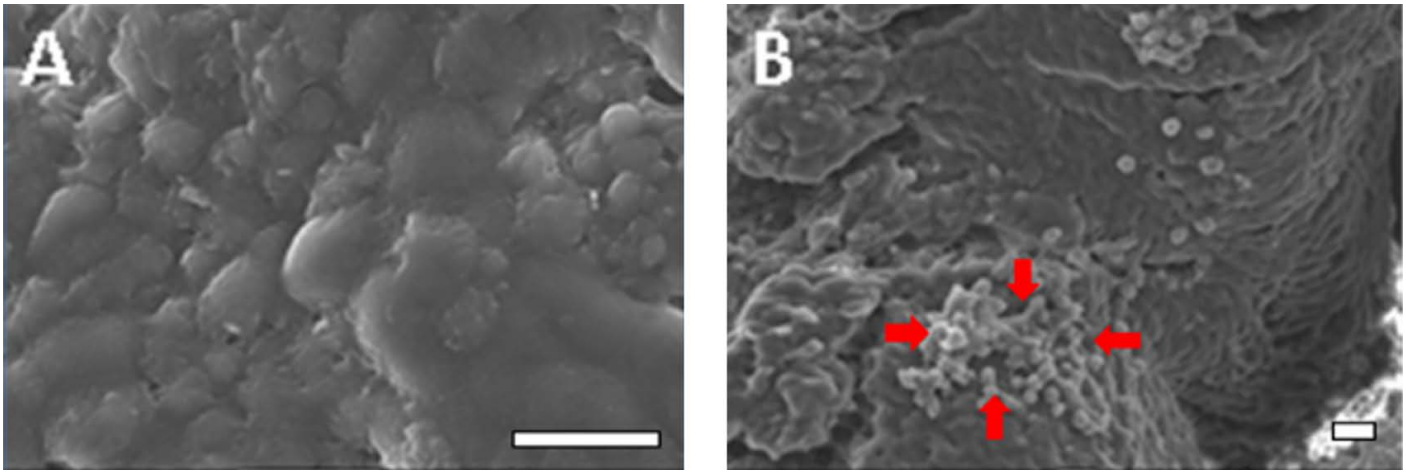

Fig. 1. Biofilms on adenoid surface. Sample without (A) and with biofilm (B) highlighted by red arrows on the adenoid surface. Scale bar 10 µm.

be used as a surrogate for biofilm presence on the adenoid surface, and adenoid core colonization, a group of isolates were further evaluated. Forty-six isolates simultaneously identified on the adenoid surface and core, namely *S. pneumoniae* (n=2), *S. aureus* (n=12), *S. pyogenes* (n=8), *H. influenzae* (n=22) and *M. catarrhalis* (n=2) were tested for biofilm assembly *in vitro*. All these isolates were able to form biofilms *in vitro* as observed in Figure 2.

Thirty-three isolates were moderate biofilm assemblers (71.7%), including all isolates of *S. aureus, S. pyogenes* and *S. pneumoniae*, one isolate of *M. catarrhalis* and 10 of *H. influenzae*. Only *H. influenzae* (11/22) isolates were classified as weak biofilm assemblers. One isolate of *H. influenzae* and another of *M. catarrhalis* were classified as strong biofilm assemblers. We were unable to identify a link between biofilm assembling ability *in vitro* and the presence of biofilms *ex vivo* (adenoid biopsies analysed by SEM). The two isolates classified as strong biofilm producers were isolated from the adenoid surface of samples without biofilms. On the other hand, weak biofilm producers were isolated both from adenoid surfaces with and without biofilms. Bacteria recovered from the adenoid surface showed an ability to assemble biofilms *in vitro* equal or higher than the respective counterpart isolated from the adenoid core.

Antibiotic susceptibility analysis showed that 44.7% (21/47) of the isolates are pan-susceptible and 55.3% (26/47) are intermediate/resistant to at least one of the tested antibiotics (Tab. II.). Antibiotic-resistant strains were found in *H. influenzae* (6 isolates from biofilm negative samples), *S. aureus* (1 isolate from the negative biofilm sample) and *M. catarrhalis* (2 isolates from the biofilm positive sample) (resistance by antibiotic presented in Tab. I.). The two *S. pneumoniae* isolates recovered from a biofilm positive sample were susceptible to benzylpenicillin, cefoxitin, ceftriaxone, ceftazidime and clindamycin. A similar result was obtained for *S. pyogenes*, where four isolates recovered from biofilm-positive and another four from biofilm-negative samples were susceptible to benzylpenicillin, cefoxitin, cefotaxime, ceftriaxone and clindamycin. Most of the isolates were susceptible to the tested antibiotics, and the presence of biofilms *ex vivo* apparently did not affect antibiotic susceptibility. Finally, *H. influenzae* capsular typing revealed that all isolates were non-typeable (NTHi).

DISCUSSION

Recently, biofilms gained relevance in the *pathogenesis* of chroni infectious diseases, namely in otorhinolaryngology-related infections. The use of different experimental designs made an inter-study comparison difficult [11, 17-19]. In the present study, no statistically significant difference was observed for the presence of biofilms on adenoid surface in the two groups (infectious and non-infectious indications for adenoidectomy). The relative small number of individuals included in the study might explain this result. On the other hand, this agrees with the fact that biofilms are ubiquitous in nature [10], and so finding biofilms in both groups is no surprise. However, we were surprised to find biofilms only in 27.4% of the samples, when comparing to previous studies that found biofilms in up to 100% of the samples [17,18]. The comparative low percentage of positive biofilm samples might derive from the use of different techniques to detect biofilms and even from different interpretations of SEM micrographs. Sample dimension and study design might also contribute to the result.

In previous biofilm studies, OME has been considered an infectious disease, and even a biofilm-associated infection. This is contrary to what has been established, for it has been shown that only approximately one in three children with OME has a bacterial pathogen identified in the middle ear fluid [20], and finding a pathogen does not necessarily mean that it is causing the effusion. Therefore, in our opinion, it is more correct to include this diagnosis as non-infectious, as we did.

The percentage of antibiotic-intermediate/resistant bacteria (45.3%) is higher than expected for community isolates of well-known commensals of the upper respiratory tract of children. In our opinion, the recent modification of the EUCAST cut-offs for *H. influenzae*/cefuroxime contributes to this unexpected result since all the isolates were either intermediate or resistant to this antibiotic [15]. The values decrease substantially when the percentage of resistant isolates is calculated except for the two *M. catarrhalis* isolates resistant to cefuroxime (Tab. I.). Since only two isolates of *M. catarrhalis*, were studied, it is difficult to interpret the relevance of cefuroxime resistance. Nevertheless, this bacterium is known to be the cause of treatment failure due to high resistance levels

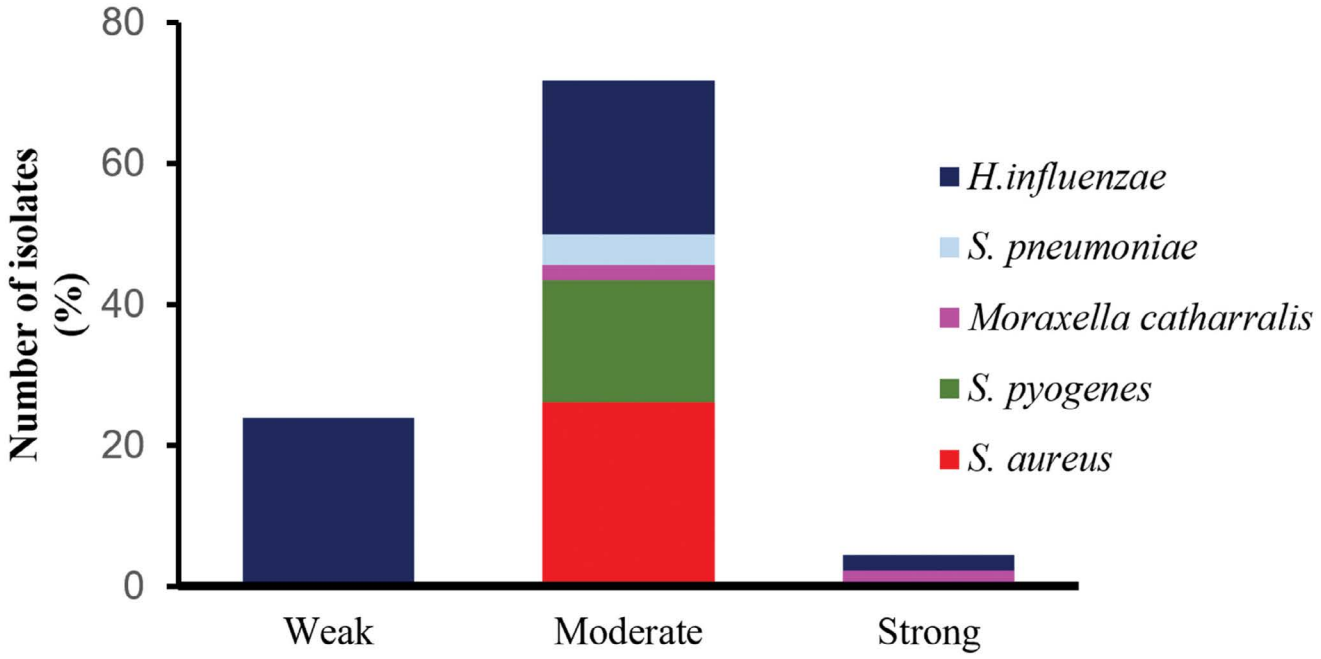

Fig. 2. Biofilm assembly *in vitro*. Ability of *H. influenzae, S. pneumoniae, M. catharralis, S.pyogenes* and *S.aureus* to assemble biofilms were evaluated using the crystal violet assay.

Tab. I. Antibiotic resistance. Benzylpenicillin (BP), ampicillin (AMP), amoxicillin (AMX), Amoxycillin: clavulanate 2:1 (AMC), cefuroxime (CXM), cefoxitine (FOX), cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), clindamycin (CLI).

BACTERIA	ANTIBIOTIC	NUMBER OF ISOLATES (N)			TOTAL PERCENTAGE OF RESISTANCE (%)
		SUSCEPTIBLE	INTERMEDIATE	RESISTANT	
H. influenzae (n=23)	AMP	19	0	4	17.4
	AMC	21	0	2	8.6
	CXM	0	19	4	17.4
	CTX	23	0	0	0
S. aureus (n=12)	BP	12	0	0	0
	CXM	12	0	0	0
	FOX	12	0	0	0
	CRO	12	0	0	0
	CLI	11	0	1	8.3
M. catarrhalis (n=2)	AMC	2	0	0	0
	CXM	0	0	2	100
	CTX	2	0	0	0
	CRO	1	1	0	0

to β-lactamic antibiotics [27]. The fact that no *S. pyogenes* isolate exhibited resistance to any of the tested antibiotics was expected since this bacterium is known to remain susceptible to almost all antibiotic classes [28]. *H. influenzae* isolates were susceptible to the β-lactam antibiotics studied, with exception of cefuroxime, although one pair of strains was resistant to ampicillin by β-lactamase production, and another pair was resistant to ampicillin without producing β-lactamase (BLNAR) (Tab. I.). As in other studies, we did not find any correlation between biofilm formation and susceptibility or resistance to antibiotics [29, 30] and these findings appear again to contradict any influence of biofilms on the pathogenesis of infectious diseases. Polymicrobial infection has been pointed as common in biofilms. In this study, we found three samples with two different bacterial species isolated simultaneously from the adenoid core and surface (Tab. I.). Since biofilm formation is associated with the persistence of bacteria in the host, this may therefore have an important role in the early phase of infection [21, 22]. Synergistic relations between *M. catarrhalis* and either *S. pyogenes* or *S. pneumoniae* account for increased biofilm formation and antibiotic resistance within medical biofilms was documented [31, 32]. Finally, since *H. influenzae* was the most prevalent bacterium in our samples, and other researchers have reported that this bacterium is one of the most commonly isolated in cases of otitis media with effusion [11, 22, 29, 33, 34], we characterized it

INDEX COPERNICUS INTERNATIONAL

Tab. II. Antibiotic resistance of each sample, comparing the origin (adenoid core and surface) and its biofilm assembling ability. Benzylpenicillin (BP), ampicillin (AMP), amoxicillin (AMX), Amoxycillin: clavulanate 2:1 (AMC), cefuroxime (CXM), cefoxitine (FOX), cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), clindamycin (CLI). Susceptible (S), Intermediate (I), Resistant (R).

BACTERIA	ANTIBIOTIC										SAMPLE		
	BP	AMP	AMX	AMC	CXM	FOX	CTX	CRO	CAZ	CLI	ORIGIN	ID	BIOFILM
H. influenzae	---	S	---	S	I	---	S	---	---	---	Surf	S3	-
	---	S	---	S	I	---	S	---	---	---	Core		
	---	S	---	S	I	---	S	---	---	---	Surf	S7	-
	---	S	---	S	I	---	S	---	---	---	Core		
	---	R	---	S	I	---	S	---	---	---	Surf	S39	-
	---	R	---	S	I	---	S	---	---	---	Core		
	---	S	---	S	I	---	S	---	---	---	Surf	S41	-
	---	S	---	S	R	---	S	---	---	---	Core		
	---	S	---	S	I	---	S	---	---	---	Surf	S45	-
	---	S	---	S	I	---	S	---	---	---	Core		
	---	S	---	S	R	---	S	---	---	---	Surf	S46	-
	---	S	---	S	I	---	S	---	---	---	Core		
	---	S	---	S	I	---	S	---	---	---	Surf	S55	
	---	S	---	S	I	---	S	---	---	---	Core		
	---	S	---	S	I	---	S	---	---	---	Core	S61	-
	---	S	---	S	I	---	S	---	---	---	Surf	S62	-
	---	S	---	S	I	---	S	---	---	---	Core		
	---	R	---	R	R	---	S	---	---	---	Surf	S64	-
	---	R	---	R	R	---	S	---	---	---	Core		
	---	S	---	S	I	---	S	---	---	---	Surf	S65	+
	---	S	---	S	I	---	S	---	---	---	Core		
	---	S	---	S	I	---	S	---	---	---	Surf	S67	+
	---	S	---	S	I	---	S	---	---	---	Core		
S. aureus	S	---	---	---	S	S	---	S	---	S	Surf	S35	-
	S	---	---	---	S	S	---	S	---	S	Core		
	S	---	---	---	S	S	---	S	---	S	Surf	S39	-
	S	---	---	---	S	S	---	S	---	R	Core		
	S	---	---	---	S	S	---	S	---	S	Surf	S40	+
	S	---	---	---	S	S	---	S	---	S	Core		
	S	---	---	---	S	S	---	S	---	S	Surf	S46	-
	S	---	---	---	S	S	---	S	---	S	Core		
	S	---	---	---	S	S	---	S	---	S	Surf	S47	-
	S	---	---	---	S	S	---	S	---	S	Core		
	S	---	---	---	S	S	---	S	---	S	Surf	S51	+
	S	---	---	---	S	S	---	S	---	S	Core		
S. pneumoniae	S	---	---	---	---	S	---	S	S	S	Surf	S40	+
	S	---	---	---	---	S	---	S	S	S	Core		
S. pyogenes	S	---	----	----	---	S	S	S	---	S	Surf	S16	-
	S	---	----	----	---	S	S	S	---	S	Core		
	S	---	----	----	---	S	S	S	---	S	Surf	S32	-
	S	---	----	----	---	S	S	S	---	S	Core		
	S	---	----	----	---	S	S	S	---	S	Surf	S63	+
	S	---	----	----	---	S	S	S	---	S	Core		
	S	---	----	----	---	S	S	S	---	S	Surf	S67	+
	S	---	----	----	---	S	S	S	---	S	Core		

Tab. II. cd. Antibiotic resistance of each sample, comparing the origin (adenoid core and surface) and its biofilm assembling ability. Benzylpenicillin (BP), ampicillin (AMP), amoxicillin (AMX), Amoxycillin: clavulanate 2:1 (AMC), cefuroxime (CXM), cefoxitine (FOX), cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), clindamycin (CLI). Susceptible (S), Intermediate (I), Resistant (R).

BACTERIA	ANTIBIOTIC										SAMPLE		
	BP	AMP	AMX	AMC	CXM	FOX	CTX	CRO	CAZ	CLI	ORIGIN	ID	BIOFILM
M. catarrhalis	---	---	---	S	R	---	S	S	---	---	Surf	S63	+
	---	---	---	S	R	---	S	I	---	---	Core		

in more detail. After the worldwide introduction of the *H. influenzae* serotype b (Hib) conjugate vaccine, most infections are now due to non-typeable strains (NTHi) [23, 35, 36]. Our results are in accordance with these epidemiological changes, with all isolates being characterized as NTHi. This was expected since this type of *H. influenzae* is frequently associated with adenoid colonization and infection. Nevertheless, what is relevant here is that no difference between infectious and non-infectious groups was found considering that apparently biofilms per se would play at most a minor role in infectious diseases of upper airways.

CONCLUSION

We found no statistical difference for biofilm presence between the two groups. The same was true for *in vitro* biofilm assembly by bacteria found on adenoid surface and core. Biofilm assembly *in vitro* did not correlate with the results obtained *ex vivo* (adenoid surface). As in other studies, no correlation was found between biofilm formation and infectious respiratory illnesses, and this contributes to questioning the influence of biofilms on the pathogenesis of infectious diseases.

1. References
2. Physicians AAoF, Surgery AAoO-HaN, Effusion AAoPSoOMW Otitis media with effusion. Pediatrics.2004; 113:1412-29.
3. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F,et al. European Position Paper on Rhinosinusitis and Nasal Polyps. Rhinology.2012; Suppl. 23:1-299.
4. Rosenfeld RM. Surgical prevention of otitis media. Vaccine. 2000;19Suppl1:S134-9.
5. Gates GA. Otitis media-the pharyngeal connection. JAMA. 1999;282:987-9.
6. Brook I, Gober AE. Dynamics of nasopharyngitis in children. Otolaryngol. Head Neck Surg. 2000;122:696-700.
7. Brook I, Shah K. Bacteriology of adenoids and tonsils in children with recurrent adenotonsillitis. Ann. Otol. Rhinol. Laryngol. 2001;110:844-8.
8. DeDio RM, Tom LW, McGowan KL, Wetmore RF, Handler SD, Potsic WP. Microbiology of the tonsils and adenoids in a pediatric population. Arch. Otolaryngol. Head Neck Surg. 1988;114:763-5.
9. World Health Organization. Global action plan on antimicrobial resistance. http:// www.wpro.who.int/entity/drug_resistance/resources/global_action_plan_eng.pdf ;2015 [Accessed 12 January 2018].
10. Douglas-Escobar M, Elliott E, Neu J. Effect of intestinal microbial ecology on the developing brain. JAMA Pediatr. 2013;167:374-9.
11. Donlan RM. Biofilms: microbial life on surfaces. Emerg. Infect. Dis. 2002;8:881-90.
12. Tawfik SA, Ibrahim AA, Talaat IM, El-Alkamy SS, Youssef A. Role of bacterial biofilm in development of middle ear effusion. Eur. Arch. Otorhinolaryngol. 2016;273 :4003-9.
13. Subtil J, Rodrigues JC, Reis L, Freitas L, Filipe J, Santos A, et al. Adenoid bacterial colonization in a paediatric population. Eur. Arch. Otorhinolaryngol. 2017; 274:1933-8.
14. Bandeira M, Carvalho PA, Duarte A, Jordao L. Exploring dangerous connections between Klebsiella pneumoniae biofilms and healthcare-associated infections. Pathogens. 2014;3:720-31.
15. Stepanovic S, Vucovic D, Hola V, Di Bonaventura G, Djukić S, Cirković I, Ruzicka F. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS. 2007;115:891-9.
16. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for Interpretation of MICs and Zone Diameters. Version 7.0. http:// www.eucast.org ; 2017 [Accessed 12 December 2017]
17. Falla TJ, Crook DW, Brophy LN, Maskell D, Kroll JS, Moxon ER. PCR for capsular typing of Haemophilus influenzae. J. Clin. Microbiol. 1994; 32:2382-6.
18. Saafan ME, Ibrahim WS, Tomoum MO. Role of adenoid biofilm in chronic otitis media with effusion in children. Eur. Arch. Otorhinolaryngol. 2013;270:2417-25.
19. Saylam G, Tatar EC, Tatar I, Ozdek A, Korkmaz H. Association of adenoid surface biofilm formation and chronic otitis media with effusion. Arch. Otolaryngol. Head Neck Surg. 2010;136:550-5.
20. Torretta S, Marchisio P, Drago L, Baggi E, De Vecchi E, Garavello W, et al. Nasopharyngeal biofilm-producing otopathogens in children with nonsevere recurrent acute otitis media. Otolaryngol. Head Neck Surg. 2012;146:991-6.
21. Poetker DM, Lindstrom DR, Edmiston CE, Krepel CJ, Link TR, Kerschner JE. Microbiology of middle ear effusions from 292 patients undergoing tympanostomy tube placement for middle ear disease. Int. J. Pediatr. Otorhinolaryngol. 2005;69:799-804.
22. Kosikowska U, Glowniak IK, Niedzielski A, Malm A. Nasopharyngeal and adenoid colonization by Haemophilus influenzae and Haemophilus parainfluenzae in children undergoing adenoidectomy and the ability of bacterial isolates to biofilm production. Medicine. 2015; 94: e799.
23. Puig C, Domenech A, Garmendia J, Langereis JD, Mayer P, Calatayud L, et al. Increased biofilm formation by nontypeable Haemophilus influenzae isolates from patients with invasive disease or otitis media versus strains recovered from cases of respiratory infections. Appl. Environ. Microbiol. 2014;80:7088-95.
24. Agrawal A, Murphy TF. Haemophilus influenzae infections in the H. influenzae type b conjugate vaccine era. J. Clin. Microbiol. 2014;49:3728-32.
25. Bernhard S, Spaniol V, Aebi C. Molecular pathogenesis of infections caused by Moraxella catarrhalis in children. Swiss Med. Wkly. 2012;142:w13694.
26. Cattoir V. Mechanisms of Antibiotic Resistance. In: Ferretti JJ, Stevens DL, Fischetti VA, editors. Streptococcus pyogenes : Basic Biology to Clinical Manifestations. University of Oklahoma Health Sciences Center, Oklahoma City. 2016 https://www.ncbi.nlm.nih.gov/books/NBK333414/. Accessed 12 January 2018
27. Moriyama S, Hotomi M, Shimada J, Billal DS, Fujihara K, Yamanaka N. Formation of biofilm by Haemophilus influenzae isolated from pediatric intractable otitis media. Auris Nasus Larynx. 2009;36:525-31.
28. Qin L, Kida Y, Ishiwada N, Ohkusu K, Kaji C, Sakai Y, et al. The relationship between biofilm formations and capsule in Haemophilus influenzae. J. Infect. Chemother. 2014;20:151-6.
29. Perez AC, Pang B, King LB, Tan L, Murrah KA, Reimche JL, et al. Residence of Streptococcus pneumoniae and Moraxella catarrhalis within polymicrobial biofilm promotes antibiotic resistance and bacterial persistence in vivo. Pathog. Dis. 2014;70:280-8.
30. Young C, Holder RC, Dubois L, Reid SD. Streptococcus pyogenes Biofilm. In: Ferretti JJ, Stevens DL, Fischetti VA,(editors. Streptococcus pyogenes : Basic Biology to Clinical Manifestations. University of Oklahoma Health Sciences Center, Oklahoma City. 2016, https://www.ncbi.nlm.nih.gov/books/NBK333419/. Accessed 12 January 2018

31. Van Hoecke H, De Paepe AS, Lambert E, Van Belleghem JD, Cools P, Van Simaey L, et al. Haemophilus influenzae biofilm formation in chronic otitis media with effusion. Eur. Arch. Otorhinolaryngol. 2016;273:3553-60.

32. Viveros MN. Biofilms in otolaryngology. Acta Otorrinolaringol. Esp. 2014;65:47-52.

33. Bajanca-Lavado MP, Simoes AS, Betencourt CR, Sá-Leão R. Characteristics of Haemophilus influenzae invasive isolates from Portugal following routine childhood vaccination against H. influenzae serotype b (2002–2010). Eur. J. Clin. Microbiol. Infect. Dis. 2014;33:603-10.

34. Van Eldere J, Slack MPE, Cripps AW. Non-typeable Haemophilus influenzae, an under-recognized pathogen. Lancet Infect. Dis. 2014;14:1281-92.

Word count: 3060 Tables: 4 Figures: – References: 32

Access the article online: DOI: 10.5604/01.3001.0012.5278 Table of content: https://otolaryngologypl.com/issue/11772

Corresponding author: Luisa Jordao, Instituto Nacional de Saude Doutor Ricardo Jorge. Departamento de Saude Ambiental. Avenida Padre Cruz. 1649-016 Lisboa, Portugal. Tel.+351217519273; ORCID: 0000-0002-5608-021X e-mail: maria.jordao@insa.min-saude.pt

Competing interests: The authors declare that they have no competing interests.

Cite this article as: Subtil J., Bajanca-Lavado M.P., Rodrigues J.C., Duarte A., Reis L., Nogueira I., Jordao L.: Prospective observational study of adenoidal biofilms in a paediatric population and their clinical implications; Otolaryngol Pol 2019; 73 (1): 22-28