

INFECTION

Biofilm formation by *Haemophilus influenzae* isolated from adeno-tonsil tissue samples, and its role in recurrent adenotonsillitis

Formazione di biofilm da parte di ceppi di Haemophilus influenzae isolati da campioni di tessuto adeno-tonsillare e ruolo dei biofilm batterici nelle adenotonsilliti ricorrenti

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SUMMARY

Aim of the present study was to identify bacterial biofilms in tissue samples obtained from paediatric patients undergoing surgical treatment, for chronic and recurrent adeno-tonsillitis, not responding to repeated cycles of selective medical antibiotic and anti-inflammatory treatment and to assay the ability of *Haemophilus influenzae* strains, most frequently identified in the culture examinations, to grow as biofilm *in vitro*. Overall, 25 surgical specimens (15 adenoids, 10 tonsils) were examined from the upper respiratory tract, from 15 paediatric patients (mean age 6 years). All patients were affected by recurrent and/or chronic adenoiditis and adenotonsillitis unresponsive to selective antibiotic and anti-inflammatory therapy. Tissues were cultured using conventional methods and subjected to scanning electron microscopy for detection of biofilm. *Haemophilus influenzae* strains, were cultured on 96-sterile well polystyrene microtitre plates (CELLSTAR-greiner bio-one) and stained with 1% crystal violet to quantify biofilm production. Bacterial cocci attached to the tissue surface and organized in colonies, with a morphology consistent with bacterial coccoid biofilms, were observed in all adenoid (15/15) and in 6/10 tonsil samples. *Haemophilus influenzae* isolates from 12/25 (48%) of our tissue samples scored a *percent transmittance* (%T_{bloc}) > 50, displaying a high capacity to form biofilms (level 4). In conclusion identification of bacterial biofilms in chronic and/or recurrent paediatric upper airway inflammatory processes and the capacity to produce biofilm *in vitro*, demonstrated by *Haemophilus influenzae* (the most frequently identified bacteria in our samples), could be related to the aetiopathogenic role of biofilms in chronic inflammatory mucosal reactions and to the resistance of these infections to selective antibiotic therapy.

KEY WORDS: Tonsillitis • Adenoiditis • *Haemophilus influenzae* • Bacterial biofilms • Percent transmittance

RIASSUNTO

L'obiettivo del presente studio è stato identificare biofilm batterici in campioni di tessuto ottenuti da pazienti pediatriche sottoposti a terapia chirurgica per adenotonsilliti croniche/ricorrenti, resistenti a cicli ripetuti di terapia antibiotica e anti-infiammatoria mirata, e determinare la capacità dei ceppi di *Haemophilus influenzae*, identificati più frequentemente nei nostri esami colturali, di formare biofilm *in vitro*. Abbiamo esaminato 25 campioni chirurgici (15 adenoidi e 10 tonsille) delle alte vie aeree, ottenuti da 15 pazienti pediatriche (età media: 6 anni). Tutti i pazienti erano affetti da adenotonsilliti ricorrenti e/o adenoiditi croniche non responsive alla terapia antibiotica e anti-infiammatoria mirata. I tessuti venivano messi in coltura utilizzando metodi convenzionali e esaminati al microscopio elettronico a scansione per l'identificazione dei biofilms. I ceppi di *Haemophilus influenzae* venivano cresciuti su 96 piastre sterili di polistirene (CELLSTAR-greiner bio-one) e colorati con cristal-violetto all'1% per quantificare la produzione di biofilm. Abbiamo osservato cocci batterici adesi alla superficie tessutale e organizzati in colonie, con una morfologia compatibile con quella dei biofilm batterici coccoidi, in tutti i campioni di tessuto adenoideo (15/15) e in 6/10 di tessuto tonsillare. I ceppi di *Haemophilus influenzae*, isolati in 12/25 (48%) dei nostri campioni di tessuto, presentavano una trasmittanza percentuale (%T_{bloc}) > 50, identificando una elevata capacità di formare biofilms (livello 4). In conclusione l'identificazione di biofilm batterici nei processi infiammatori cronici e/o ricorrenti delle alte vie aeree in età pediatrica e la capacità di produrre biofilm *in vitro*, dimostrata dall'*Haemophilus influenzae* (batterio più frequentemente identificato nei nostri campioni), potrebbero essere correlate al ruolo eziopatogenico dei biofilm nelle reazioni infiammatorie croniche mucosali e alla resistenza di queste infezioni alla terapia antibiotica mirata.

PAROLE CHIAVE: Tonsillite • Adenoidite • *Haemophilus influenzae* • Biofilm batterici • Trasmittanza percentuale

Introduction

Upper airways (UA) in paediatric patients present recurrent/chronic infections which often fail to respond to selective antibiotic and anti-inflammatory treatment.

The failure of antibiotic treatment in the eradication of susceptible organisms has recently induced microbiologists to hypothesize the presence of bacteria organised in communities, attached to organic and inorganic surfaces, identified as "biofilms"^{1,2}. A biofilm is a colony of single or multiple bacterial species embedded in a self-producing polymeric matrix (EPS), comprising nucleic acids, polysaccharides and proteins. Compared to the planktonic form, this matrix guarantees better survival and protection from macrophage action, antibiotics, temperature and pH fluctuations. The advantages gained by bacteria, in terms of survival from biofilm formation, has persuaded many Authors³⁻⁵ to search, within these formations, for the possible aetiopathogenic mechanism of antibiotic resistance and chronicization of infective processes.

The contamination of medical devices (urinary catheters, venous catheters, contact lens, artificial heart valves, intra-uterine devices) by biofilms, with periodic release of free-floating planktonic bacteria, has been described in the literature and should be considered as a new nosologic entity related to biotechnology^{6,7}.

Furthermore, biofilms can directly colonize mucosal tissues, producing chronic and/or recurrent infections that are resistant to all types of antibiotic treatment. The UA seem to be at high risk for this type of colonization since evidence has been documented in the nasal and sinus mucosa of subjects with chronic hyperplastic sinusitis⁸⁻¹² and in the tonsillar crypts¹³.

Furthermore, it has been demonstrated that several bacterial species are able to develop a biofilm, including the most frequent organisms responsible for otorhinolaryngologic disorders such as *Haemophilus influenzae* (*H. influenzae*), *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Staphylococcus aureus*^{14,15}.

Aim of the present investigation is not only to provide evidence of bacterial biofilms in tissue samples obtained from patients with chronic and/or recurrent infections of the UA not responding to repeated cycles of selective antibiotic and anti-inflammatory treatment who, therefore, underwent surgery, but also to assay the ability of *H. influenzae*, which is that most frequently identified in our cultural examinations, to grow as biofilm *in vitro*.

Materials and methods

Overall, 25 samples obtained from 15 paediatric patients (9 female, 6 male; mean age 6 years) consecutively hospitalised at the ENT Clinic, were examined. The study was approved by the Ethics Committee of the Medical Faculty of the Catholic University of Sacred Heart in Rome, and informed consent was signed by parents or guardians prior to the study.

All patients were affected by recurrent and/or chronic infectious processes of the UA, as documented by clinical history and haematochemical findings; 10 patients underwent adeno-tonsillectomy and 5 adenoidectomy.

Overall, 25 tissue samples were examined: 15 of adenoid tissue and 10 of tonsil tissue.

Each specimen was washed in a sterile saline solution and divided into two fragments, one of which was processed for bacterial culture examination and analysed with the RapID NH System (BioMerieux, Marcy-l'Etoile, France).

The RapID NH System identified and differentiated *Haemophilus* species, as well as, biochemically, *H. influenzae* and *Haemophilus parainfluenzae*. The tests used in the RapID NH System are based on microbial degradation of specific substrates detected by various indicator systems. The reactions employed are a combination of conventional tests and single-substrate chromogenic tests.

The latter fragment was fixed in Karnovsky buffer (2.5% glutaraldehyde, 1.5% paraformaldehyde, 0.1 M cacodylate, and 0.05 M sucrose) for 2 hours at 4 °C, washed in sodium cacodylate for 5 minutes, and immersed in 1% osmium tetroxide for 1 hour. After a second 5-minute wash in sodium cacodylate, the tissues were dehydrated in a graded ethanol series (50%, 79%, 95%), dried with liquid CO₂ (Dried Balzers çPD 030), lightly coated with colloidal gold, and examined with a Philips 515 scanning electron microscope.

All *H. influenzae* strains were tested by nitrocefin disk (Remel Lenexa, Kansas, USA) for detection of Beta lactamase activity.

To assay biofilm formation *Non-typeable H. influenzae* strains were grown overnight in BRUCELLA + VITK + HEM (MAIM – Spain) at 37 °C and 5% CO₂. One strain of *H. influenzae* ATCC 49766 was used.

The culture was diluted 1:20 in fresh BRUCELLA + VITK + HEM and 200 µl of this suspension was used to inoculate sterile 96 well polystyrene microtitre plates (CELLSTAR-greiner bio-one). After 24 h at 37 °C and 5%CO₂ wells were washed with phosphate-buffered-saline (PBS) solution, dried in inverted position and stained with 1% crystal violet for 15 minutes. The wells were rinsed again and the crystal violet was solubilized in 200 µl of ethanol-acetone (80:20 vol/vol). The optical density at 595 nm (Od₅₉₅) was determined using a microplate reader (Multiskan EC; Lab-system).

Each assay was performed in triplicate and repeated three times as described by Toledo-Arana et al.¹⁶. The percent transmittance (%T) value for each test sample was subtracted from the %T value of the blank reagent to define the amount of light blocked in the passage through the wells (%T_{bloc}).

Biofilm production by each isolate was scored as negative (%T_{bloc} < 5), 1 + (%T_{bloc} 5 to 20), 2 + (%T_{bloc} 20 to 35), 3 + (%T_{bloc} 35 to 50), 4 + (%T_{bloc} ≥ 50).

Results

In 15/15 adenoid samples and in 6/10 tonsil samples, bacterial cocci were found attached to the tissue surface and organized in colonies with a morphology consistent with bacterial coccoid biofilm (Figs. 1a, b, 2a, b).

At culture examination, performed by traditional methods, 3 specimens of tonsil tissue demonstrated a growth of *Staphylococcus aureus*, 5 grew alpha-haemolytic *Streptococcus*, and 2 *H. influenzae*; 10 specimens of adenoid tissue had a positive culture for *H. influenzae*, 1 for Group A *Streptococcus pyogenes* and 2 for alpha-haemolytic *Streptococcus* (Table I).

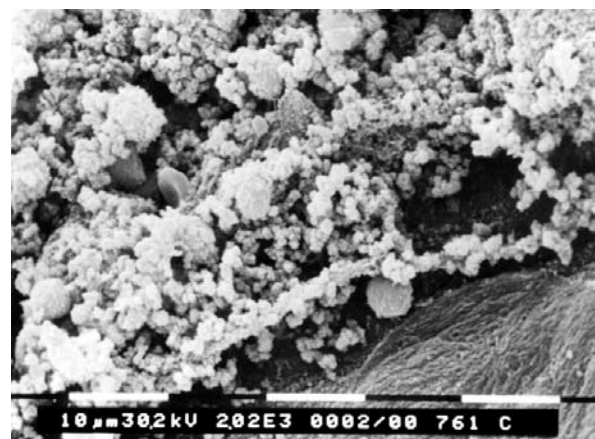
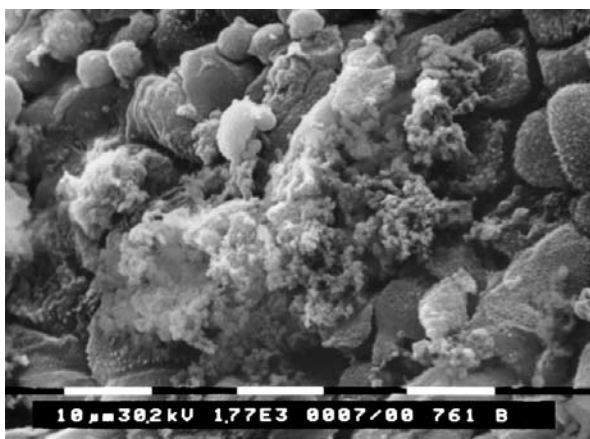
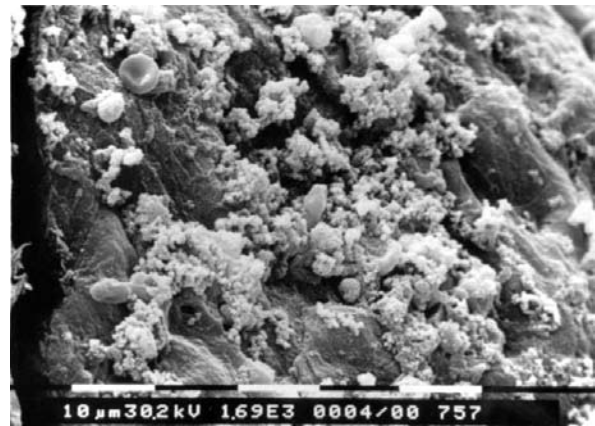
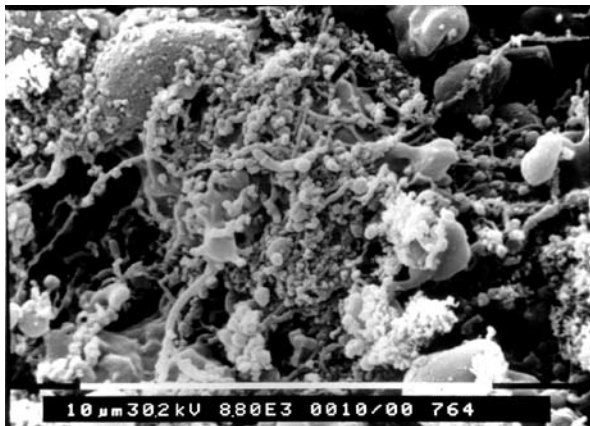


Fig. 1a, b. SEM evidence of bacterial biofilms in samples of adenoid tissue of paediatric patients with recurrent/chronic adenotonsillitis.

Fig. 2a, b. SEM evidence of bacterial biofilms in samples of tonsil tissue of paediatric patients with recurrent/chronic adenotonsillitis.

H. influenzae strains, tested by nitrocefin disk (Remel Lenexa, Kansas, USA) for detection of Beta lactamase activity, were positive in 2.9%.

H. influenzae isolates from 12/25 (48%) of our tissue samples scored a percent, transmittance (%T_{bloc}) > 50, identifying a high capacity to form biofilms (level 4).

Discussion

The introduction of scanning electron microscopy and, more recently, of confocal laser scanning microscopy has offered the possibility to perform direct and accurate observation of bacteria and their complex organization. It has

been demonstrated that about 99% of all bacteria exist in biofilms and only 1% in a free planktonic form. Recent publications by the Centres for Disease Control and Prevention estimate that at least 65% of all human bacterial infectious processes involve biofilms¹⁷.

A bacterial biofilm is defined as an assembly of microbial cells enclosed in a self-produced polymeric matrix (EPS) composed by polysaccharides, nucleic acids and proteins, with different components in relation to the growth surface.

Biofilm formation is a dynamic process that begins with the casual attachment of one or more bacteria to an inorganic or organic surface. The attachment of bacteria is facilitated, in

Table I. Results.

Tissue samples (n.)	Samples with biofilm n. (%)	Samples with positive culture exam n. (%)	Micro-organisms isolated (n.)
Adenoid (15)	15 (100)	13 (86.6)	<i>H. influenzae</i> (10) <i>S. pyogenes</i> (1) Alpha-haemolytic <i>Streptococcus</i> (2)
Tonsil (10)	6 (60)	9 (90)	<i>S. aureus</i> (3) <i>H. influenzae</i> (2) Alpha-haemolytic <i>Streptococcus</i> (5)
Total (25)	21 (84)	22 (88)	

n. = number

inorganic materials (prosthesis or medical devices), by the presence of an irregular surface, and, in organic surfaces, by cilia or flagella, or organic polymeric liquids such as blood, saliva or respiratory secretions.

Biofilm development is characterised by consecutive stages mediated by molecular messengers and by acetylated lactones, also called autoinducers, which interact with the surface receptors and regulate gene expression. This mechanism is known as "quorum sensing". These adaptations determine increased resistance to antibiotics and host defence mechanisms correlated to: 1. EPS capacity to absorb or limit antibiotic distribution; 2. the peculiar biofilm organisation, characterised by the transport system of proteinic material and the rich net of water channels in the glycocalyx (particularly elaborate in its peripheral area but limited in the biofilm core) and 3. environmental heterogeneity within the biofilm, with different pH gradients and oxygen concentrations. These differences in metabolic and environmental conditions allow bacteria to survive, within the biofilm core, without reproducing for a long period of time, as spores.

In the present study, all tissue samples were culture positive and the most frequently isolated bacteria (*H. influenzae*, *Streptococcus* alpha haemolytic (commensal), *Streptococcus pyogenes* and *Staphylococcus aureus*) were isolates usually identified in paediatric recurrent UA infections¹⁸. The high incidence of bacterial biofilms in adenoid and tonsil tissue samples may help to explain the difficult eradication of bacterial species, involved in these chronic infective processes. However, no certain conclusion can be drawn on the role of the above-mentioned bacterial species in biofilm formation in our samples.

Only recently, some Authors introduced the use of fluorescent *in situ* hybridization (FISH), and immunostaining combined with confocal laser scanning microscopy, to prove that the bacterial species isolated in mucosal biofilms are the same pathogens commonly associated with chronic infections¹⁹, and show that chronic adenotonsillitis is probably a result of multiple overlapping infections caused by different pathogens, and the spectrum of micro-organisms changes in parallel with the displacement in the lymphoid tissue and the age of the patient. The percent of *H. influenzae* identified by FISH, decreases with increasing age of children and is zero in adults¹⁸. Unfortunately, the method is still rarely available and expensive requiring a broad range of domain, group and species-specific FISH probes.

In our study, *H. influenzae* was the most commonly isolated bacteria (12/25) especially in adenoid tissue, in which biofilm organisation was identified in 100% of samples. Furthermore, it was demonstrated that *H. influenzae* strains, in the samples studied, had a high capacity to form biofilms *in vitro*. These data may be related to a physiological state which differs from that of bacteria growing planktonically²⁰, and may explain the limited antibiotic efficacy and the chronicization of the infections. *H. influenzae* is an opportunistic pathogen normally present as a commensal in the nasopharynx. Transition from commensal to pathogen occurs, probably, in response to host physiology variations such as mucociliary transport alteration and Eustachian tube dysfunction²¹.

In such conditions, *H. influenzae* colonizing adenoid tissue may be responsible also for middle ear and rhino-sinusal infections^{22,23} and biofilm formation may be a virulence factor for organisms responsible for the development of chronic rhino-sinusitis²⁴.

Even if the biofilm-forming capacity *in vitro* by *H. influenzae*, does not necessarily correlate with *in vivo* biofilm production, the high percentage of biofilm identification (100% in adenoid and 60% in tonsil tissue samples) led us to hypothesise that this bacterium survives within the biofilm core, and is responsible for the reactivation of a chronic infection, contributing to continued tissue damage and correlated systemic disorders.

Conclusions

Management of chronic and recurrent UA infectious processes in paediatric age is still a debated problem that, often, after repeated cycles of antibiotic treatment, ends in a surgical decision.

With the availability of new techniques, such as scanning electron and confocal laser microscopy, semi-quantitative methods to quantify biofilm production *in vitro*, and finally FISH, it is possible to observe the presence of mucosal biofilms in almost all cases; these microbial organisations explain the resistance to antibiotics and host immune response; and, finally, clarify the effective aetiopathogenic role of biofilms in sustaining chronic inflammatory mucosal reactions. These advances will have an enormous impact, both on the diagnostic and the therapeutic approach, in future medical practice.

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Received: January 19, 2007 - Accepted: March 15, 2007