REVIEW ARTICLE

Biofilms in Otolaryngology

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Abstract  According to the National Institute of Health of the USA, “more than 60% of all microbial infections are caused by biofilms”. This can surprise us, but it is enough to consider that common infections like those of the genito-urinary tract, infections produced by catheters, middle ear infections in children, the formation of dental plaque and gingivitis are caused by biofilms, for this statement to seem more realistic.

At present this is one of the subjects of great interest within medicine, particularly in otorhinolaryngology. Bacteria have traditionally been considered to be in a free state without evident organisation, partly perhaps by the ease of studying them in this form. Nevertheless, the reality is that, in nature, the great majority of these germs form complex colonies adhered to surfaces, colonies that have received the name of biofilms. These biofilms are more common than previously thought and almost all of the people have been in contact with them in the form of infections in the teeth or humid, slippery areas. New treatments that can eradicate them are currently being investigated.

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PALABRAS CLAVE
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Biofilms en otorrinolaringología

Resumen  Según el Instituto de Salud de los Estados Unidos de Norteamérica «un porcentaje superior al 60% de todas las infecciones microbianas están originadas por biofilms», afirmación que puede parecernos sorprendente, pero basta que consideremos que infecciones tan comunes como las del tracto genitourinario, las producidas por el catéter, las del oído medio en los niños, la formación de la placa dental y la gingivitis son originadas por biofilms, para que esta afirmación parezca más realista.

Los biofilms son conglomerados de células que viven inmersas en una matriz propia de exopolisacárido con una organización estructural y funcional compleja, resultado de una comunicación continua entre ellas. La estructura de dicha biopelícula les confiere protección contra los antibióticos y las defensas del organismo, por lo que son los responsables de innumerables infecciones recalcitrantes en todo el cuerpo humano y particularmente en oídos, senos.
Introduction

Bacterial resistance is a growing phenomenon with huge social and economic implications due to increased morbimortality and the cost of treatments, and in terms of hospital stay. It is necessary therefore, to decipher the physiology of biofilms and their relationship to a large number of recalcitrant infections.

A biofilm is an aggregate of cells of one or several species which grow and adhere to a live or inert surface and which are embedded in an exopolysaccharide (EPS) matrix of their own making that has an electrical charge and a three-dimensional structural organisation and complex function.

The Formation of Biofilms

The process of construction of a biofilm takes place in several stages starting with the formation or accumulation of a layer of organic waste which neutralises the excess charge and free energy of the surface to enable bacteria to adhere to it. When it adheres to this surface a cascade of changes is triggered which will activate a group of genes which will determine the phenotype of the biofilm.1 In order for the change of phenotype and the development of such a complex structure to be completed, the different cells forming the biofilm must communicate and coordinate. This occurs by means of a system termed “quorum sensing” which is mediated by small molecules that are released by some bacteria in order to adhere to others and this modifies the regulation of the bacterial gene expression according to their concentration. In the case of Gram-negative bacteria, these molecules belong to the acyl-homoserine lactone family and are peptides in Gram-positive bacteria. They have diverse functions such as signalling, DNA interchange, and the production of toxins.1 Quorum sensing is also crucial in determining the density of the bacteria population and, as one can guess, communication increases as more bacteria adhere.1 It has been suggested that this form of regulating gene expression is evidence of the invading pathogen’s need to achieve a critical population density and thus overwhelm the defences of the host to establish a colony or infection.3

The bacteria which are joined to the surface and to one another by reversible electrostatic attraction will then try to remain irreversibly joined, which they will achieve using the bacterial surface proteins, the cardinal protein being AlgE. They then move to the next stage, which will be aggregation into micro-colonies and the production of EPS. The chemical structure of EPS is very complex as it is comprised of polysaccharides, nucleic acids, and proteins and varies between Gram-positive and Gram-negative bacteria. The polysaccharides are mainly cationic in the former, and in the latter they are neutral or polyanionic. In addition, the biofilm will take shape depending on the stress that it is subjected to, and on the source of nutrients. They can be flat or form fungus-like towers, etc.

Once this level has been reached where the basic structure has formed, the biofilm starts a ripening process in which the cells will grow and will even reproduce inside the microenvironment which has been created and determined by the substances belonging to the EPS, and by the neighbouring cells and proximity to a water channel. These channels form the primitive circulatory system for preserving homeostasis inside the biofilm. In this ripening phase secondary colonisers-bacteria or fungi-come to this structure as the initial amount of cells diminishes and the amount of EPS increases. It is also during this phase when the cells are allocated roles in order to sustain the biofilm and create new films. Therefore the final stage is the sweeping and dispersal of the bacteria from the biofilm, either by external forces or by a physical movement such as a migrant wave, or by a self-induced process of release into the environment.2

The formation of biofilms has been clearly demonstrated for many pathogens of the mucous membranes such as Pseudomonas aeruginosa (P. aeruginosa), Staphylococcus aureus (S. aureus), Streptococcus pneumoniae (S. pneumoniae), Haemophilus influenzae (H. influenzae), and Moraxella catarrhalis. Despite the heterogeneity of bacteria in biofilms, P. aeruginosa and S. pneumoniae do not form mixed biofilms in vivo.

Bacterial Resistance

The difficulty in eradicating biofilms given their structure is obvious, establishing whether they show greater bacterial resistance than planktonic cells, is an area that has generated a great deal of controversy as, traditionally, resistance has been viewed as the skill of a microorganism to grow in the presence of a high level of antimicrobial. In other words, a species in which the minimum inhibitory concentration (MIC) has risen is resistant. Therefore, taking this conventional criterion into consideration, biofilms do not necessarily show increased resistance. To date, it has only been demonstrated that they are not easily eradicated using bactericides. This is explained by some unique features which derive from their extraordinary morphology and not necessarily by their greater intrinsic resistance. Restricted penetration is one of their particular characteristics; this is regulated by the EPS matrix which restricts the spread of substances and degrades antibiotics. It also provides very effective defence against lysosomes and the complement system. Furthermore, when the EPS charge is negative, it protects the cells of positively charged antibiotics, like aminoglycosides. This is not the case with fluoroquinolones which penetrate the biofilm and are effective in halting their
growth, but their prescription is limited by the age of the patient. Furthermore, it has been discovered recently that an AcrAB-TolC MDR transmembrane pump of Escherichia coli (E. coli) acts in synergy with a chloramphenicol (CmlA) efflux pump located in the cytoplasmic membrane. Tolerance to tobramycin, gentamycin and ciprofloxacin has also been linked to another efflux pump expressed in biofilms.

The delayed growth rate of the cells is another protective factor; although some antibiotics can enter the biofilm freely, this will only act on cells in the rapid growth phase. Therefore the stationary phase undeniably contributes towards the biofilms’ resistance and towards the increased resistance of the planktonic cells to being destroyed.

Finally, persister cells constitute a subpopulation inside the biofilm which survives the administration of antibiotics and which are preserved by the presence of an antibiotic which impedes their growth. Paradoxically, the antibiotic helps the persisters to persist. It is possible that biofilms produce more persistor cells than planktonic cells. These cells become a problem when the host’s immune system is not functioning correctly. If the concentration of antibiotic drops or if the symptoms disappear because of the eradication of the planktonic cells and the treatment is interrupted, the persisters reform the biofilm and it continues releasing new planktonic cells. This explains the relapsing nature of a biofilm and the need for prolonged periods of antibiotics. This perspective suggests that the recurrence of biofilms is not necessarily due to their greater resistance to that of planktonic cells. In fact, a biofilm of a particular species, under certain in vivo conditions, is as sensitive, or more so, to antibiotics than a population of planktonic cells, but it will survive better than them because it is invulnerable to immune system attack.

Genes which affect the persistence rate have been described in relation to these cells. The first research with the objective of searching for genes which specifically affect survival was undertaken by Moyed and Bertrand. In their research study they identified three independent loci of high-level persistence, (hip). All the hip mutants produced approximately 1000 times more persisters than the wild type. One of the loci, hipAB, was cloned and sequenced. This was the first work with the bacterial genes specifically involved in the regulation of cell death. Apparently, this hip AB locus has the capacity to act to induce or repress cell death. The mutation can in fact increase the rate of persisters to 100%. The fact that many mutations (hip, vncS, sulA, and mar) can dramatically increase the number of surviving cells in a population, without having an adverse effect on cell function, is confusing. Persisters can represent cells with inhibited apoptosis, a secure mechanism which will produce cells which survive if an antibiotic reaches the entire population, which would require the cells to be able to distinguish between an irreparable defect and dying. The development of tolerance to antibiotics in dying cells may be the result of the inhibition of apoptosis and its objective may be to prevent suicide when nutrients are limited.

Other researchers have demonstrated that under certain conditions of high stress, the bacteria in the biofilm can secrete bactericidal compounds which act on subgroups of species, thus releasing nutrients and DNA for the surviving cells.

The characteristics described have also changed the common view of biofilm resistance, altering the current operating definition of an in vitro biofilm which, at least in antimicrobial susceptibility studies, has meant that a cell aggregate on a particular surface shows a greater resistance to that of planktonic cells.

It could be said that a cell aggregate which potentially restricts the access of the host’s defence components and which produces at least some persister cells could be viewed as an infection model caused by a recalcitrant biofilm.

Parsek et al. proposed five criteria to define biofilm as the aetiology of an infection: (1) pathogenic bacteria are associated with the surface or are adhered to the substrate; (2) direct examination of the infected tissue reveals aggregated bacteria in clusters immersed in a matrix of their own constituents and those of the host; (3) the infection is localised; (4) the infection is resistant to treatment with antibiotics, despite the sensitivity of the planktonic organisms to the antibiotic; and (5) the culture is negative, despite there being a high index of suspicion of infection which has been clinically documented (as the bacteria located on the biofilm might have been lost in the blood or aspirate sample taken). Previously, in 2008, Hall-Stoodley et al. suggested a modification to the fourth criterion: in the absence of a culture, resistance to antibiotic treatment can be inferred demonstrating the presence of live bacterial aggregates, using methods such as direct staining which are associated with molecular methods such as PCR-RT or FISH. And they proposed a sixth criterion, the ineffective clearance shown by the presence of cell aggregates in clusters in discrete areas of the host tissue with inflammatory cells. The evidence of polymorphonuclears and macrophages surrounding the in situ bacterial aggregates considerably increases the suspicion of infection.

Infections of the Otolaryngological Area

Various different ear infections have recently been attributed to the presence of biofilms. In otitis media with effusion which is so frequent in children, it has been possible to demonstrate these bacterial aggregates in the mucous membranes of an experimental otitis media model and the bacterial DNA in the effusion of this otitis by polymerase chain reaction (PCR) even during periods of remission of the infection. This confirms that in children with recurring otitis, the bacteria remain in the middle ear between acute episodes. In addition, the presence of endotoxins has been compared with the presence of viable bacteria such as H. influenzae and M. catarrhalis in the effusion from 106 children’s ears with chronic otitis media and the results suggest that the Gram-negative bacteria detected by PCR which are difficult to detect by culture-could be the source of these endotoxins. Likewise, the biofilms which produce endotoxins induce an innate response from the host which is less potent, contributing to the disease chronicity. In chronic serous otitis, placing tympanostomy tubes allows ventilation of the middle ear, increasing oxygen pressure and promoting the generation of ciliated epithelium, consequently reducing the number of secreting cells. And, what is more, an
important effect of this procedure is the disruption of the biofilm which along with the above re-establishes the host’s defences and eventually leads to clearance of the biofilm.\textsuperscript{1} Furthermore, measures have been taken to prevent the formation of these films in the typanostomy tubes, bombarding the silicone with ions and coating it with albumin. This has been demonstrated to inhibit fibronectin bonding to its surfaces, thus preventing the adherence of foreign material.

Frequent flare-ups of chronic cholesteatomatous otitis media have also been associated with the presence of biofilms, as its keratin matrix seems to provide an ideal environment for them to develop. This has been confirmed on analysis of the otopathogenic \textit{P. aeruginosa} strains which were isolated from a cholesteatoma that showed firm adherence to the keratinocytes. Cochlear implants were no exception; in some cases \textit{S. aureus} biofilms have been found resulting in some having to be removed and for them to be redesigned.\textsuperscript{10-12}

Moreover, it is known that the incidence of chronic sinusitis is on the increase. This seriously affects the quality of life of its sufferers, particularly patients with chronic pulmonary obstructive disease, heart failure or back pain, yet its physiopathology remains unknown. However, given the chronic nature of its evolution, its frequent flare-ups and resistance to antibiotics, it appears logical to consider that this disease is caused by biofilms. Studies have been undertaken which have demonstrated the presence of biofilms in the mucous membranes of patients with chronic sinusitis and in the stents which have been removed from the frontal sinus of patients with this disease.\textsuperscript{13-15} Various details of this relationship have come to light thanks to confocal laser microscopy associated with specific fluorescent hybridisation techniques and to the bacterial viability equipment which enables the three-dimensional structure of biofilms to be studied and the most common species which include \textit{S. aureus} and \textit{H. influenza} to be defined. The former species is associated with more severe symptoms and with a torpid post-operative evolution and the latter with milder symptoms and a satisfactory evolution.

It has also been possible to observe that in patients where biofilms are present, in the epithelial layer of the mucous membrane (which) has been destroyed there is a total absence of cilia. This is not the case in patients where biofilms are not present, of whom a third present a normal structure and the rest present partly damaged epithelium with the remaining mucous membrane surface coated by cilia of normal structure. These changes and the response of the host to the presence of the biofilms, could account for the onset of disease due to ciliary dysfunction in the osteomeatal complex, leading to mucociliary stasis and the distal propagation of bacterial biofilms.\textsuperscript{16}

Cases of chronic tonsillitis have also been associated with the presence of these clusters of bacteria because, in addition to being covered with the clusters, resected tonsils were compared by infectious and obstructive causes and it was observed that there was significantly less biofilm present on these. Not only do they explain recurrence, they also represent a significant element in sustaining a chronic inflammatory condition.\textsuperscript{17,18}

It has been observed that biofilms were present in 90\% of tracheotomy cannulas from the seventh day after the cannulas had been placed, and the amount of colony-forming units was associated with the frequency with which the inner sleeve was changed.\textsuperscript{19} Similarly, the problem of the formation of fungi is well known in phonatory prostheses placed by tracheosophageal puncture in laryngectomised patients, with the consequent leakage of saliva and these prostheses being continually changed. It has been demonstrated that phonatory prostheses in which 7\% silver oxide has been applied to the valve are resistant to the formation of biofilms, particularly to fungal biofilms, thus lengthening the amount of time before they need to be changed and reducing costs.\textsuperscript{20,21}

### Treatment

Advances in the knowledge of the physiology of biofilms have been the result of a great amount of research undertaken in recent years which, together with the progress made in the genetic and molecular field, have enabled a better understanding of bacterial behaviour, specifically with regard to possible therapeutic targets. Thus for example, the detection of two intracellular signalling systems, R-lasi and RhIR-Rhl, which are associated with the development of \textit{P. aeruginosa} biofilms, indicates that manipulating this signal could be an objective in controlling the growth of the biofilm. Interrupting quorum sensing and inhibiting the transcription of the genes which control it or those involved in the initial adherence or in the sweeping of the mature biofilm, of individual bacteria or clusters, could be an effective strategy in the prevention of biofilm infections and even in preventing them from spreading.

From the available spectrum of antibiotics, those which maintain activity against bacteria even if they are not in the reproductive stage, such as fluoroquinolones, have proved more effective than those which require an active reproductive status, such as the betalactamics. In addition, some macrolides such as clarithromycin and erythromycin inhibit the formation of biofilms, probably because of their immunomodulating properties mediated at least in part by the effects of the activation on the genetic transcription mediated by the activation of the kappa-beta nuclear factor. Furthermore, at concentrations below the inhibitory minimums, they show a capacity to reinforce the phagocytic activity of the polymorphonuclear leucocytes against \textit{P. aeruginosa} biofilms and at subclinical doses can affect signalling inside and between bacterial communities. Because with macrolides, at current doses, appropriate concentrations can be achieved in tissues, sputum and nasal mucus, it is possible to achieve a potentially favourable effect against biofilms, at least those produced by \textit{P. aeruginosa}.\textsuperscript{1}

In the particular case of chronic sinusitis due to \textit{S. aureus}, the application of mupirocin via nasal irrigation has shown good results in destroying the biofilms of this bacteria.\textsuperscript{22} In other cases, adenoidectomy is the only effective treatment for patients with chronic rhinosinusitis and otitis, which coincides with recent work which has demonstrated that the adenoids are covered by these films which act as reservoirs of bacteria.\textsuperscript{23}

In recent studies it has been demonstrated that biofilms under the effect of electrical currents or ultrasonic radiation
are more susceptible to the effect of antibiotics\textsuperscript{24,25}; and Nd:YAG\textsuperscript{26,27} laser has also been used with good initial results. Other treatment options which are being studied, also with good in vitro results, are gentian violet which achieves disruption of the biofilm, particularly P. aeruginosa\textsuperscript{28} and honey. Alandejani et al., in a study performed in Canada, showed the antibacterial activity of honey on S. aureus and P. aeruginosa biofilms and its effectiveness in destroying them.\textsuperscript{29} However, some of these options cannot yet be used safely in human beings or guarantee destruction of the biofilm and, of course, it is also impossible to prevent them from forming again.

There is still a great lack of knowledge about some crucial stages in the formation of biofilms, such as the sweeping process of the cells of the mature biofilm and the production of persistor cells. These stages, along with the abovementioned genes which are responsible for persistence, constitute a clear objective as to where to direct future research towards the development of drugs and use of a dual treatment, where a conventional antibiotic is associated with an inhibitor of a particular persistence factor. Meanwhile, new strategies continue to be developed and tested to prevent biofilm infections, one of which is the incorporation of antibiotics into the manufacturing material of catheters.

Conclusion

The field of otolaryngology regularly and increasingly has to confront a barrage of chronic infections associated with biofilms and to date the strategies developed to treat planktonic bacteria have not proved very effective against these biofilms. It is important therefore, that known preventive measures are adopted to prevent their formation and that this issue is continually updated. It is also important to attempt to decipher their complex physiology towards the development of new rational strategies to prevent and treat these associated recalcitrant diseases, avoiding their comorbidities and high costs.

Conflict of Interest

The author declares no conflict of interest.

References

