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Role of the biofilm and therapeutic tools in infective otitis in the dog and cat

Text by G. Ghibaudo
DVM, Clinica Veterinaria Malpensa di
Samarate, Varese (Italia)

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Results

Role of the biofilm and therapeutic tools in infective otitis in the dog and cat

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Under particular conditions, many microorganisms form biofilms. When a group of bacteria or fungi accumulate on a surface and reach a specific cellular density, they star to secrete a polymeric substance composed of polysaccharides, proteins and DNA, forming a matrix to which the bacterial and mycotic cells become strongly attached.

Information about what a biofilm is and how it is formed

A **biofilm** is a complex aggregate of microorganisms characterized by the secretion of an adhesive and protective matrix, and often by:

- Adhesion to a surface, either biological or inert (e.g. rocks and prosthesis),
- Structural heterogeneity,
- Complex biological interactions,
- An extracellular matrix composed of polymeric substances, often polysaccharides.

Unicellular microorganisms usually show two distinct ways of behavior.

A) the first one is the familiar fluctuating or planktonic form, in which the cells fluctuate or float individually in an aqueous support

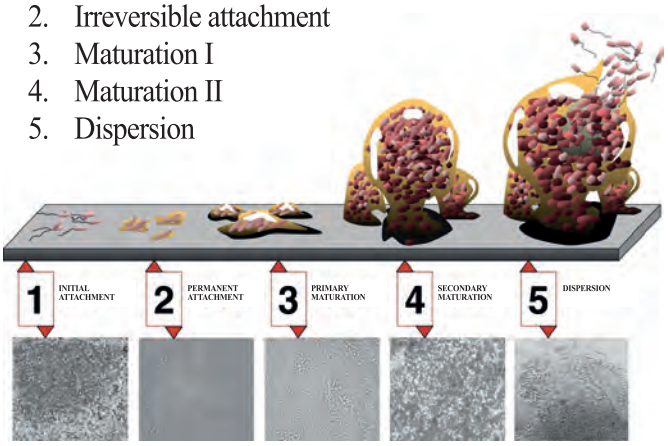
B) the second one is the aggregated or sessile state, in which the cells are closely bonded and strongly attached to one another, usually on a solid surface. The behavioral modification is activated by a chemical communication mechanism that varies among species. Some species for example can produce acyl homoserine lactone as a signal of "quiescence" that induces the surrounding planktonic cells to the phenotypical change to the sessile state, through a different expression of the cell's genes.

Formation

The formation of a biofilm starts with the anchorage of freely-fluctuating microorganisms to a surface. The first "colonists" initially adhere to the surface through weak and reversible Van der Waals forces. If the bacteria don't get immediately separated from the surface, they can attach more firmly using adhesion molecules like the pili. The first colonists promote the arrival of other cells providing more diverse sites for cellular adhesion and start to build a matrix which gives the biofilm its integrity. Some species are not able to attach to a surface by themselves, but they can often attach to the matrix or to the previous colonists. Once the colonization has started, the biofilm grows through cell division and integration of external bacteria, even of different species.

The 5 phases of the development of a biofilm.
Five phases can be distinguished in the development of a biofilm:

1. Initial attachment
2. Irreversible attachment
3. Maturation I
4. Maturation II
5. Dispersion



Properties

The biofilm is held together and protected by a matrix of excreted polymeric compounds. This matrix is so strong that, under certain conditions, biofilms can fossilize. The matrix protects the cells inside and facilitates communication through chemical and physical signals. Water channels that contribute to distribute the nutrients and the signal molecules have been found in some biofilms. At the same time, these channels carry waste products and eventual exotoxins to the periphery.

Location

Biofilms are ubiquitous. Almost every species of bacteria has mechanisms through which they can adhere to surfaces and to one another. Biofilms can be found on rocks and on the gravel in the bottom of every watercourse, and they often form on the surface of stagnant water. In general, biofilms formed by heterogeneous colonies are thicker and more stable that single-species biofilms. After some time, bacteria attached to the biofilm are able to detach, forming an individual mass that sheds periodically, and, in order to survive and colonize other niches, can give origin to a new biofilm somewhere else. This phenomenon can be enhanced by the sliding force of the fluid, the presence of specific compounds or by the characteristics of the individual bacterial species.

Biofilms allow the survival of bacterial cells in a hostile environment. In fact, planktonic cells are more exposed to many deleterious factors like bacteriophages or protozoa in nature, biocidal agents in industrial devices, antimicrobial agents in clinical situations and the action of antibodies and phagocytes. Furthermore, the complexity of their structure and the metabolic and physiologic heterogeneity, suggest an analogy between these communities and tissues of superior organisms. The biofilm can include one or multiple microbial species and can form on a wide range of abiotic or biotic surfaces.

Even though mixed biofilms predominate in many environments, those composed of a single species have a strong interest in medicine, because they cause a great variety of infections, since they can form on the surface of medical devices. *Pseudomonas aeruginosa* has been one of the most studied species among Gram negative bacteria that form single-species biofilms, but other organisms like *P. fluorescens*, *E. coli* e *Vibrio cholerae* have been widely studied as well. Among Gram positive bacteria, *Staphylococcus epidermidis*, *S. aureus* and enterococci are amongst the most studied.

Biofilms are composed mainly of microbial cells and extracellular matrix (EPS). The percentage of EPS is between 50% and 90% and it is considered to be the backbone of the biofilm. The physicochemical properties of this matrix can vary a lot, but it is mainly composed of polysaccharides. The EPS can be highly hydrated because it can incorporate great amounts of water inside its structure through hydrogen bonds, but in some cases, the EPS can be hydrophobic. The composition and the structure of the polysaccharides determines the primary conformation of the matrix. Generally, the composition of the EPS is not uniform, but it can vary in space and time, in fact the quantity of EPS grows as the biofilm ages. Furthermore, this matrix can associate with metallic ions, divalent cations and other macromolecules like proteins, DNA, lipids, humic acids. Because of its high level of hydration, the matrix prevents the drying of some natural biofilms. In addition, it can contribute to antibiotic resistance, preventing the transport and diffusion of these substances through the biofilm, probably by directly binding these molecules. Some studies have shown that the stability of the structure of the biofilm is related to the presence of neutral sugars, acid polysaccharides and amino sugars.

Clinical aspects of biofilms

Cellular aggregates

They allow the single bacterial or mycotic cells and the bacterial or mycotic colonies to exhibit a coordinated behavior and give them biological advantages like resistance to antibiotics and to the host's immune system. Biofilms are structured to allow respiration and exchange of fluids and nutritional substances, while preventing access to cells of the host's immune system, like phagocytes; at the same time, they reduce the concentration of inhibiting or lytic factors with antibacterial properties, precluding them from reaching the microorganisms. The result of these properties is that infections resulting from the formation of biofilms, are notoriously difficult to eradicate, and they require the use of high concentrations of antimicrobial agents, the removal of the affected tissues, the use of substances that are able to break or prevent the biofilm or the combination of these treatments.

Formation of the *quorum sensing*

The formation of biofilms seems to be regulated by the secretion of particular molecules following a process called quorum sensing. This is a communication process between bacterial cells, it is self-induced and it entails that when the molecules start to accumulate in the area surrounding the microorganisms, these undergo a series of physiological changes that allow the formation of the extracellular biofilm. For example, following the auto-induced quorum sensing, microorganisms can initiate the superficial production of extracellular adhesive polymers, the production of biosurfactant, sporulation, bioluminescence and the secretion of nutritional substances; with sequestration of molecules and virulence factors as a consequence of the process of formation of the biofilm. This is why sometimes bacteria present in a biofilm can be up to 4000 times more resistant to antibiotics than the same organism in a free environment[1,2]

Resistance to antibiotics

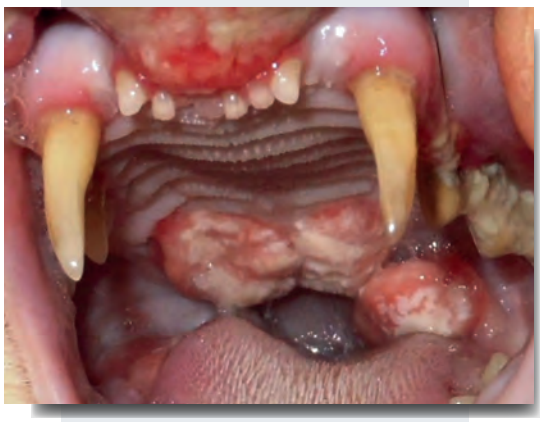
The comparison between the minimum inhibitory concentration (MIC), that describes the amount of antimicrobial necessary to inhibit free bacteria able to form biofilms, and the minimum biofilm eradication concentration (MBEC), that describes the minimum concentration of an antimicrobial agent able to inhibit the growth of a biofilm (necessary to inhibit or eliminate the growth of a biofilm), show the differences in susceptibility that exist between free and biofilm-forming bacteria

This explains why bacteria that form biofilms are much less sensitive to antimicrobial agents at the usual therapeutic schemes[3].

Besides, using antibiotics at a concentration equal to the MIC when the infection presents a biofilm, can expose the biofilm to sub-lethal doses of antibiotics, therefore causing a higher incidence of microbial resistance with serious consequences for the host.

Examples of biofilm formation in infections in dogs and cats are the dental plaque like tartar (pictures 1 and 2), on medical devices and implants (like orthopedic external fixators) and cutaneous wounds (picture 3). Particularly in small animals, infectious otitis can be caused by biofilm-forming microorganisms. (picture 4).

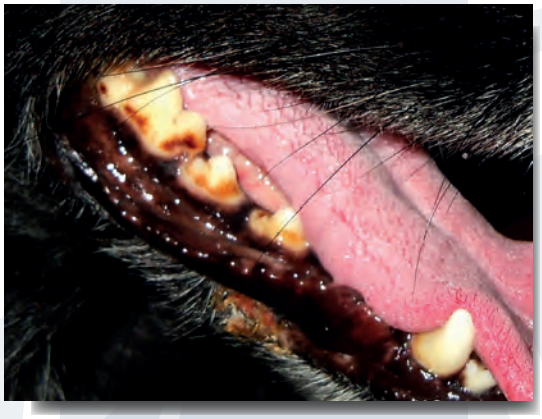
Picture 1



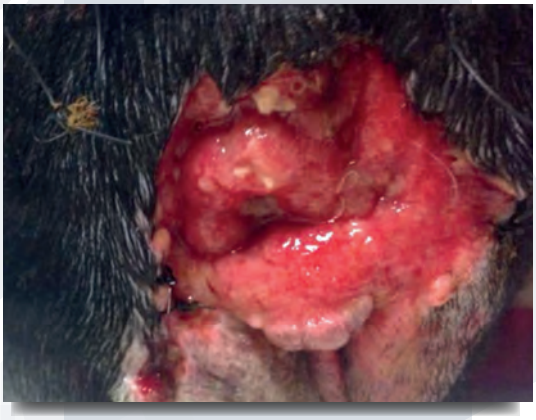
Biofilms in otitis

Biofilms have an important impact on therapy and antimicrobial resistance. They are frequent and under-diagnosed, even if they can be easily identified with otoscopy (picture 5) or cytology (picture 6). Clinically, they form an adhesive material, thick and viscous, usually dark brown or black (picture 7). In cytology they appear as an amorphous matter of variable thickness that traps and can obscure bacteria and cells (picture 8).

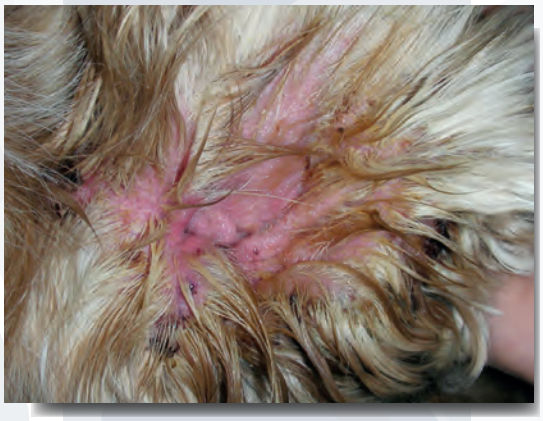
Picture 2



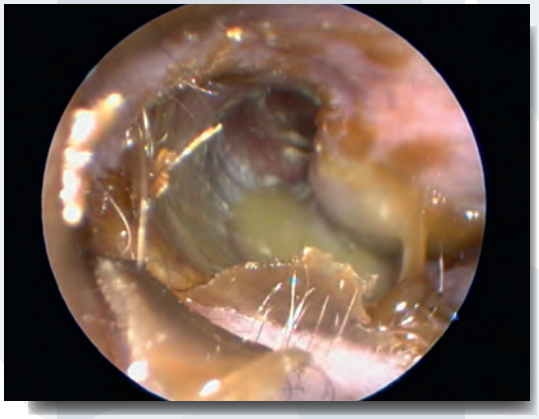
Picture 3



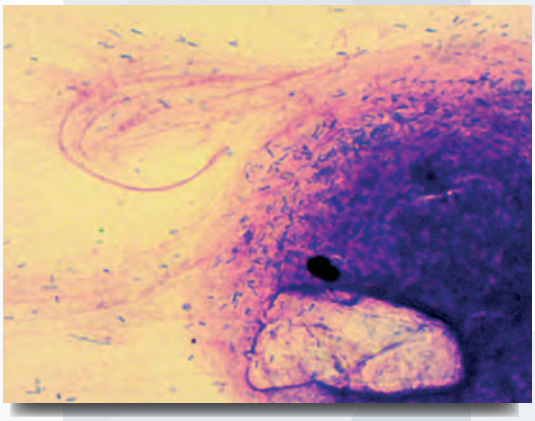
Picture 4



Picture 5

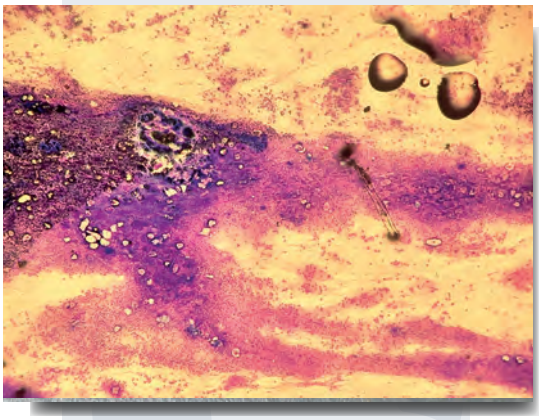


Picture 6



Picture 7

Picture 8



Biofilms are clinically important because they inactivate detergents, reduce the penetration of antimicrobials and provide a protected reservoir of bacteria. Also, antimicrobials that require bacterial division will be less efficacious because bacteria in the biofilms are usually in a quiescent state. In addition, biofilms can enable the development of antimicrobial resistance particularly in Gram-negative bacteria that acquire mutations of gradual concentration-dependent resistance to antimicrobials[4].

Biofilms generally inhibit antimicrobial penetration (Graph n.1). Wherever this results in a sudden drop in the concentration of antimicrobials, the majority of bacteria will be exposed lo low or high concentrations of antimicrobials. The main part will be eliminated or unaffected. The bacteria in the biofilm that are not reached will serve as a reserve and will lead to the failure of the therapy, but selective pressure for resistance is relatively low. Nevertheless, with a certain amount of antimicrobial penetration inside the biofilm and a gradual decrease in the concentration, some bacteria will be exposed to intermediate concentrations. This could provide a mutant selection window in which the more susceptible bacteria get killed but the more resistant mutations inside the population survive. This will lead to the failure of the treatment and a recrudescence of the infection with a more resistant isolate[5].

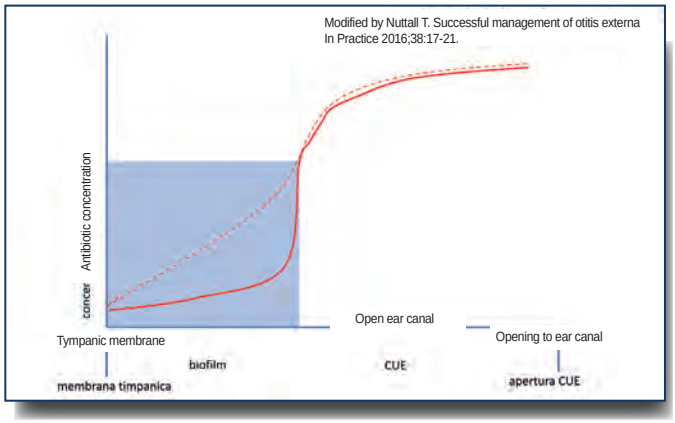


Grafico n.1

Potential impact of biofilms on bacterial resistance. There is a sudden drop in the concentration if the antimicrobial can't penetrate the biofilm (solid line). The biofilm protects the bacteria leading to treatment failure. However, if the antimicrobial can partially penetrate the biofilm, there could be a gradual drop in the concentration (dotted line). The intermediate concentrations will kill more susceptible bacteria, but they will allow the more resistant mutants to survive and proliferate. This will result in the failure of the treatment and recrudescence of the infection with a more resistant isolate. These data are from theoretical and in vitro studies.

Therapies to prevent and/or break the biofilm

Biofilms can be physically broken and removed through flushing and aspiration.

Topical therapies with molecules like N-acetyl cysteine, Tris EDTA and antimicrobial peptides have antimicrobial and preventive properties inhibiting the formation of biofilms.

N-acetyl cysteine (NAC)

NAC is used in the medical treatment of patients with chronic bronchitis. The positive effects of the therapy with NAC have been mainly attributed to its property of dissolving the mucus as well as its capacity of decreasing the formation of biofilms, with a consequent reduction of bacterial infections. A recent systematic review of the publications of 8 clinical studies that had used NAC as an adjunctive therapy to eradicate preformed mature biofilms and to inhibit the new production of biofilms, has suggested a potential role of NAC as a complementary molecule in the treatment of bacterial biofilms with an excellent safety and efficacy profile. NAC, combined with various antibiotics, has significantly increased their permeability into the deeper layers of the biofilm, overcoming the problem of resistance to the standard antibacterial therapeutic approach[6]. NAC is available as a topical otic preparation combined with Tris-EDTA (Tris-NAC © ICF). The systemic administration of NAC is well tolerated and it can help to dissolve the biofilms inside the middle ear and in other mucous surfaces. Systemic NAC and bromhexine can also liquify mucus, facilitating the drainage in cases of primary secretory otitis media in dogs and inflammatory feline otitis media (polyps).

Tris-EDTA

Ethylenediaminetetraacetic acid (EDTA) has been shown to have antibacterial activity. When combined with tromethamine (Tris) it has shown to have the capacity to damage the bacterial cell walls and increase microbial penetration[7,8]. It is well tolerated and non ototoxic[9]. It has been demonstrated that it has a synergistic effect with a wide range of antibiotics including gentamicin[10], and fluoroquinolones and also with silver sulfadiazine[11] and chlorhexidine[12] (Otodine © ICF). More recently, an in-vitro study has shown that Tris-EDTA can be a useful adjunctive treatment to topical gentamicin and neomycin for cases of chronic *Pseudomonas* otitis in which biofilms could have formed[13].

Antimicrobial peptides (AMP)

AMPs are effector molecules of the innate immune system. They represent new molecules that have been considered as potential agents alternative to conventional antibiotics. Antimicrobial peptides belong to a vast family of cationic peptides that perform their bactericidal activity destabilizing the bacterial membrane or increasing its permeability[14]. Antimicrobial peptides have been shown to have broad spectrum activity against many strains of Gram-positive and Gram-negative bacteria, including drug-resistant strains and fungi. Besides, AMPs show a synergistic effect with classic antibiotics neutralizing toxins and are effective in animal models[15]. On top of their antimicrobial effects, they have shown a potential in the management of infections in medical devices and as a coating for prosthesis[16]. The antimicrobial peptide AMP2041 (Peptivet oto gel© ICF) has recently become available as a topical veterinary product in Europe[17].

Honey, colloidal silver and povidone iodine are other molecules with synergistic properties in contrasting biofilm onset.

Conclusions

The formation of biofilms seems to be common to all cases of chronic and recurrent otitis. The combined use of agents that help to break the biofilm are useful in instances in which their presence is suspected. NAC is a systemic drug that can be useful to break down the extracellular polymeric matrix that limits the diffusion of antimicrobial agents in the area of the infection. Topical drugs effective in breaking and preventing the formation of biofilms are NAC, Tris-EDTA, as well as other substances like, colloidal silver.

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Notes:

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