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Editorial

The clinical meaning of biofilm formation ability: The importance of context

El significado clínico de la formación de biopelículas: la importancia del contexto

Relatively recent advances in our understanding of the biofilm mode of bacterial growth in various forms of infection have led to a paradigm shift in the field of microbiology and infectious diseases.[1] Far from being an exception, biofilms are wide spread in nature as a mechanism of survival and adaptation. The development of microfouling (and subsequent macrofouling), as well as being a matter of economic concern in industry, provides an illustrative example of the expression of biofilms.[2] From a clinical standpoint, biofilms can develop wherever there is a foreign body, such as a cerebrospinal fluid shunt, a prosthetic joint, or an intravenous catheter, but bacterial biofilms can also develop on organic surfaces and be part of other infections where a device is not involved, such as chronic otitis, bronchiectasis, chronic osteomyelitis, or infectious endocarditis.[1,3]

A biofilm is a complex structure constituted of microorganisms embedded in a self-produced matrix of glycoproteins and genetic material. Low concentrations of nutrients and oxygen within the biofilm lead to significant alterations in bacterial metabolic phenotype, which are reversible when eventually the bacteria are released from the biofilm structure. Indeed, the specific concentration of oxygen and substrates at a given site influences the bacterial expression of genes and proteins, as well as the excretion of molecules in the local environment. These biochemical signals serve to communicate with and influence nearby bacteria, a signaling mechanism known as *quorum sensing*. All these characteristics result in a remarkably specialized community of cells where bacteria in surface layers of the biofilm may show significant differences from bacteria in the deeper layers, even though they all share the same genome.[4]

An important consequence of these phenotypic changes is the development of antibiotic tolerance. The concentrations of antimicrobials needed to eradicate a biofilm are several orders of magnitude higher than those used for common infections caused by planktonic (exponentially growing) bacteria. As a result, the microbiological indices of antimicrobial susceptibility such as the minimal inhibitory concentration (MIC) that are to guide choice of treatment for common infections are not reliable for biofilm-associated infections.[4,5]

Apart from the problem of antibiotic tolerance, antimicrobial molecules may never reach their cellular targets, either because the physical conditions within the biofilm modify their chemical structure (such as denaturation of aminoglycosides in the acidic pH of biofilms) or because they are neutralized by extracellular enzymes (e.g. extracellular beta-lactamases). In addition, the immune system is unable to effectively clear the infection, on the contrary, the inflammatory response frequently contributes to the patient's symptoms and malfunctioning of the medical device. Finally, the biofilm structure can also harbor specialized forms of surviving bacteria, such as small colony variants or persisters.[4,5] Consequently, biofilm-associated infections are considered difficult-to-treat. Sometimes, it may be enough to remove the infected foreign body (e.g. in a catheter-related infection), but in many instances, high doses of antibiotics are given for prolonged periods and surgery is often necessary.[3]

Infective endocarditis is usually a serious infectious disease and a landmark biofilm-associated infection, affecting 1.5–9.6 cases per 100,000 inhabitants.[6] In recent years, we have witnessed a progressive change in clinical presentation, with an increase in the mean age of patients, the involvement of prosthetic valves and other intravascular devices, and an increase in staphylococcal and enterococcal episodes to the detriment of streptococci.[7,8] Mortality is approximately 20% and has remained stable in recent decades.[8] A number of guidelines and reviews have been published on the best antimicrobial therapy for a given etiology, together with the indications for surgery.[9] Prognosis is particularly bleak when surgery is necessary but the patient is not operable.[8,10]

Given the difficulties of treating biofilm-embedded bacteria and the still high mortality associated with endocarditis, it is necessary to explore other microbiological determinants apart from species and antibiogram that may be associated with the pathogenesis and prognosis of infection. We know that infections caused by specific microorganisms harboring specific genes can produce more virulent infections. Cases in point are Panton-Valentine leukocidin-producing *Staphylococcus aureus* in the setting of soft tissue infections, and bloodstream infections caused by *Pseudomonas aeruginosa* TTSS *exoU*$^{+}$.[11,12]

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In this issue, Alonso et al. analyze the biofilm-forming capacity of an impressively large collection ($n = 260$) of *S. aureus* recovered from patients with bacteremia.[13] Eventual confirmation that strains able to form more biofilm (or more metabolically active biofilm) are associated with a higher likelihood of causing endocarditis would be of enormous interest, with direct implications for the management of patients with *S. aureus* bacteremia. Biofilm characterization was performed using two different approaches: crystal violet staining, which assesses overall biofilm production including the cellular component of bacteria regardless of cell viability, along with the extracellular matrix (i.e. biofilm biomass); and the XTT-assay, which assesses the viability of cells that make up the biofilm based on metabolic activity. Both methods have been extensively validated, and are reliable and reproducible.[14]

Despite the plausibility of the hypothesis (that staphylococci producing biofilm-associated endovascular infections would have higher biofilm-producing ability), the authors report no significant differences in the production of biofilm with either high biomass or high metabolic activity. Still, it might be worth taking a second look at the statistical analysis, since a linear trend can be observed in the percentage of strains that are high biomass producers: 29.3%, 32.5%, and 46.2% in the non-device, catheter-related, and endocarditis subgroups, respectively (Mantel–Haenszel test for linear trends, $p = 0.052$). Also, the percentage of strains producing biofilms with high biomass was significantly higher in the endocarditis group (46.2%) compared with the other strains (29.9%) (X^2-test, $p = 0.045$). Finally, the authors categorized the outcome variables measuring biofilm production (high, medium, and low biofilm biomass or metabolic activity) rather than directly comparing continuous parameters (i.e. the absorbance measurements in each assay). The cut-off used for categorization was arbitrarily based on tertile range, which may have led to loss of information about the variable. A direct comparison of absorbance would have been useful.

Another factor that may have diluted a possible difference in the biofilm-forming ability of staphylococci are the definitions used for the three clinical groups. The "non-device associated bacteremia" group includes episodes that do not meet the consolidated criteria for infective endocarditis or catheter-related bacteremia. This is by far the largest of the three groups ($n = 181$, 70%) and may have included very different infections, in some of which biofilm formation is an important pathogenic feature, such as prosthetic joint infections, chronic wound infections (e.g. diabetic foot infection, vascular ulcer infection), or vertebral osteomyelitis.[1] It would have been interesting to know whether there was a subgroup with staphylococcal bacteremia due to infections with little or doubtful biofilm involvement, and whether biofilm production was significantly lower when compared with staphylococci causing endocarditis or catheter-related bacteremia.

Nevertheless, Alonso's results are consistent with previous studies on staphylococcal bloodstream infection that have set out to correlate key phenotypic (e.g., biofilm production) and genotypic (e.g., *agr* functionality) features with clinical presentation or prognosis of infection. In a previous publication, the same group of researchers found no association between biofilm production and outcome in a large number of episodes of *S. aureus* bacteremia.[15] In another prospective study involving more than 200 episodes of staphylococcal infective endocarditis, Fernández-Hidalgo et al. did not find an association between biofilm production and prognosis.[16] These clinical questions have also been explored in scenarios other than endovascular infection. In a prospective multicenter study involving more than 80 episodes of staphylococcal prosthetic joint infection, Muñoz-Gallego et al. observed higher biofilm biomass in strains causing chronic infection versus hematogenous cases, but no differences in prognosis.[17]

As Alonso et al. suggest in their discussion, *in vitro* study of biofilm may not accurately reproduce what happens *in vivo*. As previously discussed, the architecture, cell density and microbiological properties of a given biofilm is the result of a complex interplay of physical, chemical, and biological variables. Genotypic expression in the strain is influenced by the type of surface to which bacteria is attached, the type and concentration of nutrients in the environment, the concentration of oxygen, the shearing stress during biofilm development, *quorum sensing*, and the coexistence of other bacterial species.[4] Several devices have been designed to generate reproducible biofilms under very well controlled conditions. While these experimental models have provided more in-depth insights into bacterial pathogenesis, as well as reproducible 'biofilm problems' against which antimicrobial agents can be tested and compared, the transfer to actual clinical settings may not be straightforward. In this regard, measurements of biofilm antimicrobial susceptibility such as the minimal biofilm eradication concentration (MBEC) obtained with the Calgary Device and other instruments, still need to prove that they perform reliably as microbiological indices for guiding antimicrobial therapy, much as the MIC does in common planktonic infections.[17,18] In this context, it is likely that the conditions under which staphylococci were induced to form biofilm in Alonso's *in vitro* experiments were different from those existing *in vivo* at the time of bacteremia and not therefore necessarily representative of the clinically relevant biofilm production of these strains.

The staphylococcal genome is vast and includes a very large number of genes and genetic pathways, some of them redundant or alternatives.[11] A step beyond basic phenotypic responses or assessments on the presence or lack of genes is the specific expression of that genome in a given situation. Chen et al. compared two strains of methicillin-resistant *S. aureus* (MRSA) obtained from a patient with infective endocarditis: a wild-type and a small colony variant. The genome study showed only 9 mutated genes, but the transcriptome found more than 300 and 500 genes that had been upregulated and downregulated, respectively.[19] Fisher et al. evaluated two MRSA strains from another patient with endocarditis: the daptomycin-susceptible strain that originally caused the infection, and a daptomycin-resistant strain recovered at the time of relapse. As expected, both strains were isogenic, but with major transcriptomic and proteomic differences that could explain the different behavior.[20] Again, the experimental conditions under which genome expression is to be studied are key. In a catheter infection rat model, Hanses et al. found differential gene expression in the same strain of *S. aureus* depending on whether the animal was diabetic or not.[21]

In summary, Alonso et al.'s study is an impressive attempt to deepen our knowledge of the pathogenesis of staphylococcal bacteremia, infective endocarditis, and biofilm-associated infections. Their observations are useful and underline the need for further studies that assess the actual use of the bacterial genome (in other words, gene expression) in a given situation. The novel 'omics' approach and the use of models that mimic infection will enable us to move forward.

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