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REVIEW ARTICLE

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METHODS FOR STUDYING MICROBIAL BIOFILM

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ABSTRACT

Microbial biofilms are multicellular community formed by microorganisms as bacteria and/or fungi involved by polymeric substance called extracellular matrix. The extracellular matrix protects microbes against environments external threats and it facilitate the interaction between others pathogenic and symbiotic organisms. Thus, these communities of microorganismsare medical and environment importance that stimulates the study of biofilm in last years. However, the biofilm life-style is still poorly understood, and more researches are necessaries to elucidate the development of microbial biofilms. Therefore, the aim of this paper is to concatenate the methods of evaluating the development of microbial biofilm in vitro through Staining assays, techniques of Morphology and Molecular Biology.

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INTRODUCTION

Biofilms are multicellular communities composed of microorganisms cells adhered to each other on a living or non-living surfaces and embedded in a self-produced matrix of extracellular polymeric substance (Donlan and Costerton, 2002). The extracellular matrix protects microbes in adverse environmental conditions and it facilitates the interaction among others pathogenies and symbiotics organisms (Gao *et al*., 2015).

The organisms in a biofilm present differents phenotypes, motility, metabolism and transcription profile that are regulated by nutrients, oxygen, reproduction and quorum sensing. (Donlan and Costerton, 2002; Azevedo and Cerca, 2012; Gulati and Nobile, 2016).The different phenotypes are organized in layers, which established in a three-dimensional structure. So, biofilm structure is important for understanding its development, therefore it is correlated with the stages of biofilm formation of each species, as well as the aggregates and layers that constitute the biofilm (Huang *et al*., 2020).

Another important factor is the extracellular matriz, it is composed by extracellular polymeric substances (EPS): exopolysaccharides, nucleic acids, lipids and proteins (Flemming and Wingender, 2010). Nowadays, it knows that matrix acts as physical protection, but also it regulates the gene expression. Maybe, its role is similar the extracellular matrix of tissues in multicelular organismo.

In this manner, its acts in cellular proliferation, morphology and homestasy (Steinberg and Kolodkin-Gal, 2015). It is known that the exopolysaccharideexopolysaccharides contribute to the formation the biofilm three-dimensional structure (Serra *et al*., 2013; Rohde *et al*., 2010). The importance of proteins has been associated with cell adhesion and hydrophobicity. In addition, extracellular DNA is signals cell-cell communication and adhesion. Finally, the lipids in biofilm matrix is associated with the fixation and microorganism dispersion (Davies and Marques, 2009).

The study of biofilm formation and composition allows both the understanding of this microbial lifestyle and finding efficient treatments against the biofilms. It is highlighted that microbial biofilms are associated with and impact the management of over 75% of all infections. The effects of biofilms are seen primarily in 4 ways by facilitating the emergence of antimicrobial drug resistance, generating chronic infections, the modulation of host immune response, and the contamination of medical devices (Vestby *et al*., 2020). Thus, the purpose is to concatenate the techniques in order to assess the microbial biofilm development in vitro considering its morphology, composition, metabolism, gene expression and developmental kinetics.

REVIEW

Staining assays: Staining assays have also been used to assess biofilm development by metabolic activity, biomass quantification

and number of viable cells. These techniques are important in biofilm research because it enables the study of intact biofilms, as well as examination of biofilm drug susceptibility without disruption of biofilm structure (Kuhn *et al*., 2003; Azeredo *et al*., 2017). Staining assays may be the methods of metabolic activity or direct staining /dye. The methods of metabolic activity verify the quantification of viable microorganisms in biofilm using the conversion by cellular metabolic activity of specific substrate into a colored product measurable with a spectrophotometer (Kuhn *et al*., 2003; Corte *et al*., 2019).

The use of XXT as a marker of viable cells is often highlighted due to its relatively simple implementation and good accuracy. The XTT ((2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5 Car boxanilide) procedure emphasize the metabolic activity via the reduction of tetrazolium salts to strongly colored formazan in the presence of metabolic activity (Corte *et al*., 2019; Ramage *et al*., 2001). The basic principle is the XTT-to-formazan conversion by the mitochondrial succinoxidase and cytochrome P450 systems, as well as flavoprotein oxidases by (SDH), mitochondrial enzyme found in only microbial cells and in the inner mitochondrial membrane of eukaryotes. So, the number of viable bacteria or fungi in the biofilm can be deduced by measuring the absorbance of supernatant after the metabolic reduction of XTT (Kuhn *et al*., 2003; Ramage *et al*., 2001; Rocha *et al*., 2017). However, the main limitation of this method is related to the complexity and heterogeneity of biofilm structure and composition showing different metabolic gradients and to the predisposition of mature biofilm to slow down or partially retain the reduction and release of XTT and formazan, respectively (Honraet *et al*., 2005; Bandh, 2019).

Similarly, Resazurin dye (7-hydroxy-3H-phenoxazin-3-one 10-oxide), also known as Alamar Blue, has been used as an indicator of cell viability in biofilm and cytotoxicity assays. It is a stable redox indicator that is reduced to resorufin by metabolically active cells Mitochondrial enzymes, as carriers of diaphorase activities, like NADPH dehydrogenase, are probably responsible for the transference of electrons from NADPH + H^+ to resazurin, which is reduced to resorufin. Consequently, there is the conversion of the blue non-fluorescent resazurin to the pink and highly fluorescent resorufin can be monitored visually, by spectrophotometry or spectrofluorometry (for increased sensitivity) (Toté *et al*., 2008; Toté *et al*., 2009).This dye offering multiple advantages as rapidity, reliability, sensitivity, safety and cost. In addition, it keeps cells intact, which permits other parallel analyses, such as mRNA, cytogenetic, apoptosis, and immunophenotyping. Also, no requirement of the spectrophotometer for analysis qualitative of the resazurin reduction levels could be considering other advantage. On the other hand, the quantification of resazurin reduction levels are species- and strain-related, some experimental conditions are difficult to standardize. Moreover, the presence of antibacterial compounds reducing there reliability of this method in anti-biofilm researches (Azeredo *et al*., 2017; Borra *et al*., 2009; Pantanella *et al*., 2013).

Analogous to resazurin, the dye BioTimer (BTA) employs a specific reagent containing phenol red. The color of the specific reagent switches from red-to-yellow, thanks to microbial products of primary fermentative metabolism. Noteworthy, BTA has several advantages, including: does not require sample manipulation, is a low cost, easy to perform method and has been applied to count living bacteria in biofilm, to verify microbial cells growing in a biofilm and to evaluate antibiotic susceptibility of biofilm. The main disadvantage relies on the difficulty in applying BTA for the evaluation of multi-species biofilm (Bandh, 2019; Pantanella *et al*., 2013).

Crystal Violet is a most popular one and is based on the ability of this dye to color the polysaccharides (CORTE *et al*., 2019; Christensen *et al*., 1985; Fletcher, 1977). These assays stain both living and dead cells as well as some components present in the biofilm matrix, thereby being well suited to quantify total biofilm biomass (Pitts *et al*., 2003). However, it has been adapted to study biofilm formation assays. Crystal Violet method is considered versatile, since it could be used with a broad range of different bacterial species and eukaryotic cells as fungi (Reynolds & Fink, 2001). In addition, the microbial biofilm does not need to be detached from the support as required for plate counts, avoiding biased estimate of the number of cells in the biofilm due to the viable but non culturable (VBNC) state. Also, the high throughput capability of Crystal Violet technique allows testing of many different conditions simultaneously. However, there are limitations as bias of the estimate of sessile development capability of microorganisms forming loose biofilms, due to the washing steps; lack of reproducibility; does not allow species distinction in polymicrobial communities and absence of a standardized protocol (Azeredo *et al*., 2017; Corte *et al*., 2019; Pantanella *et al*., 2013). Studies to*Candida* and *Staphylococcus aureus* strains biofilms showed that there is little correlation between the Crystal violet and XTT measures. Thus, it is efficient to choose any of the techniques to study biofilm development (Corte *et al*., 2019; Rajendran *et al*., 2016). Stains other than CV, par exemple, safranin, can be used to stain bacterial biomass (Christensen *et al*., 1982).

Congo red agar (CRA) method that is a qualitative assay for detection of biofilm producer microorganism. This dye can directly interact with certain polysaccharide presents in extracellular matrixand capsidium of bacteria forming colored complexes(ARICOLA *et al*., 2001).This method described by Freeman (Freeman *et al*., 1989) is fast, cheap, reproducible, and the colonies remain viable in the medium for further analysis. The detection of biofilm producer microorganism is result of color change of colonies inoculated on CRA medium, black colonies with a dry crystalline consistency indicate biofilm producers, whereas colonies retained pink are non-biofilm producers. However, it appears more likely that some metabolic changes of the dye to forma secondary product could play a more important part in the formation of dark colonies (Aricola *et al*., 2001). Aricola *et al*. (2001) established a colorimetric scale ranging from very red to very black with 6 kinds of nuances—very red, red, bordeaux, almost black, very black, and black—for biofilm production classification (Aricola *et al*., 2001). However, it may be complicated a standardized analysis, since the evaluation criteria is based on visual analysis of the color of the colonies that grow on the agar. On other hand, CRA test is both sensitive and specific for biofilm detection when applied to Staphylococcus sp. Furthermore, this method has been used to observe the development of the biofilm matrix, number of bacteria the presence of communication channels in mature biofilm (Jain & Agarwal, 2007). There are others staining tests as 1,9-dimethyl methylene blue (DMMB), which binds specifically to Staphylococcus aureus, the fluoresceine-di-acetate (FDA) assay, LIVE/DEAD BacLight assay which allows to evaluate live and dead microbial population. Last one is associated by microscopy techniques and it will be explained better in next session.

Microscopy methods: Study of morphology of biofilm has been used to characterized composition, structural organization, cellular damage or cellular transition that allows the understanding of adhesion and biofilm formation, antimicrobial susceptibility and interspecific relation in multi-species biofilm. Light microscope, epifluorescence microscopy, confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), Transmission electron microscope (TEM), phase-contrast microscopy and atomic force microscopy (AFM) are the most important techniques (Azeredo & Cerca, 2012; Pantanella *et al*., 2013; Douterelo *et al*., 2014).

Light microscope is the most popular, since it is the easiest, cheapest, most simple, convenient and fastest method to quantitatively observe the morphology of microorganisms adhered to surfaces and to semi quantitatively estimate the amount of microorganism attached on surface by subjective analysis or counting microorganism for area. Observation with light microscopy that requires the using of dyes as Hematoxylin and eosin (H&E), Periodic-acid-Schiff (PAS), and Brown and Brenn Gram staining, and transparent, and planar surfaces on which microorganisms attach (Azeredo *et al*., 2017). The direct method of counting microorganism is by the use of a microscope and a slide with special chambers of known volume. These slides allow the counting of a small number of cells in a small volume and

extrapolating the result to determine the population. Also, It is useful to assess the transitions the pleomorphic microorganisms as dimorphic fungi. In the others word, it is possible asses the proportion the yeasts and filamentous fungi in biofilm that is associated to virulence and resilience biofilm. However, this method does not create 3D vision of biofilm. In order to evaluate the microbial morphology light microscope requires the removal of the the microorganism from the biofilm. Also, the level of magnification and resolution are limited to determine intercellular and cellular-abiotic relationships and morphotypic differentiation is relatively gross and lacks discriminatory detail, especially in thicker specimens (Azeredo *et al*., 2017; Lacaz *et al*., 2012).

In addition, the histology analysis realized in contaminated tissues uses light microscope for understanding pathophysiology of the biofilm in vivo. Observation histopathological may demonstrate evidence of tissue injuries, inflammation and invasion level by biofilm (Otha *et al*., 2007).

Epifluorescence microscopy and confocal laser scanning microscopy (CLSM) is an important tool for studying of biofilms since theses methods asses the biofilms in situ. This way, it is possible to analyze the matrix and 3D structure of biofilm thanks does not using fixation solution and dyes (Costerton *et al*., 1995). Another importante characteristic is that the live and dead cells are clearly visualized with fluorescent dye labels.

Epifluorescence microscopy is a tool used to asses number of viable cells. For this, fluorescents probes associated or not to antibody have been used to label the microorganisms enabling its observation. Acridine Orange (Hobbie *et al*., 1977), 4,6-di-amino-2 phenylindole 4 ′,6 – diamidino-2- fenil-indol (DAPI) and the 5-cyano-2,3 Dytolyl Tetrazolium Chloride (CTC) are the most popular probes (Schaule *et al*., 1993). Moreover, kit live/dead staining has been used as indicator of cell viability, as determined by the integrity of the cell wall membrane in many bacterial populations, including biofilms. This kit is composed by SYTO 9TM andPropidium Iodide. The SYTO 9TM binds to DNA and it colors in green emissions for viable microorganisms and Propidium Iodide label in red emissions by damaged cells (Saini *et al*., 2014).

There are two main types of electron microscope: scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Both uses the interaction of an electron beam with the sample to form the image, but in first the electrons are scattered electrons, in the second, they are retransmitted. These techniques have the disadvantage of damaging the matrix by processing (Rocha *et al*., 2017; Pantanella *et al*., 2013). Except, cryo-SEM and environmental SEM (ESEM) that are not tedious samples preparation to dehydrate and make them suitable for vacuum operations, since they provide the opportunity of observation of hydrated biofilm, even fluids and live cells (Stokes and Donald, 2000). In sauf, cryo-SEM and environmental SEM (ESEM) that are not tedious samples preparation to dehydrate and make them suitable for vacuum operations, since they provide the opportunity of observation of hydrated biofilm, even fluid sand live cells (Stokes and Donald, 2000).

The SEM technique may be employed to access the biofilm structure during biofilm formation at different time periods. Also, it has been used to observation of attachment, detachment, filamentation and interaction among microorganisms, principal, in multi species biofilm (Rocha *et al*., 2017; Bragadeeswaran *et al*., 2010). Transmission electron microscopy (TEM) let assess the producer-biofilm microorganism. Observation of the interior of cells (in thin sections), the structure of protein molecules (contrasted by metal shadowing), the organization of molecules, cytoskeletal filaments (prepared by the negative staining technique), and the arrangement of protein molecules in cell membranes (by freeze-fracture), the electrodensity, presence of vacuoles, membrane damage, nucleus and cellular wall are realized by TEM (Basma *et al*., 2011). However, this method is time consuming and expensive. Similar to electron microscopy, atomic force microscopy (AFM) has high resolution that we may observe cellular membranes, but it is more advantageous because it does not use vacuum and allows studying living microorganisms. AFM has been employed for visualizing biofilm development in real-time, susceptibility to antimicrobial drugs and cell to cell interaction (DUFRENE, 2008) the same is observed in phase-contrast microscopy, thus it studies live cells without dye and it presents higher resolution than light microscopy. This microscopy formed the images by conversion of small variations light -phase in amplitude of this light (Azeredo & Cerca, 2012).

Molecular Biology Techniques

The Molecular Biology is composed by techniques as in situ Hybridization, Polymerase chain reaction (PCR), Northern-blot, Southern-blot and Western-blot, that studying genetic express ion and its products (proteins). (Corte *et al.,* 2019; Pantanella *et al.,* 2013). Molecular techniques are of great matter to comprehend the signaling of biofilm development. These methods have been used to monitoring biofilm formation and detection of extracellular matrix composition. The last one is of interest to the inference of pathophysiology of microorganisms. Therefore, such a tool can be used to the development of antimicrobial drugs, which may act in inert compounds of biofilm, avoiding its formation (Azeredo *et al*., 2017). However, the physical dissection and isolation of single cells and matrix components from living biofilms is a problem with such methods.

Nucleic Acids are important to regulate kinetics of biofilm, mainly, extracellular DNA. PCR, Northern-blot and Southern-blot have been used to quantify and to analyze of nucleic acids. Another genetic assay used to detection is the Diphenylamine method, since it binds in DNA and RNA forming a blue product measurable with a spectrophotometer (Gannesen *et al*., 2019). Also, it is possible the detection of nucleic acid by nano-drop, spectrophotometer - UV, the sample should be reading to 260nm. However, these approaches might not bring out differences between RNA and DNA (Rajendran *et al*., 2016). PCR are diverse techniques applied to identification of biofilm producer microorganism. It also studies dynamics of biofilm throughout observation of genes involved in morphogenesis, metabolism, adhesion and reproduction of microorganisms (Azeredo *et al*., 2017; Pantanella *et al*., 2013; ISHII *et al*., 2013). Remarkable, the genes expression could shows effect of antimicrobial drugs in biofilm. Among techniques of PCR, Real-time quantitative-reverse transcription PCR (qRT-PCR) is a process frequently used to analyze biofilms. This process monitors a PCR reaction throughout the amplification process, granting the collection of real-time data. The fluorescent signal after each cycle can be used to indicate the amount microbial genetic information and the viability of all cells within the biofilm. Thus, it has high sensibility and assess quantitative the genes expression. It is requires samples free of contaminations and DNAse that is PCR limitation. Also, this is a high-cost process (Pantanella *et al*., 2013).

The Southern blot and Northern blot approaches are complementary techniques throughout the labeled antibody measuressemi quantitatively to estimate the amount of DNA (Southern-blot) and RNA (Northern-blot) (Águila-Arcos *et al*., 2017; Ramage *et al*., 2002). They are rarely because of the diffusion qRT-PCR which is a technique more sensitive and specific, resulting in quantitative analyzes. Another genetic assay used to monitor biofilms is in situ hybridization, which involves the labeling of probes with dyes. These probes are specific with 15-25 bp that can then be used to bind to DNA or RNA within the biofilm – allowing identification of different species. Combination to epifluorescence microscopy, confocal laser and scanning microscopy has been used fluorescence in situ hybridization (FISH). It is characterized by the labeling of probes with fluorescent dyes. Its application is to fast detection and quantification of microorganisms, which it is useful in multi-specie biofilm and in biofilm that unknown Biofilm-Producer strains (Azeredo *et al*., 2017; Amann *et al*., 1990). Lastly, genomics, proteomics, transcriptomics and metabolomics are association of techniques to understanding microbial cell regulation, its physiology

at different levels: genetic, transcriptional, post-transcriptional, translational and post-translational (Van Oudenhove & Devreese, 2013; Manzoni *et al*., 2018). The omics approach provides characterization of microbial cell behavior in biofilms, physiological differences occurring in the course of sessile development in response to interactions with its surroundings, symbiotic relationships, and environmental conditions or surfaces (Azeredo *et al*., 2017). For this, the main techniques used are 2-D electrophoresis approaches and mass spectrometry. The 2-D electrophoresis approaches allows to analyze proteomes or sub-proteomes and to perform label-free semi-quantitative comparison. Moreover, the mass spectrometry is a prominent analytical tool used to quantify known materials and to reveal chemical properties of different molecules. It plays a key role for analytical studies of metabolome, since it assesses the specific gene expression and post-translational modification in biofilm (Pantanella *et al*., 2013; SONG *et al*., 2009). However, the mass spectrometry is destructive analysis of complex because it uses high vacuum environment and aggressive chemical solvent. In other side, high-resolution mass spectrometry directly coupled to high performance liquid chromatography is a powerful tool for separating and analyzing complex protein mixtures (Song *et al*., 2009; Takats *et al*., 2004). In addition, Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) becomes a rapid, inexpensive, and accurate method for bacterial identification (Pantanella *et al*., 2013). This method obtains a protein profile, both from intact cells or cell extracts, and by comparing it to a database of microbial reference mass spectra one can obtain a rapid identification of genus, species, and in some cases the sub species level. In addition, recently, it has been increasingly used for the analysis of microorganism grown in biofilm (Pereira *et al*., 2015; Caputo *et al*., 2018). However, these methods are expensive, time consuming and require advanced equipment and skilled personnel.

CONCLUDING REMARKS

Biofilm are a very complex structures with medical and environmental importance, nevertheless it is still poorly understood microbial lifestyle that requires further investigations. Hence, using diverse techniques such as chemical, imaging and genetics methods allow understanding biofilm physiology, and, consequently, developing tools to overcome as well beneficial use of non-pathogenic biofilm. Each of them shows advantages and disadvantages and enable the evaluation of a peculiar aspect of the biofilm. The choice of method depends on the parameters that will be evaluated, and also the accuracy and its cost. This way, the knowledge of characteristics of the different methods as well as the multidisciplinary expertise of the researchers are necessary pre-requisites allowing the right choice of methodologies to be used. In conclusion, the complete analysis of biofilm needs the association of suitable methods.

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