Bacterial biofilm detection methods in the laboratory

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Biofilm formation is a key virulence factor among many microorganisms. Importantly, biofilm formation is implicated as a significant factor involved in a number of chronic human infections. Development of new technologies in molecular biology, biochemical methods and imaging techniques lead a better understanding of biofilm science. The question of using these technologies in vitro and in vivo or both makes the biofilm science a lively field of study. On the contrary the lack of standardized methods for the culture of biofilm is troublesome for many studies. The culturing techniques usually preferred might have difficulties when other laboratories attempt to confirm results produced by another laboratory. Culturing techniques designed for microscopy investigation are not always suited for other types of analyses, such as harvesting biofilm biomass for biochemical measurements. In many cases these culturing methods can be adapted to mimic certain aspects of the system in which the researcher is interested, like the replacement of abiotic attachment surfaces with pathogenically relevant biotic surfaces. Biofilm formation in microtiter plates is the most common and convenient method used in the laboratories. There are numerous methods used and planned for the cultivation of biofilm in vitro, like microfluidic devices which are designed for future bacterial biofilm studies. The biofilm biomass can be detected with microbiological, physical, chemical, microscopical and molecular methods. In this article old and new biofilm detection methods used in the laboratories will be discussed.

Keywords: Biofilm; laboratory detection methods

Introduction

Biofilms are a dynamic, heterogeneous community of microorganisms within a complex matrix of extra polymeric substance that have integrated metabolic activities and produce sessile phenotypes different from their counterparts [1].

Importantly, biofilm formation is implicated as a significant factor involved in a number of chronic human infections [1]. Development of new technologies in molecular biology, biochemical methods and imaging techniques lead a better understanding of biofilm science [2]. The question of using these technologies in vitro and in vivo or both makes the biofilm science a lively field of study [2]. On the contrary the lack of standardized methods for the culture of biofilm is troublesome for many studies [3]. The culturing techniques usually preferred might have difficulties when other laboratories attempt to confirm results produced by another laboratory [3]. In this article examples of old and new biofilm detection methods (Table 1) used in the laboratories are discussed briefly.

Table 1 Examples of biofilm detection methods

Classical & Traditional methods	New detection methods
Microtiter plate assay	Ultrasound
Tube adherence method	Photobioreactor
Congo red agar method	Sequencing technologies
Biofilm Ring test	PCR techniques
Biofilm bioreactor	CSLM*
	FISCH**
	Spectrometry
	Nuclear magnetic resonance imaging

*CSLM:Confocal laser scanning microscopy
**FISCH:Fluorescent in situ hybridization

1. Traditional/well known detection methods for bacterial biofilm

One of the first methods standardized for the quantification of biofilm formation is the microtiter plate assays. The method was developed for studying coagulase-negative staphylococci attachment to the surface of plastic tissue culture plates [4]. This method is still widely been used for various bacteria with modified protocols. In the classical procedure,

bacterial cells are grown in the wells of a polystyrene microtiter plate. At different time points, the wells are emptied and washed to remove planktonic cells before staining the biomass attached to the surface of the wells. Biofilm biomass can alternatively be quantified by detachment and subsequent plating. In the microtiter plate assay, the biofilm biomass is assessed by measuring all attached biomass. The primary advantage of this method is that it is relatively high-throughput, enabling screens for mutants defective in attachment or evaluation of the effects of different treatments or compounds on attachment or biofilm formation. The effects of surface properties on attachment can also be studied in microtiter dishes. Microtiter dish assays are less well suited to studies of biofilm structure or of antimicrobial resistance properties. This is due in part to the difficulty in microscopically visualizing the biofilms, and in distinguishing between live cells and matrix material stained by the crystal violet dye [5].

In tube adherence method nutrient media is inoculated in a test tube with a loop full of activated culture and incubated for overnight at optimum growth temperature of respective organism. The tubes are then decanted and washed with phosphate buffered saline and dried. Dried tubes are stained with crystal violet. Excess stain is removed and tubes are washed with deionized water. Dried tubes are observed for biofilm formation. Biofilm formation is considered positive when a visible film lines the wall and bottom of the tube. Ring formation at the liquid interface is not considered indicative of biofilm formation. This method does not allow for quantification of biofilm formation [6].

Another technique is the congo red agar and congo red broth method. In Freeman DJ method, the medium was composed of brain heart infusion broth including sucrose, agar and congo red. A positive result was indicated by black colonies with a dry crystalline consistency. Non-biofilm producers were pink, darkening at the centre of the colonies resembling a bull eye appearance. An indeterminate result was indicated by a darkening of the colonies but with the absence of a dry crystalline colonial morphology [7]. In the study of Hrv et al, it was found that the Congo red agar/broth method is a simple, economical, sensitive, and specific method that can be used by clinical microbiology laboratories for screening the strains for the presence of slime or slime-like substances. The strains showing positive reactions can be confirmed by the quantification of biofilm either by Christensen's method or by dye elution technique.

They concluded that between Christensen's method and other dye elution techniques, congo red method was found to be more specific and easily adaptable to routine laboratory testing [8].

In a study that compared classical bacterial biofilm detection methods performed by Panda PS et al, tissue culture plate method (Christensen method) was found to be an ideal method for the detection of bacterial biofilm formation for urinary pathogens and modified congo red agar method is superior only to congo red agar method for the detection of staphylococcal biofilm formation [9].

2. Other traditional/well known methods

A method devised specifically for the investigation of early stages of biofilm development is the Biofilm Ring Test, based on the capacity of bacteria to immobilize microbeads when forming a biofilm at the surface. The Biofilm Ring Test is based on the capacity of bacteria to immobilize microbeads when forming a biofilm at the surface. A bacterial suspension is mixed with paramagnetic microbeads before being loaded into the wells of a microtiter plates. The microtiter plates is then incubated and direct measurements can be made at different time points, without any staining and washing steps. First, the wells are covered with a contrast liquid, an inert opaque oil, allowing to read the microtiter plate with a plate reader specifically designed for the Biofilm Ring Test. Then, the microtiter plate is placed for 1 min on a block consisting of individual magnets centered under the bottom of each well. Free (unblocked) paramagnetic microbeads are concentrated in the center of the bottom of the wells after magnet contact, forming a black ring, whereas those blocked by sessile cells remain in place [10].

Another technique is the rotary biofilm reactors; there are three main types of rotary biofilm reactors including the rotary annular reactor, the rotary disk reactor and the concentric cylinder reactor [2]. These reactors are engineered for the growth of biofilms under shear stress for the reproducible evaluation of biocide efficacy [11]. A good example is the CDC Biofilm Reactor that consists of eight polypropylene coupon holders suspended from a polyethylene ported lid. In this device the coupon holders can accommodate 12.7 mm diameter coupons each and the lid with coupon holders and coupons is mounted in a 1 L glass vessel with sidearm discharge port. A liquid growth media is circulated through the vessel while mixing and shear is generated by a magnetic stir bar rotated by a magnetic stir plate. The major advantages of these reactors are that the biofilms are formed in relatively constant shear stress fields and in the case of the rotary annular reactor and the rotary disk reactor different surfaces can be tested simultaneously. Thus, coupons can be made from different materials like pvc, steel, plastics, or can be coated with biologically relevant substrates or materials that are used in implants [2].

3. New detection methods for bacterial biofilms

Ultrasound is an effective method for imaging biofilms in vitro. One method used to enhance biofilm detection is the addition of UCAs (encapsulated gas bubbles), which provide a unique acoustic scattering signature thereby significantly enhancing imaging capabilities. Anastasiadis P et al reported for the first time a combined optical and acoustic

evaluation of infectious biofilm matrices. They have demonstrated that acoustic impedance of biofilms is similar to the impedance of human tissues, making in vivo imaging and detection of biofilm matrices difficult. The study outlined that the combination of ultrasound and targeted UCAs can be used to enhance biofilm imaging and early detection. The authors suggested that the combination of targeted UCAs and ultrasound is a novel molecular imaging technique for the detection of biofilms [12].

A novel microstructured, U-shaped, fiber-optic sensor with a double-tapered structure was developed to monitor biofilm thickness in a biofilm photobioreactor (BPBR), and a model to demonstrate the biofilm thickness measurement principle was established in the study of Zhong N et al. The proposed sensor was employed to investigate biofilm growth on a polydimethylsiloxane (PDMS) support material with an appropriate groove depth (200 μm) in a BPBR with continuous and intermittent supplies of a fresh nutrient medium. Furthermore, to explain the effectiveness of the measurement, the biofilm structure and active biomass in the biofilm were examined. The experimental results showed that the senior and reference probes were highly sensitive and that the reference probe responded rapidly to changes in the glucose solution concentration. The biofilm thickness measurement results obtained during biofilm development using continuous and intermittent supplies of the synthetic medium to the bioreactor agreed well (with a maximum relative error (MRE) of 6.3%) at biofilm thicknesses of 0–195 μm, corresponding to the lag phase to the stationary phase in the biofilm culture, when a PDMS groove depth of 200 μm was used. When the PDMS groove depth used for biofilm growth was much larger than the normal biofilm thickness, the sensor still effectively monitored the biofilm growth from the adsorption phase to the exponential phase in the thickness range from 0 μm to 536 μm, with an MRE of 7.5%. Thus, the designed microstructured sensor by the authors can effectively and accurately monitor *Rhodopseudomonas palustris* (PSB) biofilm development in a bioreactor across a wide measurement range [13].

The advent of high-throughput sequencing technologies (such as Illumina, Roche 454, SOLiD, and PacBio, Pacific Biosciences) has allowed investigators to obtain genome scale information on biofilms. Some of these technologies also provide information on genome modification, which may be one mechanism that affects gene expression and physiology of biofilm cells. These new sequencing technologies have been used to obtain complete genome sequences of thousands of microorganisms, and the cost of obtaining genome sequences continues to drop, making complete genome sequences of many more strains feasible. The next-generation sequencing technologies combined with functional genomics studies (such as transposon mutant libraries and transcriptomics studies) have provided information on cellular activities within biofilms, the role of essential genes in biofilm formation, as well as the community structure of natural biofilms [14].

The global transcriptomics approaches provide an average value for gene expression over the entire biofilm population, they do not provide information on local heterogeneity of the cells. Several approaches, including microfluidics, have been developed to address the question of localized transcriptional processes within biofilm. Another approach to studying localized biofilm processes is the combination of laser capture microdissection and transcriptomics. Using these approaches, subpopulations of bacteria from different regions of biofilms are isolated and captured using laser capture microdissection. RNA and DNA are extracted from the captured cells and analyzed directly by using RT-qPCR and qPCR for individual gene analyses. Alternatively, the RNA or DNA may be amplified using multiple strand displacement for transcriptomics analysis. This approach has the advantage of excellent sensitivity and large dynamic range and therefore can be performed for quantitative analysis of gene expression for a few cells from defined regions within the biofilms [14].

One of the most important improvement in the study of biofilm structure and function since 90s, has been to visualize hydrated living biofilms in three dimensions using confocal laser scanning microscopy (CSLM) [15]. CSLM can be found in many labs and other facilities, and therefore this technology is now used in almost all biofilm studies. Important advances in CSLM for the analysis of microbial biofilms have been the optics and lasers, image analysis software, and high-speed computing power which allows image analysis of large data files and also allows time lapse imaging of biofilm developmental processes. Chemical and molecular biological advances have also improved the ability to image biofilms in three dimensions [14]. In particular, the use of fluorescent proteins and fluorescent probes allows imaging by multiple fluorochromes for assays of the individual biofilm components simultaneously [15]. The stains most widely used to label microbial cells in the biofilm are SYTO-9 and SYBR-Green [14]. Another new development in imaging technology is super-resolution microscopy such as photoactivated localization microscopy (PLAM), fluorescence photoactivation localization microscopy (FPLAM), and stochastic optical reconstruction microscopy (STORM), imagining techniques that give resolution down to tens of nanometers, below the diffraction limit of light [14].

Biofilms can also be detected with labeling the cells with fluorescent oligonucleotide probes. In biofilms, this technique has been used to link the spatial organization of microbial communities and their in situ function in complex multispecies nitrifying biofilms [16]. Fluorescent in situ hybridization (FISH) has been used to detect the abundance of multiple bacterial species in environmental samples based on hybridization to the 16S rRNA. FISH has been used on a variety of biofilm samples. Fluorescent stains are also used in combination with CSLM for imaging biofilm cellular and extracellular matrix material. Most fluorescent dyes used in biofilm studies stain cellular components. Characterizing the extracellular matrix material by CSLM has been more challenging. In fact, the extracellular matrix of biofilms has

been referred to as the dark matter of biofilms since it is difficult to image, having approximately the same refractive index as water [14].

As the extracellular matrix material of biofilms is hard to screen, spectroscopic techniques can be used. Raman spectrometry has been used to define the chemistry of matrix materials in biofilms. Raman spectrometry is based on the light scattering patterns detected after irradiation of a sample with monochromatic light. The frequency of scattered light differs among substrates and can be used to study the chemical composition of biofilm. Raman spectrometry with CSLM was used to determine spatial distribution biomass and water as well as chemical composition of wild-type and small colony variant *P. aeruginosa* biofilms. Modified Raman spectroscopy–based analysis such as surface-enhanced Raman scattering has increased sensitivity and may detect components in the EPS that are not detectable by Raman microscopy. In numerous bacterial genera, quorum sensing-regulated genes have been linked to a wide range of physiological processes such as cell differentiation, antibiotic production, stress tolerance, virulence or biofilm formation, all of which are crucial for survival and pathogenicity. Thus, understanding and controlling this chemical communication system could lead to medical and industrial applications [17]. Surface-enhanced Raman scattering (SERS) spectroscopy is an ultrasensitive analytical technique and Bodelon G et al reported rationally designed nanostructured plasmonic substrates for the in situ, label-free detection of a quorum sensing signalling metabolite in growing *Pseudomonas aeruginosa* biofilms and microcolonies. They concluded that the in situ, non-invasive plasmonic imaging of quorum sensing in biofilms provides a powerful analytical approach for studying intercellular communication on the basis of secreted molecules as signals [18].

4. Concluding remarks

According to the features of biofilm development, mature biofilms are significantly resistant to antibiotic chemotherapies and intermittently disperse planktonic bacterial cells to the environments. Routine microbiological diagnosis is important and reliable for diagnosis of biofilms, but some of the methods can be less sensitive for biofilm detection. Therefore new techniques of microbiology should be introduced. It has been proved that proper sonication of indwelling devices (implants or prostheses or catheters) from the patients with suspected infection could significantly improve the detection rate of bacteria. In microscopy and culture negative samples from the patients with clinical suspicion of biofilm infection, new techniques mentioned above could be appreciated [19].

The future of biofilm laboratory detection methods will be the question of choosing in vitro or in vivo methods. It is important to know the limitation of the current in vitro methods and to ask the right questions when correlating in vitro observations to in vivo biofilms. An important link between in vitro and infectious biofilms may be established based on compliance between observations from in vitro biofilm and from in vivo observations in representative animal models, where more variables can be controlled while maintaining a dynamic interaction with the host [20].

In conclusion, biofilms have growing importance and problem in health and environmental fields like agriculture and bioremediation. There is still a challenge in the study of abiotic and biotic surface interaction of biofilms and development of novel methods and the selection of appropriate techniques for the detection of biofilms warrants the future investigation of these important structures.

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