Methods for detection of biofilms in bacteria and fungi

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ABSTRACT

Biofilms are layer-like structures formed by microorganisms and extracellular matrix produced by them on indwelling devices. Biofilms can also readily form over catheters and devices like prosthetic valves. They help in conferring drug resistance to the biofilm-associated cells and help the microbes escape from antimicrobials as well as the immune defense of the host. There are also many methods of detecting microbial biofilms *in vitro*, which will be reviewed in this article.

Key words: *Biofilm, Matrix, Detection methods*

Biofilms formed over devices and indwelling devices are a cause of concern in nosocomial infections. They impair the diffusion of antibiotics and hence impart *in vivo* drug resistance to otherwise *in vitro* drug susceptible bugs. Furthermore, inside the host, biofilms induce pathogens to subvert the innate immune responses mounted by the host and are hence associated with long-term persistence [1]. In humans, biofilms can account for up to 80% of the total number of microbial infections, according to the National Institute of Health, including rhinosinusitis, osteomyelitis, endocarditis, cystic fibrosis, periodontitis, non-healing chronic wounds, meningitis, kidney infections, and prosthetic and implantable device-related infections [2]. They are more common in infections found in the intensive care unit or other hospital settings, This review will try to address the issues with biofilm-detection methods in medically important microorganisms.

DEFINITION OF BIOFILMS

Biofilms are complex structures formed by microbial cells inside the human body, in colonies and covered by an extracellular matrix (ECM). They confer many survival advantages to the microbes. The nature of the matrix exopolysaccharide can vary greatly, depending on growth conditions, medium, and substrates. Bacteria exist in two different forms, called planktonic state (free-floating) and sessile state (adhered to a surface), both of which have existed on earth ever since the first bacteria evolved [2]. Interestingly, bacteria display highly distinct features between these two states since attachment of the bacteria to a surface results in the rapid alteration in the expression of a number of genes that are responsible for exopolysaccharide (EPS) or "slime" production and maturation. This transformation begins almost immediately after bacterial colonization of both biotic and abiotic surfaces and leads to the production of a protective barrier that protects the bacteria against the host's endogenous defense system or from external agents such as antibiotics. Although the first observation of surface-associated bacteria was recorded by Anthony van Leeuwenhoek in 1684, the term "biofilm" was not used widely and defined till a report by Costerton *et al*. in the year 1978 and depicted by Chandki *et al.* [3].

The matrix is often composed of a polysaccharide biopolymer together with some other components, namely, proteins or DNA.

MICROORGANISMS WHICH CAN FORM BIOFILM READILY

Both Gram-positive and Gram-negative bacteria can form biofilms on medical devices, but the most common strains are *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans* (oral streptococci), *E. coli, Klebsiella pneumoniae, Proteus mirabilis,* and *Pseudomonas aeruginosa* [2]. Among bacteria, *S. aureus*, *Escherichia coli,* and *Klebsiella* spp. can produce biofilm readily. Among the fungi, *Candida albicans* and molds like *Aspergillus* can form biofilm readily.

How biofilm-associated bacteria become more resistant to antibiotics:-

This is achieved either by:

a. Preventing diffusion of antibiotics across biofilms.
b. Horizontal gene transfer.

Access this article online

Received - 08 May 2023
Initial Review -27 June 2023
Accepted - 03 July 2023

DOI: 10.32677/ejms.v8i2.4156

Quick Response code

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c. In some cases, bacteria can use multidrug efflux pumps to pump antibiotic agents out of the maturing biofilms and into the extracellular matrix, contributing to resistance
d. Interactions between bacteria and fungi have also been found to be relevant in polymicrobial biofilms. For example, it has been shown that a dual-species biofilm of S. epidermidis and C. albicans had increased resistance to vancomycin due to a fungal matrix component that acted as a barrier to the antibiotic.

Methods for detection of biofilms in bacteria and fungi: Several methods are there for studying biofilms *in vitro*. These are phenotypic methods such as the (a) test tube method, (b) microtitre plate method, and (c) Congo red agar method, and also genotypic methods. Apart from this, advanced methods like confocal laser scanning microscopy can also be used to study biofilms. Biofilms, unlike sessile and vegetative cells, adhere strongly to glass and polystyrene surfaces. For studying biofilms in microtiter plate, the inverted microscope is needed to observe the stained biofilms. Staining of the biofilms is done by alcoholic safranine or alcoholic crystal violet. In this method, optical density can also be read for quantifying biofilms.

However, the test tube method remains the easiest method to study biofilms *in vitro*. It is a qualitative method devised by Christensen *et al*. in 1995 and studied by other authors [4]. The presence of visible layer on walls and sides of tubes are taken as positive. In this method, biofilms can be graded as 1+, 2+, and 3+, depending on the transparency of the biofilms. An illustration of the test tube method of biofilms is given in Fig. 1. Furthermore, we have tried to see whether a test tube containing microbial biofilm can be viewed under ×4 or ×10 objective to visualize those biofilms that are not prominent to the naked eye. According to our experience, this is better viewed under ×4 as compared to ×10.

Fig. 2 below shows such a microscopic view of biofilm in the test tube.

The Congo red agar method is also an easy method to test biofilms. It is basically brain heart infusion agar with sucrose and Congo red dye. Biofilm-producing bacteria form black colonies with a dry crystalline consistency on Congo Red agar, and non-biofilm-producing cells form pink-colored colonies [5].

For the test tube method and microtiter plate method, liquid media that are commonly used are Peptone water with 1% glucose for bacteria and yeasts, and distilled water with 10% fetal calf serum for molds.

Advanced microscopic techniques like scanning electron micrograph can also reveal the structure of biofilms, especially fungal biofilms. Confocal laser scanning micrograph can also reveal time-resolved three-dimensional images of biofilms. It also allows real-time viewing of fully hydrated, living biofilm specimens.

Genotypic methods like polymerase chain reaction are also used often to detect and amplify the *ica* gene which regulates the extracellular matrix formation [5].

Among phenotypic methods, the transmission control protocol or tissue culture plate method has been found to possess the highest sensitivity and specificity.

METABOLISM OF BIOFILM-ASSOCIATED CELLS

Normal bacterial cells that do not form biofilms are called planktonic cells. Biofilm-associated cells are relatively slower growing as compared to planktonic cells, which can explain their slow uptake of nutrients and slow diffusion of antimicrobials through biofilms [6].

A SPECIAL MENTION OF FUNGAL BIOFILMS

Fungi, both yeasts and molds, can also form biofilms. Fungal diseases are on the rise now due to increased over-the-counter use of antibiotics and also more hospital admission than before. The persistence of fungal infections is enhanced by the ability of fungi to produce biofilms on a wide range of implanted medical devices [7].

In fungal biofilms, cells are more easily detachable from the ECM, and hence, chances of thromboembolism are more common if such biofilms form on cardiac vegetations or prosthetic valves. Furthermore, yeast cells are able to detach from adherent biofilms on the devices and cause fungemia and systemic infections. Although *C. albicans* are still more common and form biofilms readily, the incidence of non-albicans *Candida* species which

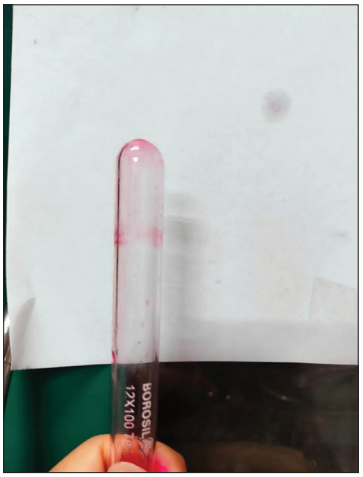

Figure 1: Test tube method of biofilm formation and observation by the naked eye

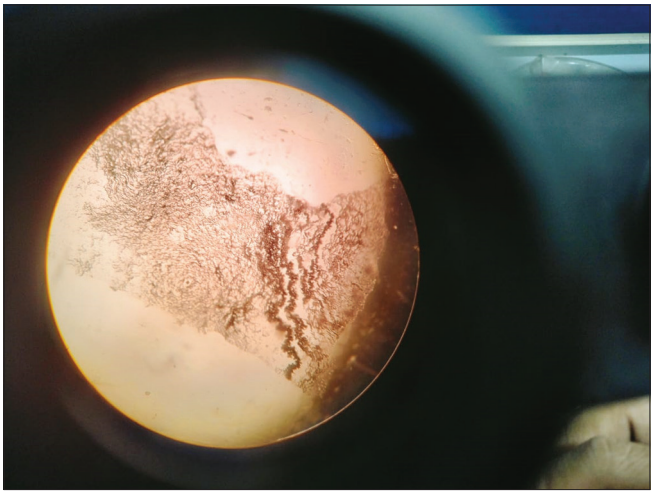

Figure 2: Microscopic view under ×10 objective of biofilm in the test tube

are capable of biofilm formation and cause device-related infections is rising steadily and, thus, is of great concern. *Candida* species that cause nosocomial infections include *C. glabrata*, *C. parapsilosis*, *C. krusei*, and *C. tropicalis*. True yeasts like *Cryptococcus neoformans* can also form biofilms over devices such as ventricular shunts and cardiac valves.

Which method is the best for studying biofilms

When compared to the microtitre plate method, the sensitivity of tube adherence method and Congo red agar method was found as 82% and 78%, respectively, in one study [8].

SOME POSSIBLE NEW METHODS FOR STUDYING BIOFILMS

New techniques such as Scanning Electron Microscopy (SEM), Optical Coherence Tomography (OCT), Fourier Transform Infrared (FTIR) Spectroscopy and Crystal Violet Staining technique are being used for study of biofilms. Slide method and borosilicate Petri dish method can also be tried for studying biofilms [9]. These methods have still not been used to study biofilms. We have tried these new methods and also comparing them with the test tube method and are obtaining interesting results.

DISCUSSION

Infections have been associated with biofilm formation on human surfaces such as teeth, skin, and the urinary tract. Bacteria in biofilms bind irreversibly with the substratum. Cells in biofilms communicate through quorum sensing by producing hormone-like molecules or pheromones [5]. It is estimated that approximately 80% of all bacteria in the environment can exist in biofilm communities, and more than 65% of human infections due to microbes do involve biofilms [7]. However, only adherent cells are not biofilms. A true biofilm is one in which the microorganisms grow as a community, and not separate surface-adherent cells [10]. Biofilm formation provides many survival advantages to the microbes, like shielding from antimicrobials, protection from protozoan grazing, and protection against human host defenses.

A relatively newer discovery is that of persister cells in biofilms. These cells are non-dividing and have been proposed to protect themselves and other cells from the action of antibiotics because they express toxin–antitoxin systems. In this, the target of the antibiotics is blocked by the toxin modules [11]. These "persister cells" are dormant variants that can exhibit antibiotic tolerance and can later become active when the therapy is withdrawn [2].

A more recent approach to preventing biofilm formation over devices is by way of silver. Colloidal silver (topical) has been found to impair biofilm formation in *S. aureus* [12]. Overall, many methods are there for studying biofilms formed by medically important bacteria, and the method one needs to follow depends on the availability of reagents and also the ease of the method to be used.

CONCLUSION

Many bacteria and fungi form biofilms over indwelling devices and biological surfaces. Biofilm formation should be studied well because even if an isolate is susceptible to many antibiotics *in vitro*, it may be refractory to treatment if it is a biofilm producer. There are many extant and upcoming new methods for studying bacterial and fungal biofilms *in vitro*.

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Funding: Nil; Conflicts of Interest: None Stated.

How to cite this article: Biswas M, Bhattacharyya S, Banik A. Methods for detection of biofilms in bacteria and fungi. Eastern J Med Sci. 2023;8(2):34-36.