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Microbial Biofilm Detection Methods: A Comprehensive Review

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Abstract—*The increasing trend of antibiotic resistance of microorganisms is the biggest challenge and a grave concern to human health, and food industries. Biofilm formation by microorganisms is considered as one of the important reasons for enhanced antibiotic resistance along with other reasons like, poor sanitation and hygiene, over-prescription and overuse of antibiotics, etc. Biofilm is a group of microorganisms (homogenous or heterogeneous) irreversibly attached to biotic and abiotic surfaces and live within a self-produced extracellular polymeric substances(EPS). Biofilm serves as the safe home of microorganisms and gives them a protection against potent antibiotics and other environmental stress conditions. It has already been reported that 65% of all microbial infections and 80% of all chronic infections, disease outbreaks and associated deaths, food spoilage, and biofouling of water, are associated with the growing capability of microorganisms to form strong biofilms. So, because of the increasing crisis associated with the microbial biofilm, researchers are focusing on development of advanced biofilm detection methods in order to devise new methodologies for inhibition and eradication of biofilm. In this review paper we highlight different biofilm detection methods used by researchers for visualization and evaluation of microbial biofilms on the surfaces including implanted devices. The different detection methods are comprised both of the qualitative and qualitative approaches. Some of the prominent detection methods include the microscopic visualization, Fourier transforms infrared, Fluorescent in situ hybridization and biochemical detection methods. This review signifies the use of detection approaches for primary evaluation of biofilm both qualitatively and quantitatively for understanding the mechanism of biofilm formation, inhibition, and eradication as well.*

Keywords*: Biofilm, detection method, qualitative, quantitative.*

1. Introduction.

Biofilm is an aggregation of microbes on biotic and abiotic surfaces formed mostly during stress conditions like presence of antibiotics, nutrient deficiency, abnormal pH and temperature. Biofilm formation is a multi-step process which starts with adhesion of microbes to the surfaces, followed by growth and maturation and finally dispersion of biofilm to repeat the cycle. It has been reported that microbes within biofilm are more than 1000 times resistant to antibiotics than free floating planktonic microbes. The biofilm forming microbes have become one of the leading causes of antibiotic resistance, recurrent infection outbreaks and associated deaths [1]. Food industries like poultry, aquaculture, ready-to-eat foods, meat, sea food, dairy products are severely affected by biofilm producing microbes and have become one of the prominent reasons for food spoilage and food borne diseases. For these rising concerns the detection and inhibition of biofilm menace has become the priority of researchers. This review describes some of the advanced and reliable methods of biofilm detection. The review describes the quantitative and qualitative nature, accuracy, advantages, disadvantages, reliability, viability and non-viability differentiating capability of different methods of biofilm detection [2].

2. Different methods used for detection of biofilm.

2.1. Light Microscopy.

It is one of the simplest, cheapest, and convenient approach to visualize the biofilm of microorganisms like *Pseudomonas aeruginosa, Candida albicans, Staphylococcus epidermidis*, *Escherichia coli* and many more. Light microscopy could be used for visual identification and quantitative evaluation of the biomass of biofilm formed on the surface of glass cover slips, polystyrene petriplates, etc. In spite of the limitations of restricted resolution as well as magnification, light microscopy is used in biofilm study as it is easy, fast and covers larger portion of sample in comparison to other microscopic techniques [3].

2.2. Scanning Electron Microscopy (SEM).

SEM is a powerful instrument for studying the detailed structure of materials using high-energy electron beams. SEM is used to visualize the 3D images, biofilm adhesion and growth patterns, biofilm spatial position on the surfaces including medical devices. Though SEM could be used to provide qualitative information to support the quantitative data but could not help in distinguishing between live and dead cells because of the incompatibility with fluorochromes. Generally, four types of SEM are used to evaluate biofilms- (i) Conventional SEM- the above discussed SEM is actually conventional SEM (ii) Environmental SEM (E-SEM)- is used

to study the biofilm structure in their natural environment (iii) Cryo-SEM- is used for evaluating topography as well as structural detail of internal structures of biofilm by using freeze-fracture technique (iv) Focused Ion Beam SEM (FIB)-employed for studying subsurface structures of biofilm [4].

2.3. Transmission Electron Microscopy (TEM).

One of the major disadvantage of using SEM as a biofilm visualizing tool is the dehydration process during sample preparation which could disrupt biofilm structure. This limitation could be solved by employing TEM technique, that skips the dehydration step for elucidation of microbial biofilm on the surfaces. TEM use a strong beam of electrons from an electron gun to magnify the image a million times or more. To study microbial samples "gold standard" coating is used as a negative stain [5].

2.4. Laser Scanning Microscopy.

It is a non-invasive in situ imaging technique for living, fully hydrated biofilms used for 3D analysis of cells, macromolecules, structure and composition, complex microbial communities, recognition of community members, immunofluorescence evaluation of environmental biofilms and in-depth study of microbial microhabitats. The advanced modifications to LSM include the use of lasers with one-photon excitation known as CLSM and two-photon excitation known as two-photon laser scanning microscopy (2PLSM) [6].

2.5. Confocal laser scanning microscopy (CLSM).

CLSM is one of the sophisticated tools to study biofilm in a non-destructive real-time manner. By using specific stains CLSM can be used to distinguish different molecules embedded within EPS, to evaluate cell density and morphology, to quantify the biofilm biomass as well as to differentiate between viable and non-viable microbes within the biofilm. The advantage of CLSM over SEM is that the former can be used to characterize biofilms up to 100 μm depth even in hydrated and undisturbed condition [7].

2.6. Atomic force microscopy (AFM).

It is a potent emerging tool used to evaluate microbial biofilms qualitatively as well as quantitatively. The advantage of using AFM as biofilm evaluating tool is that it gives a better insight of various forces influencing microbial growth and attachment of microbes on the surfaces besides providing high resolution 3D nanoscale images. It is a non-destructive, precise, requires very less pre-treatment and ambient conditions, thus, advantageous over other microscopy techniques. The limitation with AFM is that it only captures top surface images but fail to produce images between surfaces and microbes [8].

2.7. Atmospheric scanning electron microscopy (ASEM):

The detection of biofilms in liquid medium is still a tough task to perform. In this context, Sugimoto et al., 2016 [9] developed ASEM (employing Optical microscopy above the sample and inverted SEM below it) to visualize the biofilms of both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria in liquid medium.

2.8. Scanning transmission X ray microscopy (STXM):

It employs near edge X-ray absorption fine structure (NEXAFS) technique for evaluation of microbial biofilms-mapping of metallic ions, macromolecules (lipids, polysaccharides, nucleic acids, proteins), and the action of antibiofilm agents. Because of the potential of soft X-rays to penetrate water, STXM can be used for fully hydrated biological samples. The superiority of STXM over full-field transmission X-ray microscope is that the former uses high quality spectra from high-resolution beam lines [10].

2.9. Magnetic Resonant Imaging (MRI).

MRI is a non-invasive method to study living, fully hydrated biofilm structures in in-vivo, in situ and three dimensions. It is based on the phenomenon of absorption of electromagnetic radiation in an external magnetic field. MRI technique can be employed to study transport properties of biofilm, to relate mass transport and biofilm structure biofilm mapping, structural heterogeneity, water diffusion measurements, transport of macromolecules, heavy and trace metals, flow velocity, oxygen concentration. MRI is less sensitive than optical microscopy because of small energy band between the excited and non-exited states which is the only major disadvantage with it [11].

2.10. Fourier transforms infrared (FTIR).

In order to understand the pathogenicity and heterogeneity of microbial biofilms, it is necessary to know about morphological changes and differences the microbes undergo while shifting from planktonic form to sessile form. The in vitro techniques fail to provide such data so in this perspective in situ procedures come to the rescue. FTIR is one such technique that enlighten the researchers with chemical information and the dynamic processes happening within the microbial biofilms by using Synchrotron Radiation-FTIR (SR-FTIR). It is a non-invasive method but could not be applied for hydrated samples [12].

2.11. X-ray computed tomography (CT).

Most of the microscopic techniques could not explain the biofilm formation in porous media because of the opacity. To overcome this disadvantage CT could be used to study dynamic and complex nature of biofilms within porous media. It is a potent technique to visualize and differentiate biofilm formation in central venous catheters (CVCs) in a non-destructive, direct and non-invasive manner [12-13]. This technique was used by Keren-Paz et al., 2018 [14] to demonstrate detailed structure of calcium deposits within biofilm.

2.12. Fluorescent in situ hybridization (FISH).

In order to evaluate the diversity of multi-species biofilms the study of variety and variability of nucleic acids within the biofilm need to be evaluated that could be done by employing

the techniques of Denaturing Gradient Gel Electrophoresis (DGGE), FISH, and Polymerase Chain Reaction (PCR). Oligonucleotide probes are used in FISH to study DNA sequences and FISH provides a quantitative, precise data about the microbial variability of even those microorganisms embedded within biofilms that are difficult to culture [15]. Almeida et al., 2011 [16] used Peptide Nucleic Acid Fluorescence in situ Hybridization (PNA FISH) technique to study the mixed biofilm of *S. enterica, E. coli*, and *Listeria monocytogenes*.

2.13. Roll plate method.

It is one of the semi-quantitative methods used to study the microbial population within biofilms especially on medically implanted devices such as catheters. As the name depicts the catheter is rolled to and fro on the media plate for subsequent culturing. 15 CFU/catheter segment is the standard cutoff colonization for distinguishing significant colonization from microbial contamination. The major disadvantage with the method is that only the outer surface biofilm detection is possible but fail to provide any information about colonization of endoluminal or intraluminal catheter tips [17].

2.14. Sonication technique.

To overcome the disadvantages associated with roll plate method Sonication technique (standard CFU cutoff 1000≤ to 100≤/catheter segment) could be employed to assess the endoluminal as well as external surfaces of catheters and detect the microbial populations colonizing therein. However, Sonication technique is no better than roll plate method for detection of microbial populations in long-term tunneled catheters [18].

2.15. Microtiter plate assay.

96-well flat-bottomed sterile polystyrene microplates are used to quantify the biofilm formation. In this method the microbes are allowed to form biofilm in the wells of plate in a static condition and by using specific stains like safranin, crystal violet etc. the biofilm formation is quantified by analyzing under spectrophotometer. The experiment is easy to perform, inexpensive, high-throughput and does not take much time. The disadvantages associated with this method is washing off of loosely attached cells, chances of sedimentation, and only end-point measurement is possible [19].

2.16. Biofilm Ring Test (BRT).

This is a rapid and promising tool for the evaluation of biofilm forming organisms and to study the kinetics of biofilm formation. In this method the microbial culture is mixed with magnetic microbeads, the movement of which under the external magnetic field will determine the biofilm formation. If microbeads fail to move in presence of magnetic field and no central spot formation takes place, it indicates the formation of biofilm and if the visible central spot formation occurs, it indicates the absence of biofilm formation. The method is highly reproducible, high-throughput, easy, well-designed and can be used for loosely attached biofilms [20].

2.17. Congo Red Agar (CRA) Method

CRA is a qualitative approach for biofilm detection based on color change of colonies cultured on the plate. CRA is prepared by mixing Brain Heart Infusion agar (BHIA) (37 g/l), sucrose (36 g), and Congo red (0.8 g). Colony color after sub-culturing decide whether the culture is biofilm forming or not-as black colonies indicate biofilm forming microbes while the colonies that retain the pink color of the agar are non-biofilm forming. Although the accuracy of this method is not so high but with some modifications while preparing the agar increases the accuracy of the method [21].

2.18. Tube method.

This is a qualitative method for the detection of biofilm on the walls of a polystyrene test tube. Microorganisms are allowed to grow in the test tubes filled with specific media like Trypton Soya Broth or Luria broth in a static condition for 24 h. After incubation the tubes are washed with PBS to remove excessive media and free floating microbes. The tubes are stained then with safranin or Crystal violet, incubated for 30 min, and washed with PBS. A visible film on the walls of tube can be seen in case biofilm formation has occurred, otherwise no visible film can't be seen [22].

3. Conclusion and future perspectives.

Due to increase in biofilm associated infections, deaths and development of resistance against antibiotics, the scientists have shifted the focus towards understanding the mechanism of biofilm formation, detection and eradication of biofilm. Although an advancement has been made towards the detection of biofilms but researchers are still trying to develop a novel, sophisticated, reliable method for visualization and detection of strong biofilms in order to get rid of the biofilm menace.

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