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Bacterial Biofilms: A Confrontation to Medical Science

Sorabh Singh Sambyal[1], Preeti Sharma[2], Divya Srivastava[3]

[1]Ph.D Scholar, Jaipur National University

[2]Ph.D Scholar, Jaipur National University

[3]Joint Director, Life Sciences, Jaipur National University

Abstract: ***Biofilms is a consortia of microorganisms in which microorganism adhere to a substratum in an irreversible manner and are enclosed in self – produced matrix of extracellular polymeric substance. Common example of biofilm are dental plaque, device associated infection. Mostly biofilms are infective in nature and can be a source of Nosocomial infection. According to a report published by national institute of Health about 65% of all microbial infections and 80% of all chronic infections are associated with biofilms. The structural anatomy of biofilms protects microbial cells from antimicrobial agents, UV rays. These biofilms act as a barrier; antibiotics cannot reach to these microbes hindering the killing of the microbes thus posing a serious threat to public health. Management of these bacterial biofilms requires an early detection method and development of novel and efficient control measures for treating patients improving patient care and timely management.***

Keywords: Biofilm, Biofilm-matrix Antimicrobial resistance, Polymeric Substances, Tissue-culture plate

1. Introduction

In nature, microorganism subsist primarily by adhering to and growing upon biotic and inanimate surfaces. These substrates can be of distinct forms, including those found in aquatic system, soil and those on spectrum of indwelling medical devices and those of on living tissue such as heart valves or the lungs or the middle ear or dental enamel. No bacterium is alone i.e. nearly all bacteria live with; depend on other microorganism for carbon, energy and other nutrients. Thus, most of the bacteria in the world live in micro ecosystem filled with hundreds of other microorganism. Researchers have recently realized that in the natural world more than 99% of all bacteria exist as biofilm [1]. A biofilm is a well organized consortium of microorganism. Biofilm associated cell is differentiated from suspended counterparts by reduced growth rate, up and down regulation of gene and generation of extra polymeric matrix (EPSs). Primarily comprised of complex mixture of polymers comprising polysaccharides, as well as proteins, nucleic acids, lipids and humid substances [2,3]. These polysaccharides are major components of EPS and they also mediate most of the cell-to- cell and cell-to-surface interaction required for biofilm formation and stabilization.[4] It manifests an altered growth rate and transcribes gene that free floating counterparts do not transcribe.[5] Microbial adhesion to hydrophilic and hydrophobic surfaces is influenced by the EPS to some extent. It has also been showed that the formation of EPS leads to irreversible attachment with different surfaces. It also helps the microorganisms to escape their killing by antibiotics.[6,7,8]

2. A Brief History and Development

In year 1684, a Dutch named Antonie Van Leeuwenhoek displayed animalcule (bacteria) found in the plaque of teeth that he reported in his report submitted to Royal society of London[9] In year 1933, Henrici presented his recorded observations concerning biofilms in which he observed that water bacteria were not free floating, but they submerged surface[8]. In year 1940, H. Heukelekian and A. Heller wrote about the development of bacterial slime and colonial growth attached to surfaces. In year 1943, Zobell described about seawater and described many of the fundamental characteristics of attached microbial communities. In year 1973, Characklis examined microbial slimes in industrial water systems and was able to demonstrate that they were not only pertinacious, but also extremely resistant to disinfectants such as chlorine [10]. In 1978, Chesterton was instrumental in alerting the world about the importance of biofilms and coined the term biofilm. He also articulated a theory of biofilm that elucidated the mechanism by which Microorganism cohere to living and non living materials and the benefits accumulated by this ecological niche. Micro-organism constitutes the most successful form of life on earth and effect human existence directly or indirectly by carrying out processes in nature and manmade environments. [9] Mechanism of biofilm formation Biofilm may be formed on broad motley of substratum including living tissues, indwelling medical devices, Indus or portable water system piping or natural water system piping. The biofilm on the medical devices composed of a single coccid organism and the associated extracellular polymeric substance's matrix [8]. The formation process of biofilm is extremely complex, in which Microorganism cells transform from plank tonic to sessile mode of growth. It has also been insinuated that the formation of biofilm depends upon the expression of specific genes that directs the constitution of biofilm[11,12]. A fully developed biofilm is composed of many layers comprising a matrix of exopolysaccharide with vertical structures, and a conditioning film. Vertical structures of Microorganisms sometimes they took the form of towers or mushrooms, and are set-apart by interstitial spaces.

3. Formation of a biofilm occurs Step by Step

1) **Repositioning of the conditioning film** which affects the surface properties of the substratum and introit microorganism to conglutinate to the surface.
2) **Attachment** of the Microorganism (Planktonic) with the conditioning film.
3) **Bacterial colonization and Growth**. The bacteria begin to multiply while effusing chemical signals that „intercommunicate" among the bacterial cells. Once the signal intensity exceeds, the genetic mechanism underlying exopolysaccharide production is activated where production of polysaccharides helps to anchor the bacteria to the surface allowing it to multiply within the embedded exopolysaccharide matrix, thus giving rise to the formation of micro colonies. [2, 13,14,15].
4) **Biofilm formation**, whereas an amply matured biofilm will include an EPS matrix and vertical structures isolated by interstitial surfaces. It has been suggested that these interstitial spaces channels constitute primitive circulatory system, delivering nutrients to and removing waste products from the communities of cells in the micro colonies. Some cells are taken up to the surface for only a limited time, before being de adsorbed, in a process called "reversible adsorption". The initial connection is based on electrostatic attraction and physical forces, but not due to any chemical attachments. Some of these reversible adsorbed cells begin to make preparations for a lengthy stay by forming structures which may then permanently hold-fast them to the surfaces within the next few hours, the trailblazer cells proceed to reproduce and the daughter cells, from micro colonies on the surface and begin to produce a polymer matrix around the micro colonies, in an irreversible step.[16]
5) **Detachment**:-After biofilm formation, some bacteria are shed from the colony these bacteria can undergo rapid multiplication and dispersal. Detachment of plank tonic bacterial cells from the biofilm is a programmed detachment, having a natural pattern[2]. Cells of the biofilms are dispersed either by shedding of daughter cells from actively-growing cells, or detachment may occur as a result of nutrient levels or quorum sensing, or shearing of biofilm aggregates (continuous removal of small portion of the biofilms). Resistance of Biofilm to Host defense mechanisms Micro-organism within a biofilm grow in a shielded micro environment largely through production off a biofilm matrix comprised of extracellular polysaccharides, proteins and nucleic acids[18]. The fact that biofilm based infections are rarely resolved, even in individuals who have a competent innate and adaptive immune Response, Highlights the high degree of resistivity possessed by biofilms [19].

4. Resistivity towards Antimicrobial agents

This biofilm type of life style bestowed the compeered organisms a measurable dwindle in antimicrobial susceptibility. It has been founded by Ceri and his co-workers that Escherichia coli associated with the biofilm required 1500 times the MIC of ampicillin to provide a 3-log reduction [20]. Study conducted by Williams and co–worker reveals that *Staphylococcus aureus* biofilms required 110 times the MBC of vancomycin to provide a 3-log reduction [21]. The effect on susceptibility may be built-in or acquired. Antimicrobial agents must diffuse through the EPS matrix to contact and inactivate the organisms within the biofilm. Extra Polymeric substances retard diffusion either by limiting the transport rate or by chemically reacting with the antimicrobial molecules. Hoyle and his co- workers were able to demonstrate that the EPSs of *Pseudomonas aeruginosa* was capable of binding tobramycin where as dispersed cells were 15 times more susceptible to this antibiotic than were cells in intact biofilms. Also, biofilm-associated organisms have reduced growth rates, minimizing the rate that antimicrobial agents are taken into the cell and thus affecting inactivation kinetics [22]. Du Guid and his co-worker discovered that an explosive growth rate resulted in an increase in susceptibility of *Staphylococcus epidermidis* biofilms. They also demonstrated that ciprofloxacin activity was influenced by the cell cycle; newly formed daughter cells were more susceptible than other populations in the biofilm. The surrounding environment of the cells within a biofilm may provide conditions that further guards the organism.[23,24].Research had showed that plasmids can be exchanged in biofilms under a number of conditions. Plasmids are extra chromosomal circles of DNA that may encode resistance to a large number of antimicrobial agents, including b-lactams, erythromycin, amino glycosides, trimethoprim, tetracycline, glycopeptides, and sulfonamides [25]. A large number of bacterial species showed transfer of plasmids to other bacterial species [26]. Ehlers and Bower demonstrated transfer of plasmid via conjugation between different gram-negative bacteria growing in biofilms [27]. The rates of horizontal plasmid transfer were several orders higher in biofilms than in liquid cultures of the same organisms. Other investigators were also able to demonstrate similar phenomenon [28–30]. Both the greater probability of contact between cells and the negligible effect of shear forces in either disrupting cell-to-cell contact or damaging the pili required for conjugation are may be reason for enhanced transfer of plasmids in biofilms .

5. Clinical importance of biofilms in chronic infections

Modern-Day acute infections can frequently be solved efficiently with antibiotics (except for infections caused by an antibiotic- resistance strain) and are not considered involving biofilm. However, more than half of the infectious diseases that affect mildly compromised individuals involve bacterial species that are commensals and are common in our natural environment. For example, *S. aureus, S.epidemidis or S. hyicus,*which *colonize the skin, E.coli, salmonella, Streptococcus suis and S. agalictiae, which colonize the mucosal membranes; Pasteurella multocida, P.haemolytica , Actinobacillus pleuropneumoniae, MycoPlasma Spp. Or Haemophilus parasuis*, which commences upper airways etc. In a report published in 2001, it has been hypothesized that bacteria which colonize human chronic wounds may exist as biofilm communities. In 2003 a study was conducted on different specimens collected from patients having skin diseases bulbous impetigo, atopic dermatitis and pemphigus foliaceous. Akiyana and his co-workers were able to demonstrate the presence of *S.aureus*

in them by using Cong A, Safranin, Immunoflorescent staining. Kirkenterp-Moller also demonstrated *Pseudomaonas* existed biofilm rather than single cell in wound of Suspected 22 patients using PNA FISH [31]. Another study conducted by James and his co-worker, they microscopic evaluated specimens from 50 chronic wounds and 16 acute wounds. They observed that 60% of the chronic wounds have biofilm in them and the acute wounds have 6% only. [32,33]. It is difficult to eradicate such infections may lead too chronic infection which may show the presence of biofilm bacteria surrounded by exopolysaccahride matrix. These biofilm associated infections do share clinical characteristics, such as growing slowly in one or more locations, slow in producing overt symptoms The Detection of Biofilm producing Micro-organism Early biofilm formation might result in a great success in the treatment, because in long standing cases, they may be very damaging and may produce immune complex sequela.

6. Methods for the detection of biofilm producing microorganisms

There are two methods for the detection of biofilms.
a) The phenotypic methods
b) The Genotypic method.

a) The phenotypic methods

1. Test tube method:-10ml of Trypticase soy broth (TSB) with 2% of sucrose was inoculated with Loopful of bacterial colonies from overnight culture plates and incubated for 24hr-48 hrs at 37°C.The culture supernants were decanted and the tubes were washed with phosphate buffer saline (pH 7.3) and then these dried test tubes were stained with crystal violet (0.1%). Superfluous stain was poured out by washing with de-ionized water. Tubes were then dried by positioning them invertedly. Tubes were then ascertained for biofilm formation. Biofilm formation was considered positive when a viewable film bordered the wall of the test tube.

2. The Congo red agar (CRA) method:-Congo red agar was prepared as concentrated aqueous solution separately from other constituents of media and autoclaved at 121°C for 15 minutes, and then added to the autoclaved brain heart infusion agar with sucrose which is cooled at 55°C. Plates were inoculated and incubated aerobically for 24hr– 48hours at 37°C.Dry crystalline black colored colonies indicates biofilm production.

3. The Tissue culture plate method:-This quantitative assay (TCP) as described by Christensen et al. is well chosen, widely used and considered as the gold standard method for the detection of the biofilm. Isolates from pure cultures were inoculated on trypticase soy agar with 1% glucose (TSBglu) media and incubated for 18 hours at 37°C and then diluted 1 in 100 with freshly prepared medium. 96 well-flat bottom micro titer plate made of polystyrene were used. Each well was filled with 0.2 ml of BHI broth and colonies of test organism were inoculated into each labeled well. The tissue culture plates were incubated for 18 hours at 37^0C. After incubation, the plates were gently tapped and the content from each well was removed. The wells were washed with 0.2ml of phosphate buffer saline (PBS pH 7.2) four times to remove free floating "planktonic" bacteria. Biofilms formed in plate by adherent "sessile" organism were fixed with sodium acetate 2% (and stained with crystal violet (0.1%W/V). Superfluous stain was rinsed off by washing with de-ionized water and plates were kept for drying. Biofilm cells adhered with the wells were uniformly stained with crystal violet. Optical density (OD) was determined with a micro ELISA auto reader at wavelength 570nm.

4. Liquid interface cover slip assay:- Bacterial cultures were analysed for biofilm formation using the air liquid interface cover slip assay. In this assay adhered to coverslip will be visualized under light microscope. Cultures were inoculated into tubes containing 3-5ml of TSB and allowed to grow to a stationary phase. The stationary phase cultures will be diluted1:100 in TSB. Diluted cultures will be used to fill a well in a flat bottom 12 well plate. The wells will be filled to 100 µl each. Sterile glass coverslip will be inserted into each well to achieve a 90° angle relative to bottom of the well. So that, the meniscus of the medium will be at the centre of the coverslip. Plates will be covered and kept in the incubator at 37°C for a period of 18 hrs. Bacteria will be stained by submerging cover slips in 0.1% crystals violets for 10 minutes. Excess dye will be rinsed off by dipping each coverslip in two successive water bath and cover slips are allowed to dry. Bacteria at the air interface on each coverslip were visualized under a microscope. Various studies have suggested and established that TCP is a better screening test for biofilm production. This is easy to perform and to assess biofilms, both quantitatively and qualitatively. [34,35,36,37,38,]

b) The Genotypic method

Sonification and PCR amplification methods have been shown to improve the detection of biofilms. Non biofilm producers are negative for ica A and Ica D and lack the entire ica ADBC Oberon. But these methods requires specialized equipments and techniques[39,40].

7. Management of Biofilm Infection

A key factor to combating biofilm infections is to understand the physiology of biofilm development. In 2003, Davies suggested that chemotherapeutic agents could be developed to promote or prevent transition from one stage of biofilm maturation to the next by targeting unique biofilm regulatory or signaling molecules. Specific agents might be discovered or developed which will interfere with the production of virulence factors, or promote or inhibit the shedding of biofilm bacteria [41].As mentioned before, biofilm resistance depends on aggregation of bacteria into multicellular communities. Therefore, one antimicrobial strategy might be to develop therapies to disrupt the multicellular structure of the biofilm. It could be that host defences might be able to resolve the infection once the multicellularity of the biofilm is reduced, and then the effectiveness of antibiotics might be restored [42]. Other potential therapies include enzymes that 60 S.L. Percival et al. dissolve the matrix polymers of the biofilm, chemical reactions that block biofilm matrix synthesis and analogues

of microbial signalling molecules that interfere with cell-to-cell communication, required for normal biofilm formation [43]. Already a number of QS inhibitors have been identified such as the inhibitory peptide RNAIII, which inhibits the agr system of Gram-positive bacteria [44]. In *P. aeruginosa*, furanones derived from plants have been demonstrated to block AHL pathways. [45]

For in vivo indwelling device-associated infections, effective, preventive and therapeutic strategies still need to be developed. One such therapy could be the production of materials with anti-adhesive surfaces. In 2004, Tenke et al. showed that on heparin-coated catheter, stents, no biofilm formation was evident between 6 and 8 weeks, whereas uncoated tubes were obstructed within 2–3 weeks [46]. In case of IMD in non surgical patients, long term antibiotics therapy is requisite. Progress has already been made, but the future of biofilm research and management relies upon collaborative efforts to fully explore these complex systems of the microbial world.

8. Conclusion

Infectious disease progresses due to bacteria associated with biofilms such as cystic fibrosis, otitis media, periodontitis, , native valve endocarditis and chronic prostatitis all tends to be caused by biofilm-associated microorganisms. In addition, indwelling medical devices have been shown to harbour biofilms, which have been implicated in infections. Biofilms are extremely resistant to most antimicrobial agents and disinfectants. Sessile bacteria within a biofilm are able to acquire resistance by the transfer of resistance plasmids. This acquisition of resistance is particularly important in the healthcare environment for patients with colonized urinary catheters and orthopaedic patients. Many organisms exhibit plasmids encoding resistance to the multiple antimicrobial agents, particularly in the medical setting. Resulting in the persistence and long term stay in the hospital. The role of biofilm in disease is becoming understandable. However, early detection of biofilm associated infections, and newer treatment options for the management of the same are needed.

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Author Profile

Sorabh Singh Sambyal,(PhD Scholar) Jaipur National University, formely worked as Research Assistant in Viral Research and Diagnostic Laboratory in GMC, Jammu

Preeti Sharma (PhD Scholar) pursuing PhD in Clinical Microbiology having three year Research Experience in Medical Mycology.

Dr. Divya Shrivastava-(Joint Director), Jaipur National University. She has been teaching for more than 14 years.