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Detection and Control of Biofilm Produced By Pathogenic Bacteria

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ABSTRACT: *A bacterial biofilm is a structured community of bacterial cells enclosed in a self-produced polymeric matrix that is adherent to an inert or living surface. The stages involving attachment, colonization and growth that leads to establishment of a mature biofilm colony formation. There are different assay for the detection of bacterial biofilms like staining assay include FDA assay, LIVE/DEAD BacLight assay, XIT assay etc. and genetic assay include PCR technique. Control of bacterial biofilms include using phages, nanoparticles and control using anti-adhesion agents like mannosides, pillicides and curlicides and using anti-matrix agents like enzymes and chelating agents.*

Keywords: Biofilm, Pathogenic Bacteria

INTRODUCTION:

A biofilm can be defined as a population of microorganisms attached to a surface growing enclosed in a self-produced matrix of extracellular polymeric substances (EPS). Biofilm EPS, generally composed of extracellular DNA, proteins and polysaccharides, peptidoglycans, lipids and phospholipids. Biofilms can be made up of single or multiple bacterial species and can form on biotic and abiotic surfaces. They may consist of a single bacterial or fungal species, or more commonly, may be polymicrobial, i.e. contain multiple diverse species. It is estimated that only 1% of bacteria exist in the familiar, free-floating, or planktonic form, with the remaining 99% as biofilms (Molin S.*et.al*.2005).

The biofilm is usually built up of three layers:

1. The linking film which attaches to the surface of tissue or biomaterials

2. The base film of compact microorganisms

3. The surface film as an outer layer, where planktonic organisms can be released free-floating and spreading over the surface.

In humans an estimated 65% of all hospital infections are of biofilm origin. Following is the list of common bacterial biofilm forming bacteria (Christoph A Fux *et al*.2003; C.A. Fux *et al*.2005)

Sr. No.	Infection or disease	Common bacterial biofilms in human
1	Cystic fibrosis	*Pseudomonas aeruginosa* and *Burkholderia cepacia*
2	Dental caries	Acidogenic Gram-positive cocci (e.g., *Streptococcus*
3	Periodontitis	Gram-negative anaerobic oral bacteria
4.	Osteomyelitis	Various bacterial and fungal species, often mixed
5	Bacterial prostatitis	*Escherichia coli* and other Gram-negative bacteria
6	Otitis media	Non-typable strains of *Haemophilus iinfluenza*
7	Musculoskeletal infections	Gram-positive cocci (e.g., *staphylococci*)
8	Biliary tract infection	Enteric bacteria (e.g., *E. coli*)
9	Native valve endocarditis	Viridans group streptococci

Table I: Types of bacterial biofilms causing infection or disease

MECHANISM OF FORMATION OF BIOFILM:

Development of surface conditioning biofilm:

In human host, conditioning film may also be complex and determined by the site being conditioned. For e.g.-In dental plaque, the enamel of the teeth is conditioned by a "proteinaceous pellicle" composed of albumin, glycoprotein, lipid, lysozyme phosphoprotein and other components of saliva.

Transport mechanisms involved in adhesion of microorganisms to a surface:

The transport of microbial cells and nutrients to a surface is achieved by two main flows within fluid transport pipes: laminar and turbulent flow (Percival S L *et al*.1999).

Adhesion:

Adhesion was proposed in 1943 by Zo Bell, consisting of reversible & irreversible adhesion. Reversible is the weak attachment of microbial cells to surface, whereas irreversible is the permanent attachments of microorganism to a surface, generally follows.

Micro colony and Biofilm formation:

The attachment of microbials to a substratum is only initial stages of biofilm development. As the attachment occurs, the colonizing bacteria grow with the production and accumulation of extracelleular polymer. The microorganisms become embedded in the hydrated polymeric matrix and immobilized. Cells are dependent on substrate flux for the change of nutrients with neighbouring cells in biofilm formation.

Detachment and Dispersal of the biofilms:

Detachment is a interfacial process, which involves the transfer of cells to the bulk liquid, with the detachment of microbial cells occurring from the initial attachment.

DETECTION OF BACTERIAL BIOFILMS ON THE BASIS OF ASSAYS

STAINING ASSAY:

DMMB assay:

This is a colorimetric analysis used to quantify specifically *Staphylococcus aureus biofilm* using the 1, 9-dimethylmethylen blue (DMMB) (ToteK *et.al*.2008).

FDA assay:

This technique uses the colourless fluorescein-di-acetate (FDA), a cell membrane soluble dye. After bacterial uptake, FDA is hydrolyzed by cellular esterases to fluorescein which is fluorescent yellow. The signal can be measured spectrophotometrically. This method has been used to quantify *Candida albicans* biofilm growth at the surface of silicone disks. It is easy to perform and not expensive, this method is not widely used. Considering the limited field of examination and the thickness of biofilm, this method is not particularly suitable for quantitative studies on mature biofilm, yielding only semiquantitative result.

LIVE/DEAD BacLight assay:

This method is based on the use of two different nucleic acid binding stains. The first dye is the green fluorescent (Syto9), able to cross all bacterial membranes and bind to DNA of both Gram-positive and Gram-negative bacteria. The second dye is red-fluorescent propidium-iodide that crosses damaged bacterial membranes only. The stained samples are observed using a fluorescent optical microscopy to evaluate live and dead bacterial population. Live bacteria fluoresce in green and dead bacteria fluoresce in orange/red.

XTT assay:

This method uses a redox indicator to enumerate spectrophotometrically viable cells in biofilm. This method is based on the observation that microbial respiratory metabolism of viable cells is able to reduce the 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide salt (XTT) to a water-soluble formazan. (HonraetK *et al*.2005).

BioTimer assay (BTA):

BTA is a colorimetric assay allowing counting viable bacteria in biofilm life-style. BTA employs a specific reagent containing phenol red. The colour of specific reagent switches red-to-yellow due to metabolism of fermenting bacteria .BTA is a low cost, easy to perform method and has been applied to count living bacteria in biofilm, to verify microbiological quality of foods and to evaluate antibiotic susceptibility of biofilm (Pantanella F *et al*.2008). The main disadvantage relies on the difficulty in applying BTA for the evaluation of multispecies biofilm (F. Pantanella *et al*.2013)

PHYSICAL ASSAY:

Confocal laser scanning microscope (CSLM):

Confocal Laser Scanning Microscopy (CLSM) is an optical microscope equipped with a laser beam, mainly useful in biology and life sciences to study thick samples. This technique has been widely used in the study of biofilm, especially to study EPS components.

CRM (Confocal-RAMAN Microscopy)-

In Raman spectroscopy an electromagnetic laser beam with known wavelength hits the sample to be analyzed. Using Raman spectroscopy, it is used to acquire information on chemical fingerprint of different biofilms.

Electron microscopy (EM):

Techniques take advantage of the higher resolution allowed by the use of an electron beam, i.e., of short-wavelength and high-energy radiation. Transmission electron microscopy (TEM) shows unique capability for the imaging of the inner of biofilms and intracellular features, but requires the sample to be prepared as ultra-thin slices (Smirnova T A *et al*.2010, Allan-Wojtas P *et al*.2010) .Conversely, scanning electron microscopy (SEM) has been widely used to visualize the surface of microcolonies as well as old biofilm (Maclean LCW *et al*.2008).

In X-ray microscopy:

An XM technique, the sample is illuminated with a soft X-ray focused radiation, either mono- or poly-chromatic. In particular, scanning transmission X-ray microscopy (STXM) has been widely used to

find the composition of bacterial cells and biofilms with nominal resolution of 25 nm (Dynes JJ *et al*.2006)

Polymerase chain reaction (PCR):

PCR is widely used as a detection method. In biofilm, this method allows to identify efficiently the presence of specific genetic sequences related to individual bacterial specie PCR as such, is not suitable for quantitative studies of biofilm and, as amplifying indifferently DNA of both viable and dead cells cannot be used for enumeration of living cells. Moreover, due to its high sensitivity, false positive results can be expected from natural contamination.

To overcome these problems, "Real Time Quantitative-Reverse Transcription-PCR" (**qRT-PCR**) has been adopted. The qRT-PCR is one of the most powerful and sensitive gene analysis techniques available at now.

Fluorescence in Situ Hybridization:

Fluorescence in situ hybridization (FISH) is a molecular technique that uses fluorescently labelled probes to detect RNA or DNA. The most commonly used target in FISH for microbiologic applications is the 16S ribosomal RNA because it allows design of specific oligonucleotide probes for most bacteria, as well as genus-specific or species-specific probes.

Consequently, FISH has been established as an invaluable tool to investigate complex microbial communities in environmental microbiology allowing rapid detection, and visualization of the spatial arrangement in their natural environment. More recently, peptide nucleic acid (PNA) probes have been developed and optimized for bacterial detection. PNA probes have superior hybridization characteristics, including higher specificity and improved hybridization kinetics, which result from the uncharged chemical backbone of the PNA probe. PNA probes are also available and those are most costly than oligonucleotides probes. (Ly T *et al*.2008).

Combining FISH technique with Confocal laser scanning microscopy is possible the identification and visualization of different species in a multispecies biofilm.

PNA FISH with Confocal laser scanning microscopy:

It is used to detect of biofilm-forming bacteria (*Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus sp. and Micrococcus sp*.) within human chronic skin wounds generated in a constant-depth film fermenter (CDFF), on human epithelium (ex vivo) and in skin biopsies from chronic leg wounds.

CONTROL OF BACTERIAL BIOFILMS:

Control using phages:

If the phage possesses polysaccharides-degrading enzymes, cell lysis is affected by the phage, the biofilm may be destroyed. Bacteriophage was used to eliminate *P.fluorescens*, it showed that phages were important in the deletion of biofilms in the early stage of development (upto 80% of biofilm removal). More recently, engineered enzymatic bacteriophage which had the ability to attack the bacterial cells in the biofilm and reduces the bacterial count. The first bacteriophage is capable of infecting *Pseudomonas sp*. by using a polysaccharide lyase enzyme that is capable of degrading a polysaccharide within biofilm.

Control Using nanoparticles:

Medical device infections can be repeated and costly depending on the device location and the duration of use. For example, peripheral or central intravenous catheters (CVCs) resulting in bloodstream infections (BSI). The use of nanoparticles is a growing new approach against biofilm-mediated, drug-resistant, and device centered infections. Nanoparticles, which consist of metals such as silver and metal oxides, may be promising agents for antibacterial applications. Nanoparticles bind to bacterial cell walls causing membrane destruction through direct interactions or through free radical production. (Erik Taylor *et al*.2011)

Control using antimicrobial peptides:

Antimicrobial peptides are produced by the innate immune response system and have been important for the development of novel types of antibiotics (Yang *et al*. 2002). Cathelicidins comprise one of the most important classes of antimicrobial peptides. Recent work indicated that SMAP-29, BMAP-28, and BMAP-27 considerably reduced biofilm formation by multidrug-resistant (MDR) *P. aeruginosa* strains isolated from patients with CF (cystic fibrosis), and killed bacteria within preformed biofilms (Pompilio *et al*. 2011).

Control using anti matrix agent:

These studies use the direct use of naturally occurring or engineered enzymes, use bacteriophages (phage therapy) as a vehicle of enzyme delivery and expression, or take advantage of metal chelators as a means to disrupt matrix integrity.

i) Enzymes:

N-acetyl-d-glucosamine-1-phosphate acetyltransferase (GlmU), which is involved in the biosynthesis of activated UDP-GlcNAc, an essential peptidoglycan and lipopolysaccharide (LPS) precursor in Gram-positive and Gram-negative pathogens, respectively, is among the enzymes targeted for matrix disruption. The enzymes DNase I and Dispersin B have also recently gained interest as potential antibiofilm agents, mainly against Gram-positive pathogens.

ii) Chelating Agents

Metal cations, such as calcium, magnesium, and iron have been concerned in maintaining matrix integrity (Raad *et al*. 2008).Chelating agents have been shown to destabilize biofilm architecture besides interfering with bacterial membrane stability. For example, sodium citrate inhibited biofilm formation by several *Staphylococci* species in vitro.

Control using anti-adhesion agents:

i) Mannosides, Pilicides and Curlicides:

Attachment is the first step in virtually all types of biofilm formation. In UPEC (Uropathogenic *Escherichia coli*), compounds have developed that interfere with the adhesive properties or assembly of type 1 pili, because they are important for UPEC adherence during biofilm formation in vitro and within the host.

ii) **Molecules isolated from plant extracts:**

Extracts from *Rubus ulmifolius* Schott., Rosaceae (Elmleaf blackberry), which are rich in the polyphenol ellagic acid, and glycosylated derivatives, have been shown to have an inhibitory effect on biofilm formation by a number of diverse *S. aureus* strains at concentrations well below those required to limit bacterial growth. Another plant derived natural product that has recently been identified as a potential inhibitor of biofilm formation by oral pathogens is the resin acid 4-epi-pimaric acid **(**Lee J-H *et al*;2011) which was isolated from *Aralia cachemirica* L.(Araliaceae).

CONCLUSION:

Biofilms are the communities of microorganisms which are attached to a surface. Biofilm is composed of microbial cells and extracellular polymeric substance (EPS).Extracellular polymeric matrix plays various roles in structure and function of different biofilm communities. Adhesion to the surface provides advantages such as protection against antimicrobial agents, nutrient availability and metabolic co-operability. Currently, investigation of suspected infections is accomplished through a combination of diagnostic testing methods, including blood tests, microscopy, histology, optical studies, and culturing. (Shalu Mengi *et al*.2013).The eradication of biofilms depends on prolonged, high-dose antibiotics therapy and almost requires the replacement of infected foreign body material. The use of nanoparticles is a growing new approach against biof ilm-mediated, drug-resistant, and device centered infections (Erik Taylor, 2011). Novel strategies, designed to block a specific biofilm step without killing the bacteria, such as the use of antiadhesion agents like mannosides, pilicides and curlicides, use of antimatrix agents such as enzymes and chelating agents.

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