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BIOFILM PRODUCTION AND ANTIBIOTIC RESISTANCE

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ABSTRACT

Biofilms are structured communities of microorganisms attached to a surface. Microorganisms such as bacteria, algae, fungi, protozoa may be found in a biofilm consortium attached to biological or non-biological surfaces. Their formation starts from initial attachment to their dispersal to other surfaces where they colonize and sometime cause infections especially on biological surfaces like living tissues and medical devices, which is of great importance to public health. Cells in the biofilm assemblage communicate through quorum sensing, affecting biofilm processes like cell detachment and exchange of genetic materials. Biofilms cause slow and persistent infections which interfere with antibiotic therapy. They are also a major concern in food industries, oil industries and aquaculture. To assay biofilms, methods like Tissue culture plate method, tube method and Congo red agar method can be used. Chemical, physical and biological methods are being used to control biofilms. A greater understanding of biofilm processes will lead to novel and effective strategies for biofilm control and a resulting improvement in patient management, enhancing the clinical decision-making process.

INTRODUCTION

Biofilms are formed when unicellular microorganisms come together to form a community that is attached to a solid surface and enclosed in an exopolysaccharide matrix (Donlan and Costerton, 2002). This matrix contains polysaccharides, proteins and DNA originating from microbes and the bacterial family can be made up of one or more species living together (Pamp *et al.,* 2007). Non-cellular materials like mineral crystals, corrosion particles, clay and silt particles, or blood components may also be found in the biofilm depending on the environment (Donlan, 2002).

Biofilms can also be described as dynamic heterogeneous communities that are constantly changing (Hall-Stoodley and Stoodley, 2009). At the most basic level, a biofilm can be described as bacteria embedded in a thick, slimy barrier of sugars and proteins. The biofilm barrier protects the microorganisms from threats external to its environment (Phillips *et al.*, 2010).

Bacteria have been found to exist in two principal forms, that is, as free-floating planktonic replicating cells and in biofilms (Costerton *et al*., 1999). Scientists have largely focused their attention on the solitary or planktonic forms of microorganisms. However, it is now generally accepted that microbial cells exist in biofilms with about 99% of all bacteria, only 1% existing in the planktonic state (Wilson, 2005). It has also been estimated that 65% of microbial infections are caused by biofilms (Mah and O'Toole, 2001).

Antonie Van Leeuwenhoek first displayed the bacteria scraped from his teeth plaque after viewing them under his simple microscope and described it in a report to the Royal Society of London. (Heukelekian and Heller,1940). Jones *et al.,* (1969) used scanning and transmission microscopy to examine biofilms on trickling filters in a waste water treatment plant and showed them to be composed of a variety of microorganisms. The first conceptual term was "aufwuchs" meaning growth in German (1975), and later other terms were used but were seen to be inappropriate. The group of Dr. Costerton in 1978 used the term "biofilms" as a more generic term for microorganisms adhering to wet surfaces in fresh water ecosystems.

The International Union of Pure and Applied Chemists, IUPAC, defines Biofilm as an "aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhere to each other and/or to a surface". The self-produced matrix of extracellular polymeric substance, also called slime, is a polymeric conglomeration generally composed of extracellular biopolymers in various structural forms (Vert *et al.*, 2012). Biofilms may be composed of a single bacterial species (e.g., *Vibrio cholerae*) (Teschler *et al.*, 2015), but more frequently, they are formed by a complex and diverse community of microorganisms (Wimpenny *et al.*, 2000).

Biofilm formation occurs in a series of events including initial cell-to-surface or cell-to-cell attachment, micro

colony formation, biofilm maturation and dispersal. This process has been considered advantageous in biofilm protection, nutrient availability, metabolic cooperation and the acquisition of new metabolic traits (Davey and O'Toole, 2000).

Within a biofilm, bacteria communicate with each other by the production of chemical signals or inducer molecules, a phenomenon called 'Quorum sensing'. Availability of key nutrients, chemotaxis towards the surface, motility of bacteria, presence of surface adhesions and surfactants are some of the key factors influencing biofilm formation (Hassan *et al.,* 2011). Biofilms are also sites where genetic materials are easily exchanged because of the proximity of the cells, thus, maintaining a large gene pool (Donlan, 2002).

Biofilms are greatly significant to public health, they exhibit decreased susceptibility to antimicrobial agents which may be intrinsic (natural outcome) or acquired due to transfer of extrachromosomal elements to susceptible microorganisms in the biofilm (Donlan, 2001). The emergence of drug-resistant bacteria and the difficulty in killing some bacteria led to a re-evaluation of the bacterial lifestyle and it is now acknowledged that biofilms endow bacteria with mechanisms to resist antibiotics. These mechanisms may be delayed penetration of the antimicrobial agents through the biofilm matrix, altered growth rate of biofilm organism, and other physiological changes due to the biofilm mode of growth (Donlan, 2002).

Biofilms can form on many medical devices such as contact lenses, intrauterine device, catheters, prosthetic valves, due to their high resistance level to antimicrobials (Licking, 1999).

Biofilm-producing microorganisms are far more resistant to antimicrobial agents than microorganisms which do not. In some extreme cases, the concentrations of antimicrobials required to achieve bactericidal effect against microorganisms can be three-to-four-fold higher than for planktonic forms depending on the species and drug combination (Dunne, 2002). They also resist phagocytosis in the immune system. It is becoming clear that biofilms have an enormous impact on public health and medicine especially in antibiotic resistance. To face this problem both at local and global levels, a better understanding of the sources and mechanisms that contribute to the emergence and spread of antibiotic resistance is required.

This review will help to understand the role of biofilms in infectious diseases, the different mechanisms of antibiotic resistance displayed by biofilms and the contribution of biofilms in the emergence and spread of antibiotic resistance.

BIOFILM FORMATION

Formation of biofilm is a developmental process that allows bacteria to undergo a regulated lifestyle, changing from a unicellular form to a multicellular form, where subsequent growth results in structured communities and cellular differentiation (Robert, 2010). Surface-bound and free-floating microorganisms are also called sessile and planktonic forms respectively. Sessile microorganisms can be attached to either abiotic (inert) materials such as those of implanted devices like catheters, prosthetic cardiac valves, intrauterine devices (Auler *et al.,* 2001) and biotic (living tissues or cells) surfaces, prevalent in natural, industrial and hospital settings (Lear and Lewis, 2012).

The ability of microorganisms to form biofilms is closely related to infectious diseases, environmental and biotechnological processes. The structural nature of the biofilms and the characteristics of the sessile cells produce resistance towards antimicrobial agents, leading to a protected environment against adverse conditions and the host defenses.

The biofilm state is more predominant than the free-living planktonic state. This may be due to several reasons. First, biofilms can withstand harsh environmental conditions like shear forces or being washed off by water or blood stream by simply attaching to surfaces. Second, the EPS matrix protects the bacteria against antimicrobial agents. This can be possible by delaying the antibiotics from reaching their targets. Third, biofilms restrict bacterial mobility thus increasing the chances of transfer of genetic materials (Rabin *et al.,* 2015).

Stages of biofilm formation

Biofilm formation takes place in a sequence of steps or distinct events. The stages of biofilm development are as follows: Initial attachment, irreversible attachment, maturation I, maturation II and dispersal.

Initial attachment

As the conditioning layer forms, an electric charge builds on the surface and it becomes increasingly attractive to bacteria carrying an opposite charge. These first species initially form a weak, reversible adhesion to the surface via van der Waals forces and hydrophobic effects (Briandet *et al.,* 2001; Takaha Shi *et al.,* 2010) and could be easily removed and killed by sanitizers and antibiotics. Flagella and type IV pili-mediated twitching motilities enable attached cells to aggregate and form micro colonies.

For human pathogens, microbial surface components recognizing adhesive matrix molecules dependent adhesions are covalently linked to the peptidoglycan on the cell wall (Otto, 2008). Non-covalent adhesions like those mediated by autolysins also assist in the initial attachment of biofilms (Heilmann *et al.,* 1997).

Irreversible attachment

The production of exopolysaccharide (EPS) matrix signifies the irreversible phase of bacteria attachment to a surface. The EPS matrix is produced by bacteria that are attached to a surface in quantities that are several folds more than by planktonic cells (Davies *et al.*, 1993).

Some species are not able to attach to a surface on their own but are instead able to anchor themselves to the matrix or directly to earlier colonists. It is during this colonization that the cells are able to communicate via quorum sensing (QS) using products such as N-acyl homoserine lactone (AHL) (Donlan, 2002). As the bacteria replicates, they become more firmly attached and differentiate, changing gene expression patterns in ways that promote survival (Donlan & Costerton, 2002; Flemming *et al.*, 2007). This occurs within 8-24 hours.

Maturation I

Once the first layer of the biofilm is established, colonization begins. The biofilm grows through a combination of cell division and recruitment. Cells spread outward and inward from the attachment point to form clusters (Hall-Stoodley, 2002). Typically, such interactions and growth within the developing biofilm form into a mushroom-like or flat structure depending on the nutrient source which allows the passage of nutrients to bacteria deep within the biofilm (Lewandowski, 2000). The exact composition of EPS varies according to the microorganisms present, but generally consists of polysaccharides, proteins, glycolipids and bacterial DNA (Flemming *et al.*, 2007; Hall-Stoodley & Stoodley, 2009). Bacterial DNA released by living or dead bacteria is thought to provide an important structural component for biofilm EPS matrix (Flemming *et al.*, 2007).

Maturation II

After an initial lag phase, a rapid increase in population is observed, known as the exponential growth phase. This depends on the nature of the environment physically and chemically. The rapid growth occurs at the expense of the surrounding nutrients from the bulk fluid and the substrate. The physical and chemical contribution to the initial attachment ends and the biological processes begin to dominate (Barnerjee *et al.*, 2015). Excretion of polysaccharide intercellular adhesion (PIA) polymers and presence of divalent cations interact to form stronger bonds between cells (Dunne, 2002). According to Mittelman (1996), the development of a mature biofilm may take from several hours to several weeks.

Dispersal

It enables biofilms to spread and colonize new surfaces. According to Kaplan *et al.*, (2003), enzymes like dispersin B and deoxyribonuclease may play a role in biofilm dispersal. The mature biofilm sheds planktonic bacteria, micro colonies and fragments of biofilm continuously which can disperse and attach to other parts to form new colonies. Nitric oxide has been shown to trigger the dispersal of biofilms of special bacteria species at sub-toxic concentrations, so it can be used in treating patients that suffer from chronic infections caused by biofilms (Barraud *et al.*, 2006; Barraud *et al.*, 2009).

Biofilm disperse due to numerous factors such as depletion of nutrients, intense competition, overcrowding, antibiotic activity, etc. Dispersal of biofilm cells can be by shedding of daughter cells/sloughing or detachment as a result of nutrient levels or quorum sensing, or shearing of biofilm aggregates because of flow effects (Rodney, 2002).

Sloughing is more common in thicker biofilms that have developed in rich nutrient environments (Characklis, 1990) while detachment is assumed to be species specific (Korber *et al.*, 1995). Dispersal provides a mechanism for cells to migrate from heavily colonized areas that have been depleted of surface absorbed nutrients to areas more supportive of growth. Detachment can be an active process, a passively induced mechanical process or a chemical process (Hall-Stoodley *et al*., 2004).

Properties of a biofilm matrix

Biofilms can be composed of a single group of bacteria or multiple microorganisms with specialized metabolic activities. Under favourable situations, a biofilm can grow to the extent of being visible. The structure within a biofilm depends on the different species of microorganisms present (Nadell *et al.*, 2009).

Extracellular matrix

The biofilm is supported by a matrix of polymeric compounds secreted by bacteria into the environment; they are called extracellular polymeric substances (EPS). EPS are usually composed of exopolysaccharide, proteins and nucleic acids (Flemming *et al., 2007).* The EPS enclose cells within them and allow communication among them through biochemical signals as well as exchange of genetic materials (Molin and Toller-Neilsen, 2003). The EPS matrix is an external digestion system that allows for stable synergetic micro consortia of different species. It traps extracellular enzymes and keeps them close to the cells (Flemming and Wingender, 2010). Channels in the biofilm allow the distribution of water, nutrients, air and signaling molecules to all parts of the structure (Stoodley *et al.*, 1994 and Zhang *et al.*, 1998).

Exopolysaccharide found in the EPS are synthesized extracellularly or intracellularly and secreted to the outside environment (Nwodo *et al.*, 2012). In electron microscopy, they look like linear or long branched strands that are attached to cell surfaces stretching to form large networks. Exopolysaccharide act as platform for the binding of proteins, lipids, carbohydrates and nucleic acids (Rabin *et al.*, 2015).

Extracellular proteins are attached to cell surface and polysaccharides to help with biofilm formation and stabilization. An example is Glucan binding proteins

(Gbps) in *Streptococcus mutans* biofilms which helps in linking bacteria and exopolysaccharide (Lynch *et al.*, 2007). Amyloids, biofilm associated protein (bap) family and other enzymes involved in the degradation process in the biofilm are examples of extracellular proteins (Rabin *et al.*, 2015).

Extracellular DNAs (eDNAs) considered as leftovers from lyzed cells are actively secreted from cells (Hamilton *et al.*, 2005), they prevent biofilm formation in *Pseudomonas aeruginosa* (Whitchurch *et al.*, 2002). Its negative charge act as a repulse in initial attachment of biofilm, but when the cell and surface are very close, eDNA facilitates adhesion (Das *et al.*, 2010). They also coordinate twitching in *P. aeruginosa* biofilm expansion (Gloag *et al.*, 2013).

Biofilm habitat and importance

In many natural or artificial habitats, microorganisms attach themselves to surfaces, either abiotic or biotic to form a complex matrix of biopolymers called biofilm to protect them from environmental hazards (Costerton *et al.*, 1978). Biofilms form on virtually every non-shedding surface in a non-sterile aqueous or very humid environment. They can be found in aquatic habitats, streambed cobbles, sand and on floating macro and micro-aggregates (Simon *et al*., 2002) even in extreme environments ranging from very hot briny waters of hot spring (Reysenbach and Cady, 2001) to frozen glaciers. In human environments, biofilms grow in showers, water and sewage pipes, causing clogging and corrosion. When found on floors and counters, they make sanitation difficult in food preparation areas and when in soil, they cause bioclogging. In cooling or heating-water systems, biofilms cause a reduced heat transfer (Characklis, *et al.*, 1981). In marine engineering systems like pipelines of the offshore oil and gas industry (Schwermer et al., 2008) biofilms can lead to about 20% of corrosion.

Biofilms can be found in plants, they contribute to diseases in plants e.g. Citrus canker, bacterial spot in pepper and tomatoes, and they can also exist together with plants as in the case of nitrogen fixing Rhizobium on plant roots (Anderson *et al.*, 2007). Biofilms contribute to the decomposition of organic matter, biogeochemical cycling and nutrient dynamics as a key factor in ecosystem functioning (Romani, 2010). In bioremediation, biofilms help to eliminate petroleum oil from contaminated oceans or aquatic systems. The oil is eliminated by hydrocarbon-degrading activities of biofilms. Of utmost importance is the hydrocarbonoclastic bacteria (HCB) (Anderson *et al*., 2007). Biofilms are used in microbial fuel cells (MFCs) to generate electricity from different materials including complex organic waste and renewable biomass (Chua *et al.*, 2014).

Biofilms in human systems cause tooth decay as dental plaque when found on the teeth and gum disease. Biofilms can also be found in the gut, large intestine and even the appendix (Bollinger *et al.*, 2007).

Biofilm Production and antibiotic resistance

Biofilms greatly enhance survival and resistance of microorganisms embedded in the matrix to environmental and chemical stressors (e.g. Antibiotics) mainly, but not only by the protection conferred by the extracellular polysaccharide matrix (Mah and O'Toole, 2001; Stewart and Costerton, 2001; Donlan, 2002; Donlan and Costerton, 2002; Stewart, 2002; Hall-Stoodley *et al.*, 2004; Hoiby *et al.*, 2010). This resistance confers on microorganisms the ability to survive even those factors that would easily kill these same microorganisms when growing in their free living forms (Flemming *et al*., 2007). Cells in the sessile state have been found to be more resistant to antibiotics 10-1000 times than their planktonic forms and possibly 150-3000 times more resistant to disinfection (Mah and O'Toole, 2001; Patel, 2005).

Traditionally, antibiotic resistance of planktonic bacteria involves inactivation of antibiotics, inhibition of targets and antibiotic exclusion (Patel, 2005), which require the possession of specific genetic factors such as genes for β-lactamase or efflux pumps.

The mechanisms involved in altering the sensitivity of antibiotics in biofilms can be separated into intrinsic (innate) and extrinsic (induced) resistance factors.

Intrinsic or innate resistance factors

These factors are activated as part of the biofilm developmental pathway which is important to the biofilm physiology and structure resulting from conversion to a biofilm lifestyle (Costerton *et al.*, 1999).

Failure of antimicrobial agent to penetrate the barrier

To prevent or delay antibiotics from reaching their targets, biofilms can act as a diffusion barrier. Antibiotics have been shown to penetrate structures through a thick mixture of exopolysaccharide, DNA and protein to reach their targets (Donlan and Costerton, 2002; Anderson and O'Toole, 2008). The diffusion barrier is also effective against smaller antimicrobial peptides, numerous defensins and analogs (Lewis, 2001). Antibiotics have been shown to easily penetrate biofilms in some cases, but poorly in others depending on the antibiotics and biofilms. The antimicrobial activity of antibiotics will resume when the biofilm matrix is saturated with antibiotic molecules. The time needed for penetration of antibiotic treatment and the replenishment of biofilm matrix goes at a slower rate than the diffusion of antibiotic molecules (Fenggiun *et al*., 2013). The exopolysaccharide matrix retards diffusion by chemically reacting with the antimicrobial molecules or by limiting their rate of transport. Hoyle *et al.*, (1992) showed that the EPS of *Pseudomonas aeruginosa* was capable of binding tobramycin and that dispersed cells were 15

times more susceptible to this agent than cells in biofilms. Steward and coworkers investigated the penetration limitation of ampicilin and ciprofloxacin on *Klebsiella pneumoniae* and found that ciprofloxacin has a better penetration capability than ampicilin. As a result, biofilm cells could tolerate concentrated ampicilin but their resistance to ciprofloxacin is poor (Anderl *et al.*, 2000).

Establishment of microenvironments within biofilm

Limited supply of oxygen and nutrients in the biofilm especially for those cells deeply seated causes a slow metabolic rate as well as growth and division rate. That is, oxygen concentration may be high at the surface, but low in the middle of the biofilm where anaerobic conditions may be present (de Bear *et al*., 1994; Wenner *et al.*, 2004). Reduced growth rate minimizes the rate of antimicrobial intake by biofilm cells. DuGuild *et al*., (1992), found that increased growth rate resulted in increased susceptibility of *Staphylococcus epidermidis* biofilm. Increase in acidic waste products can lead to pH differences more than 1 between the bulk fluid and biofilm interior (Zhang and Bishop, 1996) causing antibiotic resistance. Aminoglycosides are more active against microorganisms in aerobic conditions than in anaerobic conditions. The β-lactams are active against dividing cells, so when used on *E. coli* biofilms, their bacteriolytic activity is reduced (Ashby *et al*., 1994).

Differentiation into persister cells

There is a subpopulation of biofilm cells called persister cells, whose growth rate is zero or very slow (Keren *et al*., 2004). Most antibiotics target actively growing cells or dividing cells, thus, not effective for persister cells which are sometimes dormant. These cells may act as disease reservoirs, reactivating into infectious particles once the antibiotics are removed (Lewis, 2001). Bacteriostatic antibiotics contribute to the growth of persister cell and biofilm preservation by inhibiting growth of sensitive cells and reshaping of biofilm into original form when the antibiotic therapy is withdrawn (Mendoza, 2004).

Increased production of oxidative Stress

Different stresses like nutrient unavailability, low oxygen, ethanol, high osmolarity and sub-inhibitory antibiotic concentrations can change the cellular functions related to the oxidative metabolism (Arce *et al*., 2011), thereby, stimulating production of reactive oxygen species (ROS) and highly reactive hydroxyl radicals (HO) generated by the presence of hydrogen peroxide (H_2O_2) and iron (Fenton reaction) by Hc superoxide anion (O_2^-) or by the superoxide anion, Hydrogen peroxide and a metal catalyst (Haber-Weiss reaction) (Aiassa *et al.*, 2011 and Paez *et al.*, 2011).

Oxidative stress is due to an imbalance between the production of oxidants like free radicals, peroxide and nitric oxide with the levels of antioxidant defenses (Becerra *et al.*, 2006). Increased production of oxidative stress causes physiological changes in the bacteria with phenotypic alterations and it has been observed that biofilm production is influenced by the balance of oxidants production (ROS and NO) and levels of antioxidant defenses. Oxidative stress is considered to cause mutations in biofilms. Findings show that micro colonies due to endogenous oxidative stress are sites within biofilms where enhanced genetic adaptation and evolution take place (Mai-Prochnow *et al*., 2008 and Conibear *et al*., 2009) and thus, promotes antibiotic resistance. Addition of antioxidants reduces the diversity in biofilms (Boles and Singh, 2008).

Antagonist action of antibiotics

Bacteria in biofilm communicate by quorum sensing. This is done by synthesizing and reacting on signal molecules to sense when a concentration of bacteria is present in a limited space in the environment, presence of microenvironment in biofilm can antagonize the action of biofilm and the degradation process (Jensen *et al*., 2007; Kolpen *et al*., 2010). Quorum sensing molecules in Gram-negative bacteria are N-acyl-l-homoserine lactones, in Gram-positive bacteria, the molecules are small peptides. Quorum sensing can regulate production of virulence factors important for the pathogenesis of infections, where the bacteria acts as a protective shield against phagocytes, it may also influence the development of the biofilm and cause tolerance to antibiotic therapy and innate inflammatory response (Bjarnsholt *et al*., 2005; Alhede *et al.*, 2009 and Van *et al.*, 2009).

Extrinsic or induced resistance factors to biofilms

Extrinsic or induced factors result from transcriptional induction by treatment with antibiotics. Mutation of biofilm bacteria is significantly increasing compared to the planktonic forms.

Horizontal Gene Transfer

Some bacteria can become antibiotic resistant due to gene mutations, others have plasmids with antibiotic resistant genes and these plasmids can be easily transferred to cells by horizontal gene transfer. Studies on *Staphylococcus aureus* biofilms showed that biofilms promote the spread of plasmid-borne antibiotic resistance genes by conjugation (Savage *et al*., 2013).

Efflux Pumps

Multidrug efflux pumps mediate antibiotic efflux to contribute to antibiotic resistance in planktonic cells. Efflux pumps allow bacteria cells to pump intracellular toxins with antibiotic drugs out. Some efflux pump genes are upregulated in biofilm indicating that they contribute to antibiotic resistance. A novel *Pseudomonas aeruginosa* efflux pump gene PA1874-1877 recognized by Zang and coworkers, which is expressed more in biofilm-growing cells than its planktonic form, has been shown to contribute to the biofilm specific antibiotic resistance of *P. aeruginosa* (Zhang and Mah, 2008). Its deletion leads to an increased sensitivity to tobramycin, gentamycin and ciprofloxacin (Zhang and Mah, 2008).

Neutralization by enzymes

Neutralizing enzymes degrade or inactivate antibiotics; they are proteins that cause resistance by hydrolysis and modification of antimicrobials. Neutralization by enzymes is enhanced by slow penetration of antibiotics and also antibiotics degradation in biofilm (Jamal *et al.*, 2015).

In biofilms, β-lactamase has been considered to come from the layer of lysed bacteria that has been exposed and is thought to release defensive enzymes into the extracellular space. Piperacillin and Impinem caused an overproduction of β-lactamases in *P. aeruginosa* biofilms which hydrolyzed β-lactam antibiotics before reaching the bacterial cells. Extracellular β-lactamase may inactivate the antibiotic as it penetrates, thereby, protecting the deep-seated cells in the biofilm (Costerton *et al*., 1999 and Costerton *et al*., 2003).

Can antibiotics stimulate biofilm formation?

When antibiotics are administered at concentration below the minimum inhibitory concentration, they induce biofilm formation in different bacterial species (Strelkova *et al.*, 2012). Cells seated deep in the biofilm may be exposed to sub-MIC level of antibiotics, thus promoting biofilm formation instead of inhibiting it (Lewis, 2005). The extensive use and misuse of antibiotics in agriculture, livestock and aquaculture may further expose bacteria to low levels of the drugs (Martinez, 2008).

Biofilms and Diseases

Biofilms play an important role in causing infections both for specific conditions like cystic fibrosis and in bloodstream and urinary tract infections due to the presence of indwelling medical devices. According to the National Institute of Health, about 80% of all infections are biofilm related. Ongoing research reveals an important role of bacterial biofilms in chronic or recurrent infections including those which are not responsive to a culture-appropriate antibiotic therapy (Costerton *et al.*, 2003). Biofilms do not only cause chronic infections but also inflammation and tissue damage.

Biofilms have been implicated in infections like periodonitis (Kolenbrander and Palmer, 2004) caused by *Porphyromonas gingivalis* which colonizes the oral cavity to invade the mucosal cells and release toxins; endocarditis (Hoiby *et al*., 1986), caused by the bacterial biofilms on prosthetic valves in patients that have undergone heart valve replacement; lung infection in patients with cystic fibrosis (CF) causing chronic pneumonia by *P. aeruginosa* (Wenner *et al*., 2004), child middle-ear infection (Romero *et al*., 2008), chronic osteomyelitis (Del and Patel, 2004), urinary tract infections (Skandamis *et al*., 2009) as a result of biofilm formation on urinary catheters; formation of dental plague (Rogers, 2008), gingivitis, coating contact lenses (Imamura *et al*., 2008) causing Keratitis, chronic wounds (James *et al*., 2008) and infections of permanent dwelling medical devices such as joint prostheses (Lewis, 2009).

Biofilm formation could be critical in immunocompromised persons whose immune system cannot fight against these invading pathogens. About 60-70% of nosocomial or hospital acquired infections are associated with the implantation of biomedical devices (Bryers, 2008). If an infection develops a biofilm, it becomes even harder to treat as the bacteria change and become more resistant to the body's host defenses and antibiotics (Lewis, 2001).

The exact processes by which biofilm-associated microorganisms cause disease in the host are not properly defined. Donlan and Costerton, (2002) suggest the following mechanisms:

- Detachment of cells or cells aggregate from indwelling medical device biofilms resulting in bloodstream or urinary tract infections.
- Production of endotoxins.
- Resistance to the host immune system
- Provision of a niche for the generation of resistant microorganisms.

The following are some of the diseases implicated by biofilms:

Dental plaque

Reports have revealed that more than 700 species of bacteria and archeae exist in dental plaque (Marsh, 2006 and Zaura *et al*., 2009). Dental plaque is an oral biofilm that adheres to the teeth and is made up of different species found in salivary polymers and microbial extracellular products (Augustin *et al*., 2010). The biofilm on the teeth surface is subjected to oxidative and acid stress (Marquis, 1995 and Lemos *et al.*, 2005). Acid is a major cause of tooth decay and innocuous bacteria species in healthy biofilms like *Streptococcus sanguis, S. gordonii, S. oralis, Actinomyces species*, often show low acid tolerance (Marquis, 1995). On the other hand, dental caries bacteria like *S. mutans* and *S. sobrinus* can tolerate high acid levels; they can form biofilms that cause oral cavity diseases like dental caries, periodonitis, gingivitis, etc. (Sbordone and Bortolaia, 2003). When the biofilm, containing *S. mutans* and related oral streptococci is subjected to acid stress, the competence regulon is induced, leading to acid resistance (Lemos *et al*., 2005). Dental caries can be prevented by preventing dental plaque from maturing through reducing the intake of sugar or fermentable carbohydrates and regular tooth brushing and flossing (Fejerskov, 2015).

Wounds

Biofilms are found commonly in chronic wounds, they are suspected to delay wound healing (James *et al*., 2008), compared with an acute wound which may not be biofilm-related. Many wound sloughs have been misinterpreted to be biofilms, thus, wound slough has

been distinguished from biofilm to be viscous, yellow and a relatively opaque layer on wound beds, while biofilm is suggested to appear more gel-like and shiny (Hurlow and Bowler, 2009). Usually, biofilms form on the outer layer of wounds although some are embedded in the deep layer of wounds such as *P. aeruginosa* biofilms and are difficult to differentiate using conventional wound swab culture (Hall *et al.,* 2014). Currently, the most reliable method to confirm the presence of microbial biofilm is by using specialized microscopy (Philips *et al.,* 2010).

Chronic fibrosis (CF)

It is a chronic disease of the lower respiratory system and can be inherited. It is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTCR) gene that results in dysfunctional electrolyte secretion and absorption (May *et al*., 1991). Decreased secretion and increased absorption of electrolyte lead to dehydration and thickening of secretions over the respiratory epithelium, destroying the epithelium and ultimately causing respiratory failure (Koch and Hoiby, 1993).

Pulmonary colonization of the lower respiratory tract of CF patients starts at early childhood, mostly by *S. aureus* and *Haemophilus influenzae*. However, by early adulthood, most CF patients have become colonized with *P. aeruginosa* (Lyczak *et al*., 2002 and Koch and Hoiby, 1993). Medical devices can also be contaminated with *P. aeruginosa*. Cases of nosocomial *P. aeruginosa* infections have been reported (Pedersen *et al*., 1986, Jones *et al*., 2001), but many antibiotics available are not good enough for treating recalcitrant *P. aeruginosa* (Romling *et al.,* 1994). Patients with *P. areuginosa* infections are often relieved of the symptoms with antibiotic treatment, but not necessarily cured. Successful treatment may depend on early therapy to prevent or delay chronic infection (Costerton *et al.,* 1999).

Urinary Tract Infection (UTI)

The presence of materials in the urinary tract increases the chance of bacterial biofilm formation leading to UTI. (Tenke *et al*., 2006). Urinary catheters are tubular, latex or silicon devices that are inserted through the urethra into the bladder to measure urine output, collect urine during surgery, prevent urinary retention or control urinary incontinence (Kaye and Hessen, 1994).

Initial colonization of catheters can be by single species of *S. epidermidis, Enterococcus faecalis, E. coli* or *Proteus mirabilis*. As the catheter remains in place, the number and diversity of microorganisms' increase and mixed communities develop containing microorganisms like *Klebsiella pneumoniae, P. aeruginosa, Providencia stuarti* and *Proteus mirabilis* (Stickler, 1996).

Cardiac valve infection

Biofilm on mechanical cardiac valve causes prosthetic valve endocarditis. The species involved are *S. epidermidis, S. aureus, Corynebacterium spp*. and *Candida spp.* (Khardori and Yassien, 1995). Accumulated biofilm can block the artificial cardiac valve. Detached biofilm cells migrate along with the blood stream to cause infections in other organs (Rabin *et al.,* 2015). The biofilm is usually treated by prolonged administration of intravenous antibiotics or surgical excision of infected valve (Parsek, *et al.,* 2003).

Biofilms in food industry

Biofilms are a major concern in food industries; they form on plants during industrial processes (Srey *et al.,* 2013) and can be gotten from water, animal or soil. The buildup of biofilms can affect the heat flow across a surface and increase surface corrosion and friction resistance of fluids. These can lead to energy loss and product loss (Kumar and Anad, 1998). Biofilm formation has caused economic problems as well as posed health risk to consumers due to the ability to make the food more resistant to disinfectants (Srey *et al.,* 2013). During food production, microorganisms attach to surfaces and develop internally in the products. In the washing process, biofilm resist sanitation causing it to spread (Srey *et al.,* 2013). This is normally found in ready to eat foods because they go through limited sanitization. Adhered microorganisms, microorganisms in biofilms or microorganisms in crevices may escape cleaning and disinfecting procedures and become sources of contaminating food products during processing, thus, a major part of the requirement of a good manufacturing plant is to ensure that microbial biofilms are removed effectively (Marriott and Givanni, 2006). It is recommended that disinfectants should be used in order to avoid cross contamination from contaminated produce to clean produce (Keskinen *et al.,* 2009).Dairy products are susceptible to biofilm contamination due to their perishability and limitations in cleaning procedure (Jessen and Lammert, 2003). In 2011, about 20 pounds of raw milk distributed in Washington were recalled due to contamination with *Listeria monocytogenes* (Srey *et al.,* 2013).

Salmonella is a major cause of food borne disease. Salmonella contamination in large quantities can be found in poultry processing industry, especially when the poultry products are not properly cleaned and cooked (Srey *et al.,* 2013). In seafood industry, salmonella biofilms form from seafood borne pathogens on the seafood itself (Rajkowski, 2009). New cleaning procedures are being tested to reduce biofilm formation and lead to safer and more productive food processing processes (Kumar and Anad, 1998).

Biofilms in Aquaculture

Biofouling species tend to block nets and cages and ultimately populate shellfish and algae forms for space and food (Braithwaite and McEvoy, 2004). In marine

environments, biofilms could reduce the hydrodynamic efficiency of ships and propellers, leading to pipeline blockage and sensor malfunction, thus, increasing the weight of appliances deployed in seawater (Qian *et al.*, 2007). Biofilms can be a reservoir for potentially pathogenic bacteria in fresh water (Cai *et al*., 2013).

Methods of Detecting Biofilm Production

A study by Donlan (2001) shows that, Gram-positive and Gram-negative bacteria are able to form biofilms, some of which include *Enterococcus faecalis, Staphylococcus epidermidis, Staph. aureus, Streptococcus viridans, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis and Pseudomonas aeruginosa*, among others.

A standard method for the study of biofilm susceptibility is not available though several methods can be used with each one having its own advantages and disadvantages. Thus, it is difficult to compare results obtained with biofilms of even the same species, when cultured and assayed under different conditions. The different methods to detect biofilm production include: Tissue culture plate (TCP) (Christensen *et al*., 1995), Tube method (Christensen *et al.,* 1982), Congo Red Agar (CRA) method (Freeman *et al*., 1989), Bioluminescent assay (Donlan *et al.,* 2001), Piezoelectric sensors (Aparna and Yardav, 2008) and fluorescent microscopic examination (Zufferey *et al*., 1988).

Invasive devices like urinary catheter tips, endotracheal tube aspirates, and intravenous catheter tips are collected. Isolates are cultured on blood or Mac Conkey agar and are identified by standard microbiological procedures like Gram staining, colonial morphology, catalase test, oxidase test, motility and biochemical tests (Bailey and Scott, 2007). Antimicrobial susceptibility testing is carried out by Kirby Bauer Disc diffusion method on Mueller Hinton agar (Myer and Koshi, 2001). The microorganisms identified are subject to at least 3 different tests to detect their ability to produce biofilms.

Tissue Culture Plate (TCP)

This quantitative test is considered as the gold standard method for biofilm detection (Mathur *et al.,* 2006). Isolates from fresh agar plate are inoculated on 10ml of Trypticase Soy broth with 1% glucose and incubated for 24 hours at 37°C. The cultures are then diluted 1:100 with fresh medium. Individual wells of sterile 96 well, flat bottom polystyrene tissue culture treated plates are filled with 0.2ml aliquots of diluted cultures and broth served as control to check sterility and non-specific binding of media.

The tissue culture plates are incubated at 37°C for 24 hours after which, the contents of each well are removed by gentle tapping. The wells are washed four times with phosphate buffered saline (0.2ml, pH 7.2) to remove free floating bacteria. Biofilms formed by adherent microorganisms in plates are fixed with 2% sodium acetate and stained with 0.1% safranine or crystal violet. Excess stain is rinsed off by thorough washing with deionized water and plates are air dried. Optical density (OD) of stained adherent bacteria is determined with a micro ELISA auto reader at wavelength of 570nm. Interpretation of result or biofilm production is done according to the criteria of Stephanovic *et al*., (2007).

Tube Method

It is a qualitative method for biofilm detection. A loopful of test microorganisms is inoculated in 10ml of Trypticase soy broth with 1% glucose in test tubes. The tubes are incubated at 37°C for 24 hours, then decanted and washed with phosphate buffered saline (pH 7.3) and air dried. Tubes are stained with 0.1% safranine or crystal violet, excess stain is washed with deionized water. Tubes are dried by inverting them and observed for biofilm formation. Biofilm formation is positive when a visible film lines the walls and bottom of the tube. Ring formation at the liquid interface indicates a negative result. Tubes are examined and results scored visually as 0- Absent, 1-weak, 2-moderate, 3-strong**.** (Hassan *et al*., 2011, Venkata *et al*., 2016).

Congo Red Agar (CRA) Method

This method is based on the cultural morphology of biofilm-forming bacteria on Congo-Red agar medium (Hassan *et al*., 2011). CRA medium is prepared with brain heart infusion broth 37g/l, sucrose 50g/l, agar 10g/l, and Congo red indicator 8g/l. Congo red stain is prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 minutes separately from the other media constituents. It is then supplemented to be autoclaved brain heart infusion agar with sucrose, when its temperature reaches 55°C (Reid, 1999). Plates are inoculated with test microorganisms and incubated overnight at 37°C.

Black colonies with a dry crystalline consistency indicates strong biofilm production, weak biofilm producers show red colonies with occasional darkening at the centre and non-biofilm producers remain pink to red.

Biofilm control

Industrially, attempts to control biofilm formation by manipulating the metallurgy and surface characteristics of pipes and vessels have failed, thus, we can expect an equal lack of success in medical devices. Mechanical cleaning and oxidative biocide are used industrially; the former removes biofilms, while the later dissolves the biofilm matrix material gradually and eventually kills the sessile cells (Donlan and Costerton, 2002). The only specific antibiofilm therapy presently in use is based on the incorporation of antibiotics into the material of indwelling catheters (Rand and Hanna, 1999; Spencer, 1999 and Woo *et al.,* 2000).

Strategies used for biofilm control will either

- Prevent initial device contamination
- Minimize initial microbial cell attachment to device

- Penetrate the biofilm matrix and kill the biofilm-associated cells.
- Remove the device (Donlan and Costerton, 2002)
-

Combination of Rifampin and Monocycline has proven to be effective. It is limited to relatively short-term catheters and not for artificial joints or heart valves nor for biofilm infections not related to indwelling devices. This approach decreases the probability of colonization and acts as a prophylactic measure (Lewis, 2001).

The genes responsible for persistence can be identified and these may serve as targets for drug discovery. Any inhibitor of a factor that causes persistence could then be combined with a conventional antibiotic such as Fluoroquinolone to eradicate biofilm. Such dual therapy is similar to the currently used β-lactam-β-lactamase inhibition combinations or MDR inhibitor-antibiotic combination (Markham *et al.*,1999; Renau *et al.*, 1999 and Stermitz *et al.*,2000).

Other approaches to biofilm eradication are discussed below:

Use of pilicides
Extracellular fibers of bacterial cells that allow binding and colonization of epithelial cells are called pili (Dodson *et al.*, 2001) and they are assembled through the chaperone-usher pathway (Surette and Bassler, 1998). Blocking this mechanism will help in biofilm eradication. The use of small synthetic compounds called plicides to inhibit the synthesis of pili has been developed (Aberg *et al.*, 2007).

Use of enzymes
Enzymes can be used efficiently to degrade biofilm. Biofilm consists of EPS and these enzymes have the potential to degrade EPS. When the biofilm is degraded by enzymes, the result is a release of the biofilm components and the planktonic cells. This makes it easy for the immune system to clear it (Xavier *et al.*, 2005).

Inhibition of Quorum sensing
Blocking the quorum sensing system by using analogs can inhibit the transfer of genetic materials which confers antibiotic resistance to other non-resistant cells in the biofilm, thus, making antibiotics effective against the biofilm cells (Bjarnsholt and Givskov, 2008).

Use of Bacteriophages
Bacteriophages have the ability to inhibit or reduce biofilm formation in-vivo (Vinodkmur *et al.*, 2008). Using genetically engineered lytic phage with biofilm degrading enzyme has shown more efficient eradication of biofilm than wild type phages (Lu and Collins, 2007). Similarly, combination of multiple phages can also be efficient for biofilm eradication (Jamal *et al*., 2015).

Surface coating
Coating indwelling devices like endotracheal tubes and catheters with metals that do not support the growth of biofilms, antiseptics or antimicrobials has proven effective in eradicating or blocking biofilm growth. (O'Grady *et al.*, 2002).

Use of Electrical currents
The application of a low intensity electric current has shown a substantial reduction in the number of viable bacteria biofilms. Electric currents with electromagnetic fields and ultrasound have shown enhanced results on biofilm eradication in studies conducted in-vivo and in-vitro (Caubet *et al.*, 2004).

DISCUSSION

Bacterial cells have grown in the biofilm phenotype for billions of years, as a part of their successful strategy to colonize most of this planet and most of its life forms. We have only recognized this distinct phenotype as the predominant mode of bacterial growth for the last 2 decades. *P. aeruginosa* infections present a global medical challenge as opportunistic pathogens which are successful at colonizing and persisting in the hospital environment. A considerable percentage of patients are at risk of being infected with isolates capable of producing biofilm. This will unnecessarily increase the hospital load and amount of time and money spent by the patients. They are able to resist desiccation and survive on inanimate surfaces for years.(Dijkshoorn *et al*., 2007; Navon-Venezia *et al*.,2005).

Characteristically, gradients of nutrients and oxygen exist from the top to the bottom of biofilms and these gradients are associated with decreased bacterial metabolic activity and increased doubling times of the bacterial cells. It is these more or less dormant cells that are responsible for some of the tolerance to antibiotics. Biofilm growth is associated with an increased level of mutations as well as with quorum-sensing-regulated mechanisms. Conventional resistance mechanisms such as chromosomal beta-lactamase, upregulated efflux pumps, and mutations in antibiotic target molecules in bacteria also contribute to the survival of biofilms. Biofilms can be prevented by early aggressive antibiotic prophylaxis or therapy and they can be treated by chronic suppressive therapy (Hoiby et al.,2010). A promising strategy may be the use of enzymes that can dissolve the biofilm matrix (e.g., DNase, F-actin, and alginate lyase) as well as quorum-sensing inhibitors that increase biofilm susceptibility to antibiotics(Hoiby et al.,2010).

The discovery of surface-attached bacteria happened over 70 years ago (Zobell *et al*.,1935). However, we are still trying to understand the significance of biofilm communities. Interestingly, to understand bacteria as a community takes us away from our traditional view of microbiology. The major challenge is to understand intercellular communications that promote stability in biofilms and usage of models that can mimic natural

communities in the laboratory. However, there is some success in this area such as development of model to study catheter- induced bladder infections(Stickler *et al*.,1993). The discovery of confocal scanning laser microscopes (CSLM) has further helped to examine the three-dimensional structure and function of biofilms. However, application of modern techniques with the collaborative efforts from scientists from various fields will help to better understand this continuous evolving dynamic world of biofilms.

It is believed that biofilm acts as a mechanism for bacteria to get a better survival, especially in cases of when resistance level is not high enough. While the mechanisms that govern this process are not clear yet, expression of the β-lactamase gene bla_{TEM-1} is known to inhibit biofilm formation of *P. aeruginosa* by perturbing cell adhesion, thereby establishing a genetic link between biofilm production and antimicrobial resistance (Gallant *et al*., 2005). The presence of plasmids was also known to be associated with both antibiotic resistance and biofilm formation. They could enhance the ability of transferring resistance markers by transformation or conjugation (*Sherley et al., 2004*). Meanwhile, genes encode the protein of flagella and fimbriae are also located in plasmids. These two structures could facilitate biofilm formation (*Karch et al., 1987).* In this study, we found out that isolates with higher level of resistance always harbored more plasmids. But no obvious difference in biofilm formation was observed among strains with different plasmid profiles. Further analyses including detailed plasmid map are needed to figure out the influence of plasmid on the relationship between these two capacities. Explorations of beta-lactamase activity in different conditions and fine genetic links between biofilm and antibiotic resistance other than bla_{TEM-1} are required to fully elucidate the mechanisms involved in these processes. A large number of indwelling medical devices or other devices used in the health care have been shown to contain biofilms, resulting in measurable rates of device associated infections (Fletcher & Leob, 1979).

In a study by Hassan *et al.,* (2011), it was reported that the majority of biofilm producing bacteria was from urinary catheter tips (26.3% of 110 isolates). Similarly, Donlan (2001) reported in his study the association of biofilm producing bacteria with urinary catheters. The longer a catheter remains in place, the more chances of biofilm.

A considerable number of patients are at risk of being infected with isolates capable of producing biofilm, thereby, increasing the hospital load and amount of time and money spent by the patients. Biofilm growth is associated with an increased level of mutation as well as quorum-sensing regulated mechanisms. Mechanisms like delayed penetration of the antimicrobial agents, altered growth rates of biofilm microorganisms and physiological changes due to the biofilm mode of growth and conventional resistance mechanisms such as upregulated efflux pumps and mutations in antibiotic target molecules in bacteria also contributes to the survival of biofilms.Some characteristics of biofilms that can help in disease formation process include detachment or dispersal of cells, exchange of resistance plasmids between cells in biofilm, reduced susceptibility of cells to antimicrobials and resistance of biofilms to host immune system.

CONCLUSION

Bacterial cells have grown in biofilm phenotype for billions of years as a part of their successful strategy to colonize most of the earth and its life forms, making it difficult to control. Thus, the field of microbiology has come to accept the universality of biofilm phenotype.

Biofilm producing bacteria are responsible for many recalcitrant infections and are notoriously difficult to eradicate. Some methods of resistance appear to be intrinsic to biofilm growth or extrinsic in nature. Due to the heterogeneous nature of biofilms, it is likely that multiple mechanisms of antimicrobial resistance are useful to explain biofilm survival with antibiotic resistance being the result of an intricate mixture of extrinsic and intrinsic factors. Wise usage of indwelling devices in patients and their timely replacement will help to prevent the formation of biofilms. Although methods to detect biofilm-growing bacteria have been developed which have helped to prevent potentially fatal and persistent infections, some still await confirmation of their clinical relevance with regards to the prediction of clinically successful therapies. A major setback in the treatment of biofilm-related infections is the ineffectiveness of existing antibiotics to penetrate the protective layers of the biofilm and reach the target, thus, the community of dormant cells persist even in the presence of antibiotics which are effective against their free-living counterparts. Formation of new biofilms could be inhibited by preventing initial processes like attachment of cells to surfaces. This would be crucial in developing medical and dental implants as they are easily colonized by biofilm-forming pathogenic bacteria. Antibiofilm therapies may need to thwart more than one mechanism simultaneously to be clinically effective. Potential therapies include enzymes that dissolve the matrix polymer of the biofilm, chemical reactions that block biofilm matrix synthesis and analogues of microbial signaling molecules that interfere with quorum sensing required for biofilm formation.

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