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Bacterial Biofilm Formation by Clinical Isolates and their Clinical Impacts in Chronic Infections

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ABSTRACT

Bacterial biofilm is a structured community of bacterial cells adherent to a surface and enclosed in a self-produced Exopolymeric substance (EPS) matrix which is mainly consists of polysaccharides and other biomolecules. Advantages for bacteria associated with biofilm formation include protection from the environment, nutrient availability, metabolic cooperation and acquisition of new genetic material. Many several pathogenic species have been reported to be able to form biofilms such as Listeria monocytogenes, Streptococcus mutans, Staphylococcus aureus, Staphylococcus epidermidis, E. coli, Klebsiella pneumoniae and Pseudomonas aeruginosa. The formation of biofilms depends on physical factors, such as composition of the nutrient media, pH, temperature and biological factors. Bacterial biofilms are extremely linked with and controlled by bacterial quorum sensing (QS) which is a cell-to-cell communication system that allows bacteria to monitor their population density and control the physiological processes by releasing and receiving small signal molecules called auto inducers (AIs). Biofilm formation is a significant virulence mechanism in the pathogenesis of many medically important bacterial pathogens causing serious life-threatening infections. The importance of biofilm-related infections arising from indwelling medical devices and implants such as catheters, artificial joints and contact lenses has been highlighted. Bacteria within the biofilm can persist, causing chronic and recurrent infections and developing antibacterial and immunological resistance. Several mechanisms contribute to biofilm resistance to antimicrobials, such as low penetration of the antimicrobial agent due to biofilm matrix barrier function, presence of persistent dormant cells, and small, highly resistant variant colonies. In spite of multiple mechanisms of biofilm resistance to antibacterial agents which vary with the bacteria present in the biofilm and the antibiotic being applied. Thus, new strategies for the prevention, dispersal and treatment of bacterial biofilms are urgently required.

Key words: Biofilm, Quorum sensing, Infections, Medical device, Antibacterial, Resistance

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INTRODUCTION

Most bacterial communities grow in 3-dimensional biofilm structures on many different surfaces in natural, clinical, and industrial settings. Bacterial biofilm is a structured community of bacterial cells stuck together, irreversibly attached to a living or non-living surface and embedded within a consequent self-produced matrix of extracellular polymeric substance (EPS) [1-5].

This bacterial phenotype is an example of physiological adaptation, which is more difficult to eliminate [6]. Biofilms can be either single or multilayered [4] and contain either homogenous (single species) or heterogeneous (multiple species) populations of bacteria which remain in the matrix [4,7].

The microorganisms account for less than 10% of the dry mass, whereas the matrix (EPS) can account for over 90%. EPS has been called 'the dark matter of biofilms' because of the large range of matrix biopolymers and the difficult to analyzed. EPS attaches biofilm cells firmly to surfaces and protects them from harsh conditions [8,9]. It mainly consists of polysaccharides (homo- and heteropolysaccharides) and other biomolecules like proteins, lipids and nucleic acids etc. Polymers like glycopeptides, lipids and lipopolysaccharides form a scaffold and hold the biofilm layers together [4].

Biofilm development is a complicated process that requires the collective behavior of bacteria and is useful

for the bacteria compared to their single life [2,3]. Advantages for bacteria associated with biofilm formation include protection from the environment, nutrient availability, metabolic cooperation and acquisition of new genetic material [10]. Bacterial cells in biofilms express genes in a pattern that differs profoundly from that of their planktonic counterparts. Thus, biofilm bacteria are different from planktonic bacteria in terms of gene expression and cellular physiology and morphology [2,3,6]. Genetic studies including various Gram-negative bacteria have identified genes involved in the biofilm formation and development [2,3].

Bacteria exist in two different forms; Planktonic state (free floating) and sessile state (adhered to a surface), both of which have existed since the first bacteria evolved. Interestingly, bacteria display very distinct characteristics between these two states, as attachment of the bacteria to a surface result in the rapid alteration in the expression of a number of genes responsible for exopolysaccharide (EPS) or "slime" production and maturation. This transformation begins almost immediately after bacterial colonization of both biotic and abiotic surfaces and results in the production of a protective barrier that protects the bacteria against the organism's endogenous defense system or from external agents such as antibiotics. This barrier is in some cases referred to as "slime" or the exopolysaccharide matrix [1]. Direct observations have showed that biofilms constitute the majority of bacteria in most ecosystems comparing with the planktonic cultures [3].

Bacterial biofilm history

The first description of biofilm dates back to the 17thcentury in 1683, when Anton Von Leeuwenhoek - the inventor of the Microscope, saw microbial aggregates on scrapings of plaque from his teeth (now known to be Biofilms) [5]. Secondly and not surprisingly, came the pioneer of microbiology Louis Pasteur (1822-1895) who proved that the spoilage of wine was due to aggregates of microorganisms, and also reported membrane formation which he noticed in vinegar barrels. This fact was already reported by Arthur Henrici in 1933, when he observed that most aquatic microorganisms were not in the form of individual cells swimming freely but aggregated over solid submerged surfaces [11]. In addition, In 1970s it is observed the existence of biofilm in the sputum of cystic fibrosis in patients. Since then, the relationship between bacterial biofilm and human infections was documented. The term 'Biofilm' was coined by Bill Costerton. Almost 15 years later, in 1993, the American Society for Microbiology recognized the significance of biofilms. Between 1999 and 2002, Donlan et al. offered the last and most salient description of a biofilm [5].

Biofilm producing bacteria

Many several pathogenic species have been reported to be able to form biofilms such as Listeria monocytogenes, Streptococcus pyogenes, Streptococcus mutans, *Staphylococcus aureus, Staphylococcus epidermidis, E. coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [3,12,13, 14].

Pseudomonas aeruginosa produce Glycocalyx that facilitates bonding between bacteria and host cells, thereby forming micro colonies, contributing to the biofilm formation and protecting the bacteria from the phagocyte system and antimicrobial materials [2]. Listeria monocytogenes is another example of pathogenic bacteria that able to persist for years in environments through biofilm formation. Another pathogenic species of great importance is enter pathogenic *Escherichia coli* (EPEC), which is a major causative agent of childhood diarrhea and has been linked to outbreaks of food-borne infections also through biofilm formation [15]. Furthermore, *E. coli* biofilms are frequently described for catheter associated chronic and recurring Urinary Tract Infections (UTIs). These structures protect the bacteria against the mechanical flow of urine, host and antibiotics [16].

Biofilm formation

Bacteria adsorbed on surfaces grow in microcolonies and secrete EPS, becoming encapsulated in a hydrogel layer that forms a physical barrier between the microbial community and the extracellular environment. The initial bacterial adhesion to surfaces is mediated by reversible interactions whose associated physical forces; van der Waals forces and steric electrostatic interactions. Subsequently the bacterial cells adhere irreversibly to the substrates through hydrogen bonds, ionic bonding, and dipole-hydrophobic interactions. Bacterial cell surface structures such as lipopolysaccharides (LPS) and exopolysaccharides also participate in these irreversible interactions. The secretion of an extracellular polymeric substance (EPS) also facilitates the adhesion between cells and surfaces [17].

Literature review showed that both genetic and environmental factors contribute towards the microbial biofilm formation process. This includes two component systems of extra-cytoplasmic function (ECF) signaling pathway and quorum sensing (QS) events [4]. Biofilm formation is a developmental dynamic and multicellular process that involves a series of steps mediated by combination of adhesion mechanisms, bacterial motility and quorum-sensing (QS) phenomenon [6]. So, bacterial biofilms are extremely linked with and controlled by bacterial quorum sensing (QS), which is responsible for biofilm growth, differentiation and determination of bacterial physiology [6]. Pathogenic and opportunistic microorganisms are able to survive and adapt in different environments by QS that regulate their genes which are responsible for many physiological processes such as virulence, biofilm formation, swimming, motility, genetic transfer and pathogenicity [18].

Quorum sensing (QS) is a cell-to-cell communication system that allows bacteria to monitor their population density and control the physiological processes by releasing and receiving small signal molecules called autoinducers (AIs) to trigger the expression of that specific genes [6,18,19]. For QS to be possible, a

minimum number of bacteria must be aggregated within a specific volume. The bacterial cells can determine the local density of cells by sensing when signaling molecules (autoinducers) that are generated by neighboring cells in small microcolonies reach a critical threshold [1].

There are several classes of AIs, based on common molecular features. These include acyl homoserine lactones (AHLs), autoinducing peptides (AIPs) and autoinducer-2 (AI-2). Gram-positive bacteria are mediated by AIPs, and Gram-negative predominantly employ AHLs as AIs. AI-2 is involved in inter-specific communication in both Gram-negative and positive bacteria [6]. They are synthesized by specific enzyme and produced by bacteria diffusing out and accumulating in the surrounding environment. Once a threshold concentration has been reached, they diffuse back into the bacteria binds to a cognate receptor (transcriptional activator protein) and regulate the transcription of specific virulence genes and expression of biofilm formation [20]. This multicellular behavior results in biofilm formation which greatly contributes to bacterial pathogenesis [20]. Thereby, affecting the function of the quorum sensing system, significantly inhibiting the formation of biofilm [21].

In biofilm formation, planktonic bacteria become sessile cells capable of forming community of microcolonies after adhering to a surface, secreting biomolecules that make up the EPS where they are embedded and encased. In the mature biofilm, bacteria can establish communication with one another, receive nutrients and water through channels, contributing to their survival on the biomaterial surfaces until they detach and become free-living cells capable of contaminating or infecting other locations [17].

In more detail, biofilm formation comprises the following stages: 1-Reversible attachment of the bacterial planktonic cells to the surface by adhesion mechanism; 2-starting cell adsorption and multiplication; 3-Irreversible connection of cells, aggregation and early microcolony formation; 4-Production of cell-cell signaling molecules within micro colonies and consequent production of extracellular polymeric substance forming the matrix layers; 5- final formation of biofilm and maturation with maximum cell density and a three-dimensional community; and 6-dispersion of single planktonic cells from the mature biofilm to migrate to new surfaces spreading the infection to other locations [2,4,22].

Molecular basis of biofilm formation

The development of a biofilm and the release of cells (either individually or in clusters) can be regulated by population density dependent gene expression controlled by cell-to-cell signaling molecules. There is evidence that during this attachment phase of biofilm development, the transcription of specific genes is activated after attachment to a solid surface for synthesis of the extracellular polysaccharides and perhaps after microcolony formation, receptor-like proteins or secondary messengers triggering biofilm formation. PIA is polysaccharide intercellular adhesin that helps in biofilm formation and is encoded by specific gene (operon). Regulatory and biosynthetic genes are important for the formation of biofilms and impart virulence to the bacteria [4].

Factors that affect biofilm formation

The formation of biofilms depends on physical factors, such as composition of the nutrient media, pH, temperature and biological factors. Most of the bacterial biofilm formation is growth dependent. Hence, it is important to know the biofilm formation is growth dependent or growth independent [23,24]. Motility seems to be critical for transition from planktonic to surface-associated life-style. Other factors that are involved in biofilm development, such as the initial adhesion process. Bacterial adhesion to surfaces has been studied extensively over the past decades in many diverse areas. Adhesion is a complex process that is affected by many factors including the physicochemical characteristics of bacteria (hydrophobic interactions), the material surface properties, and the environmental factors. Bacteria with increased hydrophobicity facilitate biofilm formation by reducing repulsion between the extracellular matrix and the bacterium [25]. The biological properties of bacteria, such as the presence of fimbriae and flagella and the production of EPS, also influence the attachment to surfaces and the consequent biofilm formation [6].

Clinical significance and risks of biofilm

After more than 70 years of the first report on biofilms, still an alarm in a broad range of areas like food industry, environmental and biomedical fields and everyday life is associated with biofilm as a source of diverse major problems [2,8,4].

Biofilm formation is a significant virulence mechanism in the pathogenesis of many medically important bacterial pathogens, such as *Pseudomonas aeruginosa* [26], *Staphylococcus aureus* [27], and *Escherichia coli* [28]. The opportunistic human pathogen *Staphylococcus aureus*, for example, which has recognized importance in severe hospital infections, a well-known the capacity to form biofilms and the ability to acquire resistance to antibiotic, is still a challenge for researchers in the search for agents with efficacy against it and in particular against their biofilms [29]. One biofilm-related infection of particular medical concern is *P. aeruginosa* biofilms in the lungs of cystic fibrosis patients. This opportunistic pathogen has been known to cause acute and chronic lung infections that can result in significant morbidity and mortality [30]. This bacterium is also implicated in the mixed biofilm infections of burn victims, chronic wounds and diabetic pressure ulcers [31].

Biofilms have been implicated as the cause of other serious infections [10]. In fact, it is estimated that over 60% of microbial infections and ~80% (two-thirds) of all human bacterial infections are caused by the biofilms [32,33] and also associated with 65% of nosocomial

infections [34]. It has been estimated that the maximum bacterial infections treated in hospitals are associated with bacterial biofilm [35]. The treatment of these biofilm-based infections costs >$1 billion annually [36,37,38]. Bacteria within the biofilm can persist, causing chronic and recurrent infections [39]. Biofilm growing bacteria can cause overwhelming chronic infections in compromised hosts and can occur in these cases; endocarditis, periodontitis, lungs of cystic fibrosis (CF) patients causing chronic bronchopneumonia, chronic and secretory otitis media, urinary tract infections, and chronic Rhinosinusitis [4]. Moreover, the presence of biofilms in food processing environments is a potential source of contamination that may lead to food spoilage and disease transmission [40].

Biofilm and dental infections

The most common medical problem of biofilms which occur on almost every individual is plaque that caused by oral commensal bacteria on teeth and if left untreated leads to decay or caries, gingivitis and periodontitis [41]. The oral cavity may also disseminate biofilm growing bacteria through dental works to distant body sites, leading to systemic diseases like infective endocarditis, bacterial pneumonia, etc. [41].

The human oral cavity harbors more than 700 microbial species and they are composed by both commensal and pathogenic species. Some of these adhere to the teeth and initiate formation of a dental biofilm, which is the major cause of dental caries and periodontal disease. Bacterial biofilm formation on some orthodontic appliances and dentures may also cause serious dental infections [42].

Streptococcus mutans (S. mutans) is the most common pathogen associated with tooth caries. The cariogenic potential of S. mutans is associated with its ability to form biofilms on both soft and hard oral surfaces such as the palate, tongue, restorations and teeth [43]. It was shown that S. mutans strains in biofilms are up to 70,000 times more acid tolerant than their planktonic counterparts [44].

Biofilm and eye and wound infections

There is a provided evidence of the presence of biofilm on contact lenses samples collected from patients with a clinical diagnosis of microbial keratitis. In a study of patients with corneal infections, bacterial biofilms were found in 17 of 20 examined contact lens storage cases. The latter rate was found to be higher than the rate of biofilm formation on the contact lenses themselves. Another area of considerable concern is that of chronic wound infections. Recent analysis from chronic wounds has identified the presence of biofilm-growing bacteria, thereby explaining why these wounds persist. Many wound pathogens are very difficult to culture (even if grown anaerobically) and persistent cells from the biofilm might even be impossible to culture [4].

Biofilm formation is an important pathophysiology step in diabetic foot ulcers (DFU). It plays a main role in the disease progression and chronicity of the lesion, the development of antibiotic resistance, and makes wound healing difficult to treat [45].

Highly persistent biofilm-related wound infections, which commonly involve the pathogens *P. aeruginosa* and *S. aureus* [46], are suggested to be responsible for over 80% of the 100,000 limb amputations carried out on diabetic patients in each year [45,47]. In DFU, pathogenic and commensal bacteria co-aggregate symbiotically in a pathogenic biofilm and act synergistically to cause and maintain a chronic infection. In a 2008 study assessing wound tissue biopsies using electron microscopy, James et al., suggested that 60% of chronic wounds presented biofilms versus 6% for acute wounds [45].

Biofilm and medical devices infections

Although therapeutic prosthetic devices are common medical procedures and a life-saving treatment for many patients, yet, several complications are associated with their use and can lead to serious illness and death when colonized by bacterial biofilms [48,49]. Despite efforts to maintain sterility, implantable and prosthetic medical devices can easily become contaminated with bacteria. Major challenges in treating biofilms are their difficult diagnosis, difficult eradication due to a high tolerance to antibiotics and lack of suitable biomarkers [1].

Biofilm related infections arising from indwelling medical devices and implants such as such as intravascular catheters, urinary catheters, artificial hips and knee joints , endotracheal tubes, prosthetic heart valves, contact lenses and ortho-dental devices or appliances including dentures, orthodontic brackets and retainers [50; 68] have been highlighted and increased tremendously [10,11,14] since the biomaterials provide surfaces for bacteria to adhere to and subsequently form biofilms.

Bacteria may adhere to form biofilms in foreign bodies placed in patients establishing infection [17]. Both Gram-positive and Gram-negative bacteria can form the biofilm on various medical devices. In this regard, *Staphylococcus aureus* and Proteus vulgaris are biofilm-forming pathogens on medical implants able to produce severe biofilm-associated infections such as urinary tract infection, musculoskeletal infection and respiratory tract infection. In fact, the number of implant-associated infections near about 1 million/ year in the US alone and their direct medical costs exceed $3 billion annually [35]. The complications include bacteremia, systemic infections, damage of the artificial device, antibiotic resistance and high morbidity and mortality. As millions of indwelling devices are implanted in patients yearly, as many as are accompanied by the formation of biofilms that adhere to the surfaces of the medical devices, subsequently leading to treatment failure.

The most common pathogenic microorganisms that can form biofilm on medical devices are Enterococcus faecalis, *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, E. coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas*

aeruginosa. Amongst them, *S. aureus* and *S. epidermidis* are estimated to cause about 40-50% of prosthetic heart valve infections, 50-70% of catheter biofilm infections and 87% of bloodstream infections. Two-thirds of infections associated with implantable devices such as orthopedic and breast implants are caused by the staphylococcal species with the majority being associated with *S. aureus* and coagulase-negative staphylococci [1]. They synthesize primary polysaccharide called polysaccharide intercellular adhesin (PIA) by the expression of genes. Deacetylation of this adhesin promotes the adhesion of these bacteria to the biomaterial surfaces and the consequent biofilm formation an infection [17]. In addition, *P. aeruginosa* is associated with many hospital-acquired infections due to colonization and biofilm formation on medical equipment [31].

Furthermore, infections associated with prosthetic heart valves conferred several problems some with reported fatality. Unfortunately, once bacterial biofilm is established, bacterial communities in these biofilms become resistant to antimicrobial treatment and host defense, thereby become the source for recurrent infections. Above all, such infections are difficult to eradicate because these bacteria live in well-developed biofilms.

Multidrug-resistant nosocomial pathogens are the most common micro-organisms in medical device infections [17].Owing to biofilm increased resistance to antimicrobial agents, all these infections can often only be treated by removal of the implant, thus increasing the trauma to the patient and the cost of treatment [4,17,34]. Also, another strategy to eradicate implant infections is prolonged treatment with high doses of antibiotics, often using antimicrobials that act through different mechanisms. However, in clinical practice, infected implants usually require their surgical removal in addition to long-term antibiotic therapy [17].

Prosthetic vascular graft infection (PVGI) can be disastrous and cause an increase in morbidness and mortality risk. The yearly cost burden related to PVGI is $640 million in the United States. Morbidity of PVGI ranges from 1–5% of patients varies according to the location of anatomical implantation, the using biomaterials, and the patient's comorbidities. The mortality rate is about 10–25% within 30 days after the detection and almost 50% after 1 year. The risk of amputation is about 4–14%. Besides, when the infection occurs in transplant location where blood density low, they must be treated with high doses of antibiotics and for a long time. Thus, it is toxic to the patient's body and causes antibiotic-resistant bacteria. Biofilm develops on the surface of graft materials plays an important role in the difficulties of treating PVGI. When biofilm is formed, treatment with removal of infected device is compulsion [50].

Catheter-associated urinary tract infections due to biofilm formation are rising rapidly, and have a high mortality rate especially in long term catheterized patients. Moreover, catheter associated urinary tract infections (CAUTI) have been reported as the most common nosocomial urinary tract infection associated with indwelling urinary catheter around the world causing bacteremia.

Multidrug-resistant nosocomial pathogens colonize the external and internal region of the catheters and proliferate at a rate of 0.5 cm of surface area per hour, being able to form a thick biofilm in 24 h on the surface of these plastic devices, from an inoculum with a small number of bacteria. The most usual route of infection in short-duration catheters is by migration of microorganisms from the skin at the insertion site to reach the catheter tip. Catheter hub contamination by contact with contaminated hands, fluids or devices may also lead to an intraluminal colonization of the device. More rarely the catheter may be contaminated via the hematogenous route; occasionally, contaminated infusate may introduce microorganisms into the catheter lumen [17].

The duration of catheter use differs from patient to patient, as it depends on how severe their condition occurs. Some patients may use catheters for 14 days or less (short term catheterization) while others could extend its use for about 30 days or more (long term catheterization). However, catheter insertion and duration have been a major concern because of its tendency to harbor harmful microorganisms including *Escherichia coli*, *Staphylococcus aureus, Proteus, Klebsiella, Enterobacter, Pseudomonas* and *Candida spp*. They are involved in biofilm formation on catheter surfaces [51]. Biofilms can colonize a whole catheter and move along the internal lumens of catheters into the bladder, kidney and sometimes the blood stream. This poses a public health problem for patients who depend on urinary catheters. Some of the nosocomial urinary tract infections that could arise due to catheters include urethritis, cystitis, pyelonephritis, renal scarring, and bacteremia and in severe cases death [51].

Biofilm and host immune system

Even in individuals with excellent cellular and humoral immune reactions, biofilm infections are rarely resolved by the host defense mechanisms. Complex sticky polysaccharides of biofilm matrix promote protection from Immune responses. Bacteria embedded within biofilms are resistant to both immunological specific and non-specific defense mechanisms of the body. Biofilm sessile bacterial cells release antigens and stimulate the production of antibodies or defensins, but they are not effective in killing bacteria within biofilms and may cause immune complex damage to surrounding tissues. biofilm also evade host immune-responses and phagocytic cells seem not only to be unable to physically engulf the biofilm structures but also to be impaired in their activities. This causes the phagocytes to release large amounts of cytokines, leading to inflammation, destruction of surrounding tissues and delayed healing

[4]. Biofilms that cause inflammation rarely grow to sizes larger than 100. Mm [4].

Biofilm and resistance to antimicrobial agents

The resistance of the cells in a biofilm system to antimicrobial agents including antibiotics, disinfectants and preservatives has been widely reported [40; 41]. The ineffectiveness of antibiotic treatment in the biofilm diseases may cause serious problems in the eradication of infections [9]. However, the world is currently faced with the dilemma of a decline in the number of new therapeutic agents to treat various diseases due to the resistance. Current estimates reveal an annual death toll of 700,000 people due to antibiotic resistance and a projection that by 2050, 10 million lives may be at risk if nothing is done to halt the drift towards increasing antimicrobial resistance. In 2017, a comprehensive list of priority biofilm producing pathogens was released by the World Health Organization (WHO), including microbes such as *Staphylococcus aureus*, Acinetobacter baumannii, *Streptococcus pneumoniae*, *E. coli, Klebsiella spp*., *Enterobacter spp*. etc. These pathogens have high levels of resistance to most existing antibiotics such as carbapenem, vancomycin, penicillin, ampicillin, and the third-generation antibiotic cephalosporin [33].

Biofilm renders bacteria highly resistant to conventional antibiotics and host defenses more than planktonic cells; In fact, when cells exist in a biofilm, they can become 10–1000 times more resistant to the effects of antimicrobial agents making them more difficult to treat than their planktonic counterparts [33]. Thus, the major problem caused by biofilms is increased tolerance towards antimicrobial agents that impairs the treatment of biofilm-related infections [4]. The increase of microbial resistance to antibiotics threatens public health on a global scale as it reduces the effectiveness of treatments and increases morbidity, mortality, and health care costs [52].

Bacterial resistance and tolerance are differently defined by authors; however, according to most, bacteria are said to be tolerant when they are unable to proliferate (yet continue to persist) under antimicrobial therapy, whereas proliferation under the same conditions is considered as resistance. It is reported that sessile bacteria are 500-5000 times more tolerant towards antibiotics in comparison to their planktonic state. Tolerance, an active and adaptive process, typically occurs when bacteria aggregate at high density, while resistance is a result of intrinsic and external factors such as mutation [1].

Most antimicrobial treatments available are generally developed and evaluated against microorganisms in the planktonic (free-living) mode of life. Consequently, these treatments are often ineffective against pathogenic biofilms [53], Antibiotics are able to kill the planktonic cells released by the biofilm after its maturation stages, but bacteria within the biofilm can persist, causing chronic infections [39]. Antibiotic therapy typically reverses the symptoms caused by planktonic cells released from the biofilm, but fails to kill the biofilm. For this reason biofilm infections typically show recurring symptoms, after cycles of antibiotic therapy, until the sessile population is surgically removed from the body [4]. Biofilm is therefore difficult to eradicate and is a source of many recalcitrant infections [12]. For example, it was shown that biofilm-grown Propionibacterium acnes cells are more resistant to various antimicrobial agents than planktonic cells and the biofilm phenotypes have been invoked to explain therapeutic failure [13].

Biofilms increase the opportunity for gene transfer between bacteria. Gene transfer can convert non-virulent commensal organisms into a highly virulent pathogen [4]. Bacteria embedded in biofilms exhibit greater resistance to environmental conditions as result of the high degree of enabling horizontal gene transfer among them, including antibiotic resistance genes, within members of the biofilm micro-community [24] which can lead to increase in the number of virulent strains [4] favoring the infections [17]. Biofilms represent an ideal niche for plasmid exchange among bacteria. In fact, the conjugation frequency appears to be higher in bacteria growing in the sessile mode than in the planktonic mode. Thus, because some plasmids contain genes coding for multidrug resistance, microbial biofilms provide a suitable environment to amplify both naturally occurring and induced antibiotic resistance phenomena [48,50]

The conventional mechanisms of antibiotic resistance, such as efflux pumps, modifying enzymes, and target mutations, do not seem to be the only responsible for the protection of bacteria in biofilms from antimicrobials [6]. Possible reasons of this feature that may be due to the EPS layer include the limitation of the transport of the agents to interior bacterial cells in thick layers and the reduction of available agents by adsorption into or reaction with the EPS matrix [40]. In addition, planktonic bacteria that are found outside of biofilm display strong metabolism; so, they are very sensitive to antibiotics. Alternatively, bacteria that are deeply embedded in biofilm display slow metabolism; so their antibiotic sensitivity decreases [50].

However, it was strongly suggested that multiple mechanisms are required and involved for in biofilm tolerance and resistance, including: 1-slow penetration of the antimicrobial agent through the biofilm changes in the chemical microenvironment rapidly within the biofilm which leads the malfunction of the antibiotics [4] because most of the chemicals are active only against unattached microorganisms [8]; 2-the failure of an agent to penetrate the full depth of the biofilm; 3-Polymeric substance of the biofilm matrix are known to retard the diffusion of antibiotics; 4- antibiotic gets degraded while penetrating the biofilm and its action declines rapidly; 5-Antibiotics may get adsorbed on the extracellular polymeric surfaces of the biofilm which can decrease the penetration of the antibiotic; 6-Occasionally, antibiotics which are positively charged in nature can bind to the negatively charged molecules of the biofilm

matrix retarding the passage of the antibiotic to the biofilm depth; 7- In subterranean layers of the biofilm, there is no consumable oxygen and becomes anaerobic environment and It has been proved that a class of antibiotics namely aminoglycosides are not active in anaerobic environmental condition; 8-In response to high dose of antibiotics, bacteria can accumulate high levels of beta-lactamases enzymes; 9-DNA-binding regulatory protein involved in the biofilm-specific antibiotic tolerance acts as a repressor of specific antibiotic sensitivity genes; 10- In biofilms, a small subpopulation of bacteria can reversibly enter a slow-growing or starved state due to nutritional stress and these cells are known as per sisters or dormant cells (in a stationary phase) and highly resistant to killing by antibiotics and can become active when the therapy is withdrawn. In spite of multiple mechanisms of biofilm resistance to antibacterial agents which vary with the bacteria present in the biofilm and the antibiotic being applied, The main mechanism is briefed and confined to: A-physical or chemical diffusion barriers to antimicrobial penetration into the biofilm, B-slow growth of the biofilm owing to nutrient limitation, C-activation of the general stress response and D-the emergence of a biofilm-specific phenotype [1,4,54].

Failure of the antimicrobial to penetrate the biofilm

The production of an exopolysaccharide matrix, or glycocalyx, is one of the distinguishing characteristics of biofilms. It has been suggested that this matrix, beside other functions, can prevent the access of antibiotics to the bacterial cells embedded in the community. Either reaction of the compound with, or sorption to, the components of the biofilm can limit the transport of antimicrobial agents to the cells within the biofilm. It was suggested also that the antibiotic was binding to the biofilm components [55]. However, the exopolysaccharide matrix does represent an initial barrier that can delay penetration of the antimicrobial agent [54].

5B-Slow growth and heterogeneity

Slow growth of the bacteria has been observed in mature biofilms [56,57]. Because cells growing in biofilms are expected to experience some form of nutrient limitation, it has been suggested that this physiological change can account for the resistance of biofilms to antimicrobial agents [58-60]. The slow growth rate of biofilm cells protects the cells from antimicrobial action. At slow growth rates, both the planktonic and intact biofilm cells were equally resistant to the antibiotic [23]. Other studies have suggested that mechanisms differ for different antibiotics. For example, although the slow growth rate in a *P. aeruginosa* biofilm seemed to account for biofilm resistance to tetracycline, it did not seem to affect resistance to tobramycin [61]. Cells within the biofilm will experience a slightly different environment compared with other cells within the same biofilm, and thus be growing at a different rate. Gradients of nutrients, waste products and signaling factors form to allow for this heterogeneity within the biofilm [54]. This heterogeneity within biofilms has also been shown for protein synthesis and respiratory activity, whereas DNA content remained relatively constant throughout the biofilm [62,63]. The environmental heterogeneity might promote the formation of a heterogeneous population of cells, such that different levels of resistance can be expressed throughout the community. For example, the cells closest to the liquid–biofilm interface might be protected to a small degree by the exopolysaccharide matrix and by enzymes that inactivate certain antimicrobial agents. The cells in an intermediate position might be growing slowly and could also be protected by the outermost layer of cells [49]. In one study, when biofilm cells were treated with the antibiotic fleroxocin, cell elongation was observed and was most extreme in cells located close to the exposed side of the biofilm [42]. These studies reveal that the response to antimicrobial agents can greatly vary, depending on the location of a particular cell within a biofilm community [54].

5C-General stress response

Recently, it has been suggested that the slow growth rate of some cells within the biofilm is not owing to nutrient limitation, but to a general stress response initiated by growth within a biofilm [65]. The stress response results in physiological changes that act to protect the cell from various environmental stresses. the cells are protected from the detrimental effects of heat shock, cold shock, changes in pH and many chemical agents [66]. The central regulator of this response is specific alternate factors; σ factor and RpoS, originally thought to be expressed only in stationary phase [33]. However, recent studies suggest that RpoS is induced by high cell density and that cells growing at these high densities seem to have undergone the general stress response [67].

5D-Induction of a biofilm phenotype

A biofilm-specific phenotype is induced in a subpopulation of the biofilm community that results in the expression of active mechanisms to combat the detrimental effects of antimicrobial agents [68,69]. When cells attach to a surface, they will express a general biofilm phenotype and work has begun to try to identify genes that are activated or repressed in biofilms compared with planktonic cells [70]. Furthermore, it is possible that all or just a subset of these biofilm cells could express increased resistance to antimicrobial agents. This resistant phenotype might be induced by the particular environmental factors influencing these cells such as nutrient limitation, certain types of stress, high cell density or a combination of these phenomena [54]. General biofilm resistance phenotype includes: Multidrug efflux pumps or alteration of the membrane-protein composition in response to antimicrobial agents [71,72]. Multidrug efflux pumps can extrude chemically unrelated antimicrobial agents from the cell. In one study, it was shown that, at low concentrations of ofloxacin, biofilms lacking the pump were more

susceptible to this drug than biofilms that overexpressed the pump [61]. Bacteria in a biofilm are indeed living in an environment of increased osmotic stress. Thus, the environmental conditions within the biofilm can lead to alterations within the cell envelope that protect the bacteria from the detrimental effects of antimicrobial agents [54]. However, not only are the bacterial cells in biofilm resistant to antibiotics, they are also able to defend themselves against a number of physico-chemical aggressions, including acidity, salinity, heavy metals, ultraviolet light, and phagocytosis. The phenomenon of biofilm recalcitrance makes them extremely difficult to treat and eradicate effectively. Thus, new strategies for the prevention, dispersal and treatment of bacterial biofilms are urgently required.

CONCLUSION

Most bacterial communities grow in 3-dimensional biofilm structures on surfaces in natural, clinical, and industrial settings with many important risks and negative impacts. Due to the high rate of biofilm related infections with highly resistance to antimicrobial agents, a number of research studies about biofilm have been performed in the last two decades. Now the biofilm is considered as major target for the pharmacological development of medication and control. Therefore, there is an urgent need to rethink about both the biofilm prevention and treatment novel strategies.

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