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BIOFILM INFECTIONS: A NEW THERAPEUTIC CHALLENGE

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ABSTRACT: Biofilms are multicellular communities of bacteria. The ability to form biofilm is a universal attribute of bacteria and as bacteria can form biofilm in medical, industrial and environmental settings, it impacts human lives in many different ways. Bacteria undergo profound physiologic, morphologic and genetic changes as the transit from planktonic (free swimming, non-biofilm) form to surface attached biofilms. As bacteria in biofilm exhibit enhanced resistance to antibiotics and clearance by the host immune system, biofilm has been an intensely researched area for microbiologists, immunologists and pharmaceutical scientists alike. This review focuses on current knowledge on biofilm science, clinical relevance and virulence of biofilm, host response to it and therapeutic options to eradicate biofilm.

INTRODUCTION: Microbial biofilm is a community of either single or multiple microbial species enclosed in a self-produced extracellular polymeric substance (EPS) and adherent to an inert or living surface [1, 2]. EPS is comprised of exopolysaccharides, proteins, and DNA. EPS serves as the adhesive material cementing cell surface and intracellular interactions and also as scaffold for proteins that mediate cell-cell interactions in biofilm (**Figure 1**) [3, 4]. In nature, bacteria do not exist as free floating organisms, but grow upon submerged surfaces as biofilms and all the bacteria that have been investigated produces biofilm [5]. Microorganisms undergo profound changes during their transition from planktonic (free swimming, non-biofilm) organisms to a complex, surface-attached community of biofilm.

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This transition results in changes in a variety of phenotypic characteristics [6, 7]. These changes are reflected in the new properties such as capacity to withstand nutrient limitation, pH changes, oxygen radicals, disinfectants, antibiotics and phagocytosis [7, 8].

Biofilm represent a heterogeneous population of bacteria. Bacteria in biofilm exhibit chemical, physiological and metabolic and genetic heterogeneity [9, 10]. Biofilms constitute a protected mode of growth that allows microorganisms to survival in hostile environments, being their physiology and behavior significantly different from their planktonic counterparts [11, 12].

Differences exist throughout the biofilm community. Thickness of biofilm range from a single cell layer to multiple layers and depending on environmental conditions, can assume various colony architecture including pillar or mashroom shaped structures [1, 12]. The existence of cells of the same bacterial pathogen in different metabolic phases in a single biofilm is typically exemplified by the gram positive pathogen *Staphylococcus*

aureus, which is one of the extensively studied bacteria for biofilm (**Figure 2**). Studies have shown that *S. aureus* cells can exist in four distinct stages in biofilm; aerobic, fermentative growth, dormant and dead [13, 14]. As functionality of many antibiotics vastly depend on metabolic activity of bacterial cells, the existence of single bacterial pathogen in different metabolic stages in a single biofilm shows how complex and challenging is eradication of biofilm in clinical settings, especially when causing chronic infections.

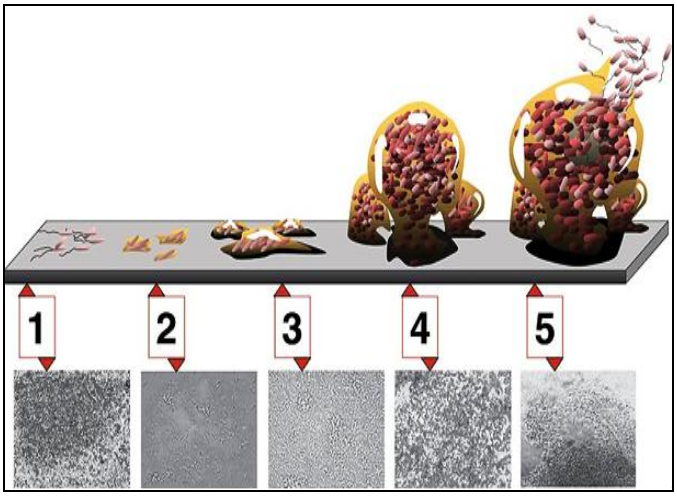

FIGURE 1: FIVE STAGES OF BIOFILM DEVELOPMENT: [1] INITIAL ATTACHMENT, [2], IRREVERSIBLE ATTACHMENT, [3] MATURATION I, [4], MATURATION II, AND [5] DISPERSION. Each stage of development in the diagram is paired with a photomicrograph of a developing *P. aeruginosa* biofilm [15].

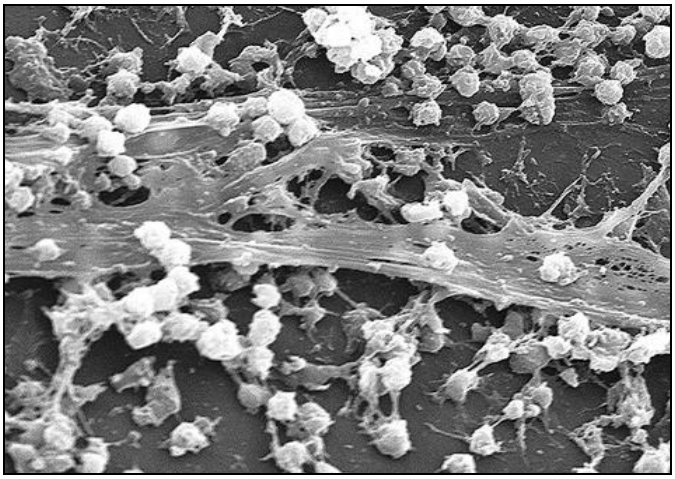

FIG. 2: BIOFILM PRODUCED BY *STAPHYLOCOCCUS AUREUS* ON AN INDWELLING CATHETER [19]

Gram negative opportunistic pathogen *Pseudomonas aeruginosa* can form three different types exopolysaccharides which can form the EPS matrix encasing the biofilm [15, 16], is another example complex nature of biofilm.

Various environmental factors influence biofilm formation which include pH, temperature, osmolarity, iron, oxygen and growth medium composition [5, 11]. *Escherichia coli* 0157:H7 form biofilm in low nutrient medium [16]. *Vibrio cholera* and *E. coli* K12 does not form biofilm in minimal medium unless it is enriched with amino acids [17, 18]. On the other hand, *P. aeruginosa* produces biofilm in any medium under any growth condition [18].

Cations (sodium, calcium, lanthanum, ferric iron) influence biofilm formation. Higher amounts of biofilms were produced as the concentrations of these ions increased, presumably by reducing the repulsive forces between the negatively charged bacterial cell surface and the solid surface onto which biofilm is formed. Increase in nutrient concentration correlated with an increase in the number of attached bacterial cells forming biofilm [19].

Hossain (**Figure 3**) [20] showed that that growth of *P. aeruginosa* in biofilm enhanced its potential to form new biofilm, presumably indicating that passage in biofilm induces gene expression cascade which results in increased amount of biofilm formation (**Figure 4**). EPS constitutes the primary matrix of biofilm and it may account for 50% to 90% of the total organic material of a formed biofilm. ESP may vary widely in chemical and physical property depending the microorganism(s) concerned, organic material available and subtratum involved onto which biofilm is formed. The level and type of ions bound by the EPS depends on its ionic properties, which in turn contributes to the structure and strength of the biofilm [3, 4, 5].

FIG. 3: FORMATION OF BIOFILM *IN VITRO* BY *STAPHYLOCOCCUS AUREUS*, STAINED WITH CRYSTAL VIOLET [20]

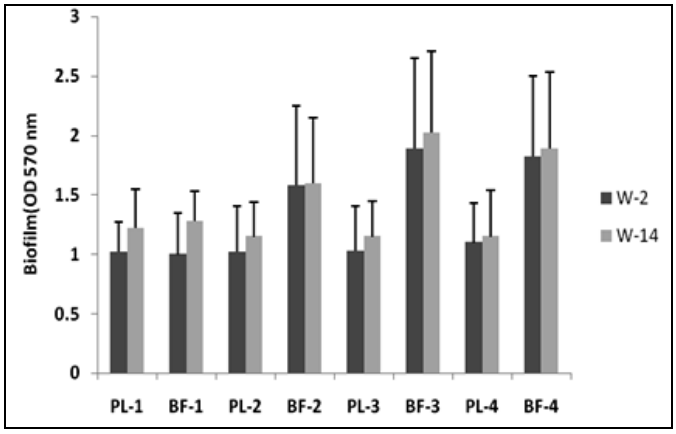

FIGURE 4: PRODUCTION OF BIOFILM BY WOUND ISOLATES OF *P. AERUGINOSA* W-6 AND W-14 AT DIFFERENT PASSAGE LEVELS (PL, passage in planktonic stage; BF, passage in biofilm stage). Passage in biofilm resulted in enhancement in the potential of formation of new biofilm [20].

Clinical relevance and Virulence of Biofilm: Growth of pathogenic organisms in a biofilm result in an infectious disease process. Numerous studies carried out by physician, public health microbiologists and biomedical scientists showed that microbial biofilm as the pathogenic principle of many infectious diseases such as cystic fibrosis, native valve endocarditis, otitis media, periodontitis, and chronic prostatitis [5, 21].

Bacteria infecting humans are capable of transiting between environment and human host and are able to adapting to sudden changes in nutrient availability. In addition, in order to maintain their existence in the host, the pathogens must encounter the innate and adaptive immune response of the host [22]. It is now established that formation of biofilm contributes to the chronicity of infection by creating an environment that permits enhanced antibiotic resistance and surviving the clearing effect of the immune system [22, 23].

The chronic infection caused by the opportunistic gram negative human pathogen, *P. aeruginosa* in the lungs of the patients suffering from the genetic disease cystic fibrosis (CF) is a classic case of biofilm acting as the pathogenic principle [22, 23]. *Burkholderia cepacia* has also been found to be too associated in the infection of the lungs of the CF patients [24, 25].

As the bacteria exist as biofilm in the lungs of the infected patients, they are less accessible to therapeutic antibiotics that are used. In addition, host immune response is not able to clear the biofilm completely as cells and molecules of the immune system are often not capable to reaching the entire depth of the biofilm. Moreover, constant stimulation of the immune system by the bacterial antigens and repeated failure of the immune system to clear the pathogen completely results in a state of constant inflammation, resulting in further damage of the infected lung tissue [2, 26]. During chronic *P. aeruginosa* and *B. cepacia* infections, CF patients experience a continuous degradation of lung tissue. This is caused in part by the infection and in part by the inflammatory processes. The consequence is a decline in the lung function, which is the primary cause of death in CF patients.

So, because of the organization of the bacteria as biofilm in the lungs of the infected patients it becomes incurable, resulting in death of the cystic fibrosis patients. The severe consequences of the patients chronically infected with biofilm bacteria particularly patients with cystic fibrosis, resulted in a focused, multidisciplinary effort to delineate the role of biofilm in the infection and disease process and design of appropriate therapy [5, 23, 25].

Safadi *et al* [27] showed that correlation exists between *in vivo* biofilm formation and virulence gene expression *in E. coli* O104:H4 in mice model. Biofilm formation helps *V. cholera* to persist during inter-epidemic periods. Biofilm formation also helps to protect the pathogen during passage through the stomach and enhance its infectivity upon oral ingestion. Biofilm *V. cholera* colonized more efficiently than their planktonic counterpart suggesting that infectious dose is much lower than planktonic cells [28].

Biofilms exhibit an inherent resistance to all classes of antimicrobial agents such as antibiotics, disinfectants and germicides. EPS, which encases the biofilm, functions as a diffusional barrier to antimicrobial agents [1, 29]. The nutrient availability gradually decreases in the depth of biofilm as the EPS interferes with flow of nutrients, just the way it does with the diffusion of antibiotics.

The result is the existence of slow growing or starvation state of bacteria in biofilm. Most antimicrobials require at least some degree of cellular activity to be effective; since their mechanism of action usually relies on disrupting different microbial metabolic processes.

So, existence in biofilm of bacterial population in a wide variety of metabolic states and the fact that slow growing and non-growing cells are less susceptible to antibiotics in comparison to actively growing cells, contributes significantly to resistance of biofilm bacteria to antibiotics [21, 23].

Biofilm bacteria in general exhibit higher levels of resistance to all classes of antibiotics [29, 30]. In comparison to their non-attached, individual planktonic counterparts, biofilm bacteria are in the range of 10-2000 times more resistant [29, 30]. Multiple mechanisms are involved in resistance of bacteria in biofilm to antimicrobial agents. First, depending of the type of biofilm and the poor penetration of biofilm by antimicrobial agents as the EPS which constitute the biofilm retard the diffusion of the antibiotics and the drugs cannot penetrate the full depths of the biofilm matrix [31, 32].

Rate of penetration also varies with the nature of the drug and structure of the biofilm. Antibiotic ciprofloxacin required 21 minutes versus 4 sec to reach a surface when the surface was coated with a *P. aeruginosa* biofilm or when no biofilm was present. Comparative analysis of susceptibility to antibiotic tobramycin revealed that biofilm cells were 15 times more resistant to the drug than their isogenic, planktonic counterparts [32].

Another factor that also contributes to resistance is the gene expression profile of bacteria in biofilm. A large number of genes are modulated as bacteria transit from free floating state to biofilm state and also gene expression of bacteria in different region of biofilm is different [2, 6]. This differential gene expression leads to modulation of a wide range of phenotypic characteristics including susceptibility to antibiotics. In addition, development of persistors and the biologically programmed response to growth on a surface are also considered to add to enhanced resistance of biofilm bacteria to antimicrobial agents [19, 24].

According to a report from National Institute of Health (NIH), USA, biofilm accounts for 60-70% percent of microbial infections in human [35]. Biofilm poses a great challenge as bacteria in biofilm exhibit varied protein expression profile as they are morphologically, physiologically and genetically heterogeneous [9, 21]. To make things more complicated with regard to the action of antibiotics, it has been found that certain antibiotics such as aminoglycosides induce biofilm formation at sub-inhibitory concentration [5, 36]. In a recent study Cook and Dunny[37] showed that biofilm growth increases plasmid copy number and expression of antibiotic resistance genes in *Enterococcus faecalis*. The plasmid copy number and the expression of resistance gene levels reverted to pre-biofilm formation state, once the bacteria were grown as planktonic culture. These findings further highlight the complexity and diversity that bacteria in biofilm life style acquire as the transit from planktonic state, which may interfere with effective drug development.

In addition to attempts to develop anti-biofilm therapeutic agents, two approaches are now in active perusal attempting to resolve chronic infection; modulating the host immune response and use of genomic and proteomic techniques to identify vaccine candidates [3, 6].

As the antimicrobial resistance of biofilm is higher, use of antibiotic at recommended dose is often unable to eradicate biofilm infection. Challenging biofilm with such sub-lethal dose often leads to partial disruption of biofilm, facilitating repopulation and formation of biofilm at newer locations [22]. As bacteria from a biofilm have enhanced potential to form new biofilm in comparison to their isogenic, planktonic counterparts [20], the eradication of the newer biofilms thus formed may be more difficult.

Biofilm can be made up of single species or multiple species of microorganisms. For example biofilm in the lung of patients with cystic fibrosis, *P. aeruginosa* is the primary organism. On the other hand dental biofilm may contain more than 500 species of bacteria [30]. In addition to chronic infection, biofilm also represent a cause of infections associated with the use of indwelling medical devices such as shunts, catheters, sutures, prosthesis and contact lenses [38].

Biofilm formation on medical devices is considered as a virulence factor and they pose a challenge in clinical settings as biofilm protect bacteria from antibiotics and host immune system. It is often impossible or undesirable to remove prosthetic device in use which may be necessary for eradication of biofilms [39].

Various approaches are at different investigational stages for development of methodology for prevention or reduction of biofilm formation on medical devices in clinical settings. These include coating implantable medical devices with trimethylsaline (TMS) which has been found to markedly reduce biofilm formation [40]. Biofilms of potable water distribution systems have the potential to harbor enteric pathogens, *L. pneumophila*, nontuberculous mycobacteria, and possibly *Helicobacter pylori* [41].

Host Immune Response to Biofilm: Numerous *in vitro* and *in vivo* studies unraveled multitude of mechanisms of host immune response to infecting bacterial pathogens; however, in vast majority of cases the infectious bacteria used was planktonic bacteria. As majority of infections are caused by bacteria in biofilm stage, the real scenario of host pathogen interaction remains largely unknown. Many host defense strategies which are highly lethal against single, planktonic bacteria are not effective gains biofilm bacteria leading chronic infections which are difficult to treat [3, 6, 8].

To formulate better antimicrobial strategies to eradicate biofilm bacteria from chronic infection settings, it is essential to understand the extremely complex and varied interactions between host defense systems and biofilms. It is now apparent that our standing of host-pathogen interactions needs to be re-evaluated with biofilm bacteria as most of the studies which cumulatively formed our understanding of antibiotic resistance and virulence of a pathogen came from studies with planktonic bacteria.

The bacteria embedded within clinically-relevant biofilms use quorum sensing based cell-cell communication system and often express new, more virulent phenotypes [42]. The structure of biofilms is such that host immune responses may be directed only at those antigens found on the outer surface of the biofilm [29].

In addition, bacteria have evolved and adopted numerous strategies to counteract the action of both innate and adaptive arms of the immune system. To make the scenario more complicated serum and salivary antimicrobial factors such as complement proteins, lysozymes are rendered ineffective as they fail to penetrate the biofilm [35, 42].

Studies have shown that interaction of neutrophils with biofilm is varied and complex. Intense accumulation of neutrophils at the site of biofilms has been demonstrated recently in biopsies from chronic wounds [43]. In addition, induction of biofilm formation was observed during the interaction between normal human neutrophils and *P. aeruginosa* [44].

Studies directed towards understanding of the correlation between biofilm formation and virulence of pathogens and how the immune system reacts to bacteria in biofilm revealed important finding and identification of putative drug targets for development of potential therapeutic strategies to control and eradicate such microbial communities [23].

Extensive research is being carried out to determine the mechanistic detail of persistent infections caused by pathogens. Recently, it has been found that *S. aureus* biofilms are capable of attenuating traditional host proinflammatory responses, which may explain why biofilm infections persist in an immunocompetent host [45].

In order to improve patient health and survival, understanding the complex interactions between the biofilm communities and the host defenses is essential. Meyle *et al* [46] showed that PMN recognize biofilms and activate defense-associated reactions, including phagocytosis, degranulation of lactoferrin and elastase, and DNA release resulting in destruction of biofilms showing that biofilms are not inherently protected against the attack by phagocytic cells.

Macrophage killing of *P. aeruginosa* in biofilm was less efficient in comparison to their isogenic, planktonic counterparts demonstrating that these attributes of biofilm may contribute to chronicity of *P. aeruginosa* infection for example as in the case of patients with cystic fibrosis [47].

Bacteria forming biofilms *in vivo* cause persistent infection and bacteria in biofilm causes inflammation which leads to stimulation of the immune system. So, interaction of immune system with the bacteria in biofilm plays a critical role in clearance of biofilm and resolving chronic infection [23, 25].

Moreover, because biofilm infections are often persistent, an odd situation appears with the simultaneous activation of both arms of the host immune response, neither of which can eliminate the biofilm pathogen, but instead, in synergy, causes collateral tissue damage [9, 22].

Nevertheless, more recent *in vivo* and *in vitro* studies on host-biofilm interactions have revealed that infected host mount both innate as well as adaptive immune responses to biofilms. On the other hand, studies have also shown that biofilm bacteria also adopt various immune evasion strategies to avoid clearance by the host. The mechanism of resistance of biofilm to host resistance includes;

a) Limited penetration of leukocytes and their products into the biofilm,

b) Global response regulators and quorum sensing that protects biofilm bacteria,

c) Decreased phagocytic capacity of host cells against biofilm bacteria,

d) genetic switches that increase resistance of biofilm bacteria,

e) Suppression of leukocyte effector function, including magnitude of respiratory burst [46, 37].

Bacterial cell surface components also play a role in susceptibility of biofilm bacteria to host immune system. Experiments have shown that in *P. aeruginosa* biofilm cells that lack flagella, neutrophil-secreted lactoferrin kills these bacteria efficiently [25]. Several bacterial pathogens form biofilms having complex interactions with components of the innate host defense system [23, 47]. Understanding the mechanistic detail of these interactions could lead to novel, biofilm-specific therapies.

Biofilm and Therapeutic Targets: Various physical, chemical and biological agents are being investigated for their effectiveness for in controlling biofilm both in *in vivo* and *in vitro*. Through understanding of the mechanism of anti-biofilm effect of various agents is important to formulate effective biofilm control methodology, as many common antimicrobial agents are effective against planktonic bacteria but are only partially or totally ineffective against the same bacteria in biofilm. An interesting observation that became evident through investigation of various researchers is that many antimicrobial agents also possess antibiofim activity (ability to inhibit biofilm formation and to disperse a preformed biofilm).

In addition, there are chemical compounds exhibiting profound antibiofilm activity with little or no antimicrobial activity. For example, aryl rhodamine efficient inhibited biofim formation by gram positive pathogen *S. aureus* but possessed no antibacterial activity [48].

Antidiarrheal agent nitazoxanide has been shown to successfully inhibit biofilm formation by the diarrhoeagenic pathogen enteroaggregative *E. coli* by inhibiting formation of fimbriae [49] which is one of the cell surface components of bacteria found to be involved biofilm formation in several pathogens. Lactoferrin, which also possess antimicrobial activity inhibited growth and biofilm formation by periodontopathic bacteria *Porphyromonas gingivalis* and *Provotella intermedia* exhibiting potential in prevention and treatment of periodontal diseases [50].

Other chemicals showing promise as antibiofilm agents include oxantel against *Porphyromonas gingivalis.* Dashper *et al* [51] showed that oxantel, a cholinergic anthelmintic and fumarate reductase inhibitor, significantly inhibited biofilm formation by *P. gingivalis* and disrupted established biofilms at concentrations below its MIC against planktonic cells. Histidine kinase inhibitor wakmycin C was found to be active against biofilm of another dental pathogen *Streptococcus muta* [52].

Slow growing bacteria and biofilm are extremely tolerant to antibiotics; but oritavanin kills stationary phase *S.aureus* and inhibits biofilm formation [53]. Sodium salicylate and antibiotic vancomycin in combination was highly efficient in eradicating biofilm of *S. epidermis* [54].

Antimocrobial peptides are also investigated as potential antibiofilm agent. A 9 amino acid long synthetic cationic peptide inhibited biofilm formation at 1/30 MIC is a significantly development in this regard.

Microarray analysis of *P. aeruginosa* exposed to this cationic peptide showed that 11 genes were involved in biofilm formation by this pathogen [55]. In biofilm, bacteria remain encased in a polymeric matrix synthesized by the bacteria themselves. Enzymes that degrade biofilm matrix polymers have been shown to inhibit biofilm formation, detach established biofilm colonies, and render biofilm cells sensitive to killing by antimicrobial agents. Although several enzymes have been tried, two enzymes deoxyribonuclease I and the glycoside hydrolase dispersin B particularly holds potential as biofilm matrix-degrading enzymes for the treatment and prevention of device related infections [56].

As c-di-GMP is found only in bacteria and this regulatory system has an essential role in biofilm formation, it constitutes and excellent target for drug development [57]. As quorum sensing has been shown to play a role in biofilm formation many bacteria, identification of quorum sensing antagonists is an attractive target for widespread antimicrobial strategy for biofilm control [58].

In an interesting study, Fu *et al* [59] demonstrated that a cocktail of *P. aeruginosa* bacteriophage could reduce the biofilm cell density of the pathogen on catheters by 99% in an *in vitro* model. Biofilm poses a formidable challenge in clinical settings. As the biofilm composition varies with the pathogen concerned in addition to surface, presence of other pathogens, availability of nutrients, and as the antimicrobial resistance of the pathogen concerned also varies within a single biofilm, it is difficult to assign a single therapeutic dose for the pathogen.

A completely novel approach to combat antibiotic resistance of bacteria in biofilm is underway. Instead of searching for new antibiotics, the researchers have questioned whether it is possible to rejuvenate older antibiotics so that these become more effective against the resistant bacteria. Oroidin, ageliferin and mauritiamine are secondary metabolites of marine sponges belonging to the genus *Agelas*. These metabolities have been found to possess the unique capacity of inhibiting formation of biofilm and more remarkably the capacity to disperse preformed biofilm [60]. These compounds have a core 2-aminoimidazole structure with different moieties and side groups in different compounds.

Derivatives of the core structure 2-aminoimidazole recently been developed and analyzed for antibiofilm activity [61].

Rogers and Melandar [62] synthesized 2-aminoimidazole/triazole (2-AIT) conjugate which exhibited potent antbiofilm formation and biofilm dispersion activity against common pathogens such as S. *aureus, P. aeruginosa, Acinetobacter baumannii* and *Bordetella bronchiseptica*. Interestingly, bacteriological studies indicated that inhibition of biofilm formation and dispersal of preformed biofilm activity of 2-AIT conjugate did not result in killing of bacteria but mediated the transition of bacteria from biofilm state to planktonic state. This creates an interesting scenario for effective eradication of bacteria in biofilm.

The anti-biofilm agent maintains bacteria in planktonic state by inhibiting biofilm formation or mediated transition of bacteria in biofilm to planktonic state and the antibiotic, which was ineffective against bacteria in biofilm, efficiently eliminates the sensitive, planktonic bacterial population. In addition to their antibiofilm properties, some of these molecules are able to resensitize resistant bacterial strains to previously ineffective antibiotics and are being assessed as adjuvants [59, 60].

Molecules that renders otherwise resistant bacteria into sensitive bacteria offer therapeutic strategy of enormous potential given that fact that bacterial strains exhibiting total resistant to all know antibiotics are being reported with increasing frequency from different parts of the world. Studies directed towards different aspects of such antibiotic rejuvenating molecules showed that these molecules augment the activity of conventional antibiotics by acting synergistically with the antibiotics and cooperate advantageously to overcome infections [60].

Additionally, such molecules were not toxic to various *in vitro* and *in vivo* toxicity assays including *Caenorhabditis elegans* (a free living nematode) fecundity assay until they reached well above their active (biofilm dispersion/ inhibition) concentrations. Further investigation revealed that this compound in combination with different antibiotics maintained the same low level of

toxicity when used alone in *C. elegans* assay further highlighting the potential use of this compound as antibiotic adjuvents at active concentration [63].

The explosion of multidrug resistance is the driving force of pharmaceutical research to develop newer, more effective antimicrobial agents. Considering the fact that majority of clinical infections are caused by bacteria in biofilm, there is a growing realization that approaches should be directed to biofilm based model of bacterial diseases and biofilm based assays should be used in determining the potency and efficacy of the antibiotics [64].

CONCLUSIONS: There is dynamic research activity in the emerging field of biofilm as it has been identified to be of paramount importance in public health because of their critical role in many infectious diseases and in a variety of infections related to medical devices. As is the case of many areas of biological sciences, *in vivo* biofilms are much more complex and difficult to study. However, current knowledge in bacterial biofilm provides a strong foundation to undertake a broad multidiscipilanry approach that is needed to fully rationalize the clinical significance of biofilm, understand the molecular basis of the disease caused by biofilms and rational approach to eradicate biofilm.

Studies are carried out directed at the kinetics of release of endotoxin, lipopolysaccharide (LPS) from biofilm *in vivo*, as LPS is a major source of chronic inflammation, which in turn is one of the major predisposing factors of many important diseases such as arthritis, obesity, chronic fatigue syndrome, diabetes and many other medical conditions. Detailed understanding of biofilm biology *in vitro* and *in vivo* settings and the genetic basis of these processes are the key requirement for development of anti-biofilm therapeutic agents by pharmaceutical and biomedical industries.

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