ACTA SCIENTIFIC DENTAL SCIENCES (ISSN: 2581-4893)

Volume 3 Issue 4 April 2019

Literature Review

# Current Molecular Perspectives in Biofilm Mediated Antimicrobial Resistance: A Literature Review

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**Received:** March 19, 2019; **Published:** April 01, 2019

## Abstract

Periodontal diseases are chronic complex diseases with a polymicrobial aetiology and marked inflammatory response leading to destruction of periodontal ligament and alveolar bone.

Periodontal treatment aims to eradicate pathogens associated with the disease and revert the periodontium to a healthy state. Periodontitis is initially treated by nonsurgical mechanical debridement and routine oral hygiene maintenance. However, mechanical debridement alone may not be helpful in all cases due to the microbial invasion into the underlying tissues. In such cases, adjunctive systemic antibiotic therapy along with mechanical debridement remains the treatment of choice. The emerging risk of empirical antibiotic prescription as a part of periodontal therapy, without evidence from microbiological evidence, is a major cause for antimicrobial resistance. Understanding how antimicrobial resistance mechanisms work from the molecular level will identify new targets for potential inhibition of multi-drug resistance and thus restore clinical utility of chemotherapy for treating infectious diseases caused by serious periodontal pathogens. The aim of this review is to provide an overview of the myriad molecular mechanisms that contribute to biofilm associated antimicrobial resistance in pathogens.

**Keywords:** Periodontitis; Systemic Antibiotics; Antimicrobial Resistance

## Introduction

The term ''periodontal disease'' includes various degenerative and inflammatory states of the gingiva, periodontal ligament, alveolar bone and dental cementum. The current model of etiopathogenesis for periodontitis suggests that the complexity between the host-microbial interactions may lead to overproduction of destructive enzymes and pro-inflammatory mediators that determine the extent and severity of tissue and alveolar bone destruction even though periodontal disease is said to be pathogen and site specific [1]. Understanding these host- microbial interactions has led to the better appreciation of the host immune responses and to the development of treatment strategies that may improve clinical outcomes and help in the overall management of periodontitis patients. Treatment of periodontal disease is mainly aimed towards the reduction or elimination of pathogens present in the subgingival biofilm matrix [2]. Mechanical periodontal therapy is usually the first line of treatment for most infections involving the periodontium and includes subgingival scaling and root debridement procedures. These traditional treatment methods has its own restrictions owing to its inability to completely eradicate the periodontal pathogens from the soft and hard tissue surfaces and from within non dental niches in the oral cavity which may cause recolonization of these pathogens leading to reinfection [3]. To overcome these limitations in the traditional mechanical periodontal therapy, the adjunctive use of antimicrobial agents either systemically, locally or topically becomes an imperative modality of treatment [1].

However as Haffajee AD et.al suggested, there are many aspects related to the selection and administration of systemic periodontal antibiotic therapy that remain unresolved [4]. Currently, most

systemic periodontal antibiotic treatment regimens are empirically prescribed by clinicians prior to any evidence from a microbiologic analysis of subgingival bacterial biofilm populations [5].

Antibiotic resistance is one of the emerging risks in systemic antibiotic therapy, compromising the efficacy of the treatment and increasing the likelihood of a clinical treatment failure. Those pathogens that develop an antimicrobial resistance are occasionally referred to as "superbugs". These superbugs have resulted in ineffectiveness of certain chemotherapeutic agents and hence infections persist in the body, increasing the risk of spread to others.

The aim of this review is to provide a summary on the countless molecular mechanisms that contribute to biofilm associated antimicrobial resistance in pathogens.

## Biofilm matrix

Biofilms are complex microbial communities that adhere to the biotic or abiotic surfaces, and the cells within the biofilm are contained in a self-produced matrix [6]. Biofilms are a medically important entity because their formation has been implicated in the pathogenesis of numerous bacterial infections that are difficult to successfully eradicate with the use of antibiotics [7].

The phenotypic switch from a free-swimming, planktonic lifestyle to a sessile existence in a biofilm is a highly regulated multifactorial developmental process which relies on numerous environmental and genetic factors differing from species to species. The classic developmental pattern of biofilm formation suggest that motile planktonic cells attach to a surface in response to a variety of environmental signals [8].

The structural and spatial arrangement of the biofilm bacteria can have an overwhelming impact on biofilm ecology. The bacteria within a biofilm uses a process of chemical communication known as "quorum sensing" that helps them to coordinate their metabolism and other complex interactions in order to adapt to the dynamic changes in the environment [9]. The maturation of the biofilm depends on certain cell-to-cell interactions known as "co-aggregation". The three dimensional structural organization of a biofilm allow cells to fix their locations with respect to each other and in turn, help in release of environmental mediators present within the biofilm providing added benefits for metabolic cooperation and niches [10]. For instance, cells that are situated at the centre of a micro colony are more probable to experience low oxygen tensions. This may provide a better growth medium for strictly anaerobic methanogens that are embedded in extrapolysaccharide [11].

## Need for Antibiotics in periodontics

The mechanical debridement of a dental biofilm and elimination of localized irritating factors are the basis of any periodontal therapies. Certain longitudinal studies have demonstrated the effectiveness of this approach based on mechanical debridement procedures, reinforcement of the patient's routine oral hygiene practices and periodic follow-up [12]. The effectiveness of this treatment is reflected by the disappearance of clinical symptoms, the reduction or elimination of periodontal pathogens and the return of beneficial bacterial flora. However, this treatment procedure has its own limitations. It should be noted that patients/sites respond varyingly to conventional mechanical therapy. Reduced effectiveness of the therapy may be explained by a set of patient-related factors which can be local or generalized, the extent and nature of attachment loss, local anatomic variations, and the form of the periodontitis and the composition of the biofilm formed [13].

The most commonly used systemically administered antibiotics (Table 1) show their action by invading the periodontal tissues and the pocket epithelium via serum. There they can destroy microorganisms that are inaccessible to mechanical debridement. Systemic antibiotic therapy also has the potential to suppress certain periodontal pathogenic bacteria and is therefore advantageous for the eradication and prevention of infections by periodontal pathogenic bacteria that invade the subepithelial periodontal tissues or that colonize non dental niches [14].

As noted by Slots J, these antibiotic treatment regimens, prescribed by clinicians appear to be without evidence of a microbiologic analysis of subgingival bacterial biofilm populations, even though periodontal disease is considered as a polymicrobial infection [5]. One of the most common and emerging risks being antimicrobial resistance, compromises the efficacy and clinical outcome of the systemic antimicrobial therapy.

## Resistance and tolerance

The study of how *in vitro* planktonic bacteria escape antibiotic treatment led to the definition of two different concepts: resistance and tolerance.

Resistance: growing in the presence of an antibiotic. Resistance is defined as the capacity of a microorganism to grow and multiply in the presence of a toxic compound (antibiotic or antiseptic). It is applicable to both bacteriostatic and bactericidal antibiotics. Resistance can be assessed by measuring the MIC of a compound, i.e., the lowest concentration inhibiting growth of a standardized inoculum of exponentially multiplying bacteria.

| Drug | Mechanism of action | Clinical use | Dosage |
|---|---|---|---|
| Tetracycline | Act by inhibition of protein synthesis by binding to 30 S ribosomes in the susceptible organism | • Adjuncts in the treatment of localized aggressive periodontitis (LAP).<br>• Arrest bone loss and suppress A. actinomycetemcomitans levels in conjunction with scaling and root planing. | 250 mg four times daily |
| Doxycycline or minocycline | Act by inhibition of protein synthesis by binding to 30 S ribosomes in the susceptible organism | • Effective against a broad spectrum of microorganisms.<br>• Suppresses spirochetes and motile rods as effectively as scaling and root planing, with suppression evident up to 3 months after therapy. | 100–200 mg q.d. for 21 days |
| Metronidazole | Acts by inhibiting DNA synthesis. | • For treating gingivitis, acute necrotizing ulcerative gingivitis, chronic periodontitis, and aggressive periodontitis. | 250 mg tid for 7 days |
| Penicillin | Interfere with the synthesis of bacterial cell wall, inhibit the transpeptidases so that cross linking does not take place | • Management of aggressive periodontitis, in both localized and generalized forms.<br>• Exhibits high antimicrobial activity at levels that occur in GCF for all periodontal pathogens except *E. corrodens, S. sputigena* and Peptostreptococcus, inhibits the growth of the gram positive facultative anaerobes | 500 mg tid for 8 days. |
| Clindamycin | Inhibition of protein synthesis by binding to 50 S ribosome. | • Assisted in stabilizing refractory patients<br>• Achieves higher levels of antimicrobial activity than other antibiotics. | 300 mg bid for 8 days (OR)<br>150 mg qid for 10 days |
| Azithromycin | Inhibit protein synthesis by binding to the 50 S ribosomal subunits of sensitive microorganisms and interfere with translation. | • Effective against anaerobes and gram negative bacilli.<br>• Penetrates fibroblasts and phagocytes in concentrations 100-200 times greater than that of extracellular compartment.<br>• Actively transported to sites of inflammation by phagocytes, then directly released into the sites of inflammation as phagocytes rupture during phagocytosis. | 250 mg/day for 5 days after initial loading dose of 500 mg |

**Table 1:** Commonly used systemic antibiotics in periodontal therapy.

Antimicrobial resistance can be broadly classified into 3 groups: (a) intrinsic, (b) mutational and (c) acquired resistance. An inherent resistance to an antibiotic, commonly referred to as Intrinsic resistance, is a feature that is a naturally occurring within the microorganism. In certain oral bacteria such as many streptococci, the lack of nitroreductases is inevitable to convert the metronidazole to its active metabolites and therefore are not affected by the drug [15]. A spontaneous chromosomal mutation that produces genetically-altered bacterial population which are resistant to the drug is referred to as mutational resistance. The change in a single nucleotide base can result in and mutations that lead to the development of resistance has been well documented for aminoglycosides and rifampin [15]. And finally, acquired resistance is referred to as the horizontal acquisition from another microorganism of a genetic element that encodes antibiotic resistance. Such acquired resistance process can occur by transduction, transformation or conjugation. Transduction is a process by which exogenous DNA is transferred from one microorganism to another by the action of a bacteriophage, while transformation is the process by which bacteria acquire segments of DNA that are free in the environment. In the process of conjugation, the passage of genetic material occurs by direct cell-to-cell contact, through a sex pilus or bridge. Conjugation is considered as the most common mechanism of transferring antibiotic resistance genes.

Tolerance: avoiding antibiotic-induced cell death. Tolerance can be defined as the absence of growth but the existence of bacterial survival in the presence of a bactericidal antibiotic. In contrast to resistance, tolerance can only be related with the use of bactericidal antibiotics. The two types of tolerance which have been described are namely genotypic and phenotypic. In case of genotypic tolerance the presence of a genetic modification leads to a reduced ability of the antibiotic to kill the bacteria and can be transmitted to the successor cells. In the case of phenotypic tolerance, the environment is unfavourable to the action of antibiotics, thus leading to a reduced ability to kill [16].

This phenomenon of combined resistance and tolerance is known as bacterial recalcitrance. Bacterial recalcitrance is multifactorial in nature and, depends on the class of antibiotic used, and involves different mechanisms [17].

## Biofilm mediated antimicrobial resistance

Almost without any exception, microorganisms in a biofilm is 1000 to 1500 times more resistant to antibiotics than in their planktonic state. The mechanisms of this increased resistance differ from species to species, from antibiotic to antibiotic and for the biofilm growing in different environmental conditions.

It has been proven that the resistance of bacteria to antibiotics is affected by their nutritional status, growth rate, temperature, pH, and prior exposure to subeffective doses of antimicrobial agents [18]. Changes in any of these parameters will thus lead to a variegated response to antibiotics within a biofilm. An important mechanism of resistance appears to be the slower rate of growth of bacterial species in a biofilm, which makes them less susceptible certain antibiotics [6]. Even though a biofilm matrix is not a significant physical barrier to the diffusion of antibiotics, they have certain properties that can retard antibiotic penetration. Certain strongly charged or chemically active agents can fail to reach the deeper zones of the biofilm since, the biofilm usually acts as an ion exchange resin removing such molecules/agents from the solution.

In addition, extracellular enzymes such as β-lactamases, formaldehyde lyase, and formaldehyde dehydrogenase may become trapped and concentrated in the extracellular matrix, thus deactivating certain antibiotics (especially those which are positively charged hydrophilic antibiotics).

"Super- resistant" bacteria have been identified to have multidrug resistance pumps and they bring about extrusion of the antimicrobial agent from the cell. Antibiotic resistance may also be spread through a biofilm by intercellular exchange of DNA. The high density of bacterial cells in a biofilm aids in the exchange of genetic information among cells of the same species and among different species. Conjugation (the exchange of genes through a direct interbacteria connection formed by a sex pilus), transformation (movement of small pieces of DNA from the environment into the bacterial chromosome), plasmid transfer, and transposon transfer have all been shown to occur in biofilms.

## Mechanisms of biofilm mediated antimicrobial resistance

The molecular insights of antimicrobial resistance has been discussed under the following headings (Table 2):

| |
|---|
| Biofilm matrix |
| • Antibiotic penetration |
| • Capsules or glycocalyx |
| • Antibiotic modifying enzymes |
| • Extracellular DNA |
| Nutrition and stress response |
| • Heterogeneity in metabolism and reduced growth rate |
| • Oxidative stress responses |
| • Efflux pumps |
| • Quorum sensing |
| Antibiotic resistance genes |
| • Horizontal gene transfer |
| • Mutation frequency |
| Persister cells |
| Multiple species interaction |
| Target modification |

**Table 2:** Mechanisms of biofilm mediated antimicrobial resistance.

## Biofilm matrix associated Antibiotic penetration

Although antibiotic penetration has been widely discussed as an antimicrobial resistance mechanism, it is generally accepted in literature that decreased antibiotic penetration through the biofilm matrix does not, for certain antimicrobial agents, adequately explain the increased resistance or tolerance of biofilms. Vrany and colleagues highlighted factors such as culture conditions and biofilm thickness on the capability of antibiotics to penetrate the biofilm [19]. All of the findings from the study suggested that the importance of decreased antibiotic penetration depends on several variables, namely the bacterial species or strain, the antimicrobial agent in question and the biofilm growth conditions. However, the role of reduced antibiotic penetration in promoting biofilm resistance is yet to be unleashed since even antibiotics that rapidly penetrate the biofilm do not cause significant cell death. It has been stated that slower antibiotic penetration, may give time for an adaptive phenotypic response that could possibly increase tolerance [20].

Reduced antibiotic penetration into the biofilm is likely a combination of various mechanisms and involve most of these factors working side by side as the biofilm matures. Antibiotic inactivation may be due to secretion of certain enzymes, such as β-lactamases, or binding of the agent by the exopolysaccharide

matrix. The exopolysaccharide could inhibit antimicrobial penetration by either binding the antimicrobial [21] or serving as a protective coating that prevents or delays diffusion through the biofilm [22]. The complex heterogeneity and interactions within biofilms has been evidenced in a study analysing different microenvironments throughout the biofilm which differ in metabolic activity, pH, and oxygen distribution [23].

### Capsules or glycocalyx

Glycocalyx is an inherent part of the biofilms, and its thickness varies from 0.2 to 1.0µm. This forms the outer membrane structure that is seen both in gram-positive and gram-negative bacteria [24]. The composition of glycocalyx is flexible and is usually regulated with biofilm growth that supports the pathogenic bacteria to survive within extreme adverse host environments [25]. The biofilm capsule components such as glycoprotein and polysaccharides are influenced by a number of environmental conditions. The adsorption sites of the matrix limit the transportation of the antibiotics and hence affecting its ability to inhibit the pathogenic bacteria and they serve as an adherent for exoenzymes [26]. The exoenzymes protect the motility of certain specific agents of antibacterial activity and provide a source of substrate for drug metabolite degradation that in turn resulted in slowing down the activity of susceptible drugs [27].

### Antibiotic modifying enzymes

The transformation of an antimicrobial agent to the nontoxic form is mediated by enzymes that provide resistance to biofilm. Certain bacterial species have been reported for degradation of toxic compounds such as aromatic, phenolic and other heavy metals (nickel, cadmium, mercury, antimony, silver, copper, zinc, lead, cobalt, etc) [28]. The process of detoxification usually occurs by the enzymatic reduction of ions and metal resistance genes. The presence of heavy metals were found to have induced the broader spectrum of resistant phenotype [29]. Enzymes present in the biofilm matrix, namely secreted β-lactamases, can degrade or cause inactivation of antimicrobial agents, thereby preventing these agents from reaching their cellular targets.

### Extracellular DNA

Extracellular DNA (eDNA) is an important and universal component of the bacterial biofilm matrix. While it is an important component of most bacterial biofilms, the molecular roles of eDNA in biofilm resistance and tolerance have been most thoroughly investigated in *P. aeruginosa*. eDNA may be endogenously derived from quorum-sensing-mediated release, outer membrane vesicle and autolysis of biofilm cell subpopulations, and exogenously from polymorphonuclear leukocytes at the infection sites [30].

The ability of DNA to transform bacterial cells growing in the oral biofilms has not been clearly understood. With many microbial species populating the oral cavity, including *Streptococcus* spp., *Neisseria* spp. and *Actinobacillus* spp., it is highly possible that transformation of DNA is a major contributor in the transfer of genetic material.

Saad Hannan and colleagues in a study, showed that *Veillonella dispar* transfers Tn916 to four *Streptococcus* spp. in oral biofilms, and moreover, the purified genomic DNA from *V. dispar* 34.2A can change *S. mitis* to tetracycline resistance. They also found that pAM120 was unable to transform any of the biofilm members to tetracycline resistance, showing that the source of DNA in the biofilm may determine its transforming host range [31]. The presence of uptake signal sequences (USS) makes *Neisseria meningitides* a suitable candidate for DNA transformation [32]. Various Gram-positive bacteria have no such USS requirement; but homologous DNA from related species is normally recombined into the cell's chromosome [33].

While the predominant source of eDNA in biofilms is likely to be derived from dead bacterial cells, there is recent evidence that some of the eDNA is actively transported from intact cells, as is the case for streptococci [34]. It was demonstrated that dna can be isolated from bacterial species [35]. Furthermore, transfer of tetracycline resistance from a transient Bacillus subtilis to a member of an oral biofilm [36] has also been demonstrated. The persistence of eDNA in the environment is dependent on many variables such as the presence of nucleases, but it has been demonstrated that they can survive in various environments for a considerable length of time [37]. Therefore, it is possible that this source of DNA could also be a source of genetic information, which can be used by the oral microbiota.

A study by Chiang WC and co-workers showed that regardless of whether the source of the eDNA is endogenous or exogenous, eDNA increases biofilm resistance to certain antimicrobial agents [38]. Release of eDNA by cells in a biofilm may also be affected by treatment with certain antibiotics. For instance, in some strains of *S. aureus*, release of eDNA in biofilms was improved by exposure to subeffective concentrations of methicillin in a process that is dependent on the AtlA autolysin; however, the sensing and signalling mechanisms that are required for eDNA to be released in response to methicillin are not entirely clear [39].

## Nutrition and stress response

### Heterogeneity in metabolism and reduced growth rate

The heterogeneities within an adapting population increases the probability of at least some individuals meet immediate or future challenges. The growth and metabolic rate activity of the bacteria are affected by the differences in nutrition and oxygen availability within biofilms. The level of bacterial growth and activity within the biofilm is proved by the different concentration of metabolic substrates and products [22]. The metabolic activities of cells were promoted by nutrients and oxygen in the peripheral region of biofilm, which helps in bacterial proliferation. In contrast, due to the poor diffusion of nutrients, the metabolic potential inside the niche is retarded and resulted in slow growing of the cells inside the biofilm matrix [40]. This is performed by the accumulation of guanine nucleotide-guanosine 3,5'-bis-pyro-phosphate (ppGpp) and the reduced level of RNA (tRNA and rRNA) synthesis. Information on metabolic and growth rate heterogeneity of cells were demonstrated comes from the cellular enzyme synthesis within the biofilm. The changes in the bacterial growth cycle influences the level of enzyme synthesis that is proportional to cell mass [41]. In stationary phase or slow growing bacteria, there is cessation of cellular enzyme synthesis. Antimicrobial agents kill the metabolically active bacteria, whereas at the dormant growth phase, bacteria are less susceptible to the antimicrobial agents and protect them from the antimicrobial action [42].

### Oxidative stress responses

There is evidence in literature to suggest that, in addition to contributing to cell death via their classically accepted mechanisms of action, bactericidal antibiotics kill bacteria by inducing the production of lethal levels of ROS [43]. Oxidative stress can be functionally defined as the excess production of pro-oxidants such as reactive oxygen species (ROS) in the cells and pose a significant threat to cellular integrity [44]. Studies have suggested that bactericidal antibiotics increase cellular respiration [45,46]. An increased oxidation rate of tricarboxylic acid (TCA) cycle-derived NADH by the electron transport chain results in enhanced superoxide radical formation, which upsets iron homeostasis by damaging iron-sulfur clusters. Ferrous iron from iron-sulfur clusters is oxidised to ferric iron in the Fenton reaction by hydrogen peroxide, which yields reactive and harmful hydroxyl radicals as by-products [47]. These hydroxyl radicals then contribute to cell death by oxidising substrates, such as deoxyguanosine triphosphate as well as macromolecules such as DNA [47]. Some other research groups have, put forward contradictory results, showing no increase in ROS production in antibiotic-treated cells [48]. It is much appropriate to propose that the mechanisms involved are complex and that the degree to which bacteriostatic antibiotics cause cell death by ROS will likely vary depending upon experimental set up, the bacterial strain and species, and the antibacterial agent being studied. Studies using addition of certain antioxidants, such as glutathione, at the time of antibiotic treatment significantly improved biofilm cell survival [49]. In addition to the novel mechanism for oxidative resistance of individual species for survival in periodontal pockets [44].

### Efflux pumps

Efflux pumps are found in almost all bacterial species and genes encoding them are located on the chromosomes or plasmids. Antimicrobial efflux pumps confer resistance to cells by moving antimicrobial agents away from their intracellular targets and back out into the extracellular space [50]. The mechanisms of efflux systems facilitate bacterial survival under adverse conditions, including antimicrobial agents. Efflux pumps exerts both intrinsic and acquired resistance to various antibacterial agents that belong to same or different families [51]. Overproduction of efflux pump can lead to multidrug resistance among biofilm bacteria. Along with other resistance mechanisms such as target modification and antibiotic inactivation, bacterial efflux pumps also exert multidrug resistance (MDR) phenotypes [52].

According to their composition, sources and substrates, bacterial efflux pumps are broadly classified into five different families such as the major facilitator superfamily (MF), the resistance-nodulation-division family (RND), the small multidrug resistance family (SMR), the ATP-binding cassette family (ABC) and the multidrug and toxic compound extrusion family (MATE) [53]. To activate antimicrobial agent efflux, the ABC family system cause hydrolysis of ATP, whereas the MF family, MATE family, and the RND family functions as secondary transporters, causing catalysis of drug ion antiproton (H+ or Na+) [54]. RND family transporters are the first line of defence in bacteria by serving as target mutation or drug modification and are species specific that is they are only found in Gram negative bacteria whereas the other four: MFS, ABC, SMR and MATE are found in both Gram positive and negative bacteria [55].

Exposure of the bacterial biofilm to lower concentrations of antibiotics, such as chloramphenicol and tetracycline, induces the expression of multi-drug resistance operons and efflux pumps [56]. Multidrug resistance phenotype in *E. coli* biofilm is regulated

by mar and acrAB encoding genes. A resistance phenotype can be developed by up regulation of mar encoded genes in planktonic bacteria by several antibiotics such as penicillin's, cephalosporin's, rifampicin, nalidixic acid and fluoroquinolones and oxidative stress agents [57]. In addition, subeffective concentrations of several commonly used medicinally important antibiotics such as tetracycline; chloramphenicol, salicylate and paracetamol can induce mar encoded gene expression level [58].

### Quorum sensing (cell to cell signalling)

Quorum sensing (QS) is a process of the cell-to-cell interaction or communication that regulates the behaviour of bacteria. It depends upon extracellular signalling molecules including its detection, production, and the presence of autoinducers. Quorum sensing includes the formation and secretion of an acyl homoserine lactones (AHL), which diffuse through the cell wall from the cell to the medium [9]. In gram-positive bacteria, quorum sensing secretes peptides as signalling compounds and a two regulatory system (membrane-bound histidine kinase receptor and an intracellular response regulator) to detect the required changes in gene expression pattern and the peptides [59]. Other than this, autoinducer-2 is another form of quorum sensing mechanism. This mechanism is found in both gram-positive and negative bacteria [60]. The signalling molecule production such as S-adenosyl methionine and acyl-carrier proteins are regulated by the active cells in bacterial growth and are influenced by the glycocalyx matrix and the degradation enzymes. The mechanism of quorum sensing has also been reported in the control of biofilm maturation [61]. The role of signalling molecule-mediated quorum sensing in biofilm formation has been demonstrated in many bacterial species. Quorum sensing systems influence the heterogenic nature of the biofilm for regulation of the degradation enzymes synthesis. Also, in suitable nutrient supply and environment, the expression of quorum sensing mediated phenotype is vital in cell migration and also protects from the deleterious environment of new modes of growth [62].

One way to assess the impact of quorum sensing on biofilm resistance is to inhibit, or 'quench', the quorum-sensing system in pre-established biofilm mattrices. Quorum quenching with small molecules was shown to enhance biofilm susceptibility to antimicrobial agents. In a study by Brackman and coworkers, inhibition of the TraP quorum-sensing receptor by hamamelitannin in pre-established *S. aureus* biofilms by reducing peptidoglycan synthesis, cell wall thickness and matrix eDNA content led to an increase in the antimicrobial efficacy of various cephalosporins, vancomycin, daptomycin, linezolid, tobramycin and fusidic acid [63].

### Antibiotic resistance genes

### Horizontal gene transfer

In addition to the ability of some bacteria to pick up eDNA from the biofilm matrix, horizontal gene transfer may also occur through the transfer of plasmids between cells in a biofilm via conjugation. Studies suggest that plasmid transfer between cells may be more efficient in biofilms compared to planktonic cultures, and this is likely aided by the sessile nature and spatial proximity of cells within a biofilm [64]. For example, in *Staphylococcus aureus*, the transfer frequency of a multidrug resistance plasmid by conjugation was greater in biofilms than in planktonic cultures, suggesting that biofilms may serve as a site of resistance-owing to plasmid transfer [65]. Integrons are genetic elements consisting of an integration site, a promoter that drives expression of mobilisable gene cassettes, and a gene encoding an integrase, which catalyses excision and integration of the gene cassettes. The gene cassettes in some classes of integrons frequently encode antibiotic resistance determinants, and these have been implicated in the dissemination of resistance genes between bacteria in the clinical setting [66]. Mobilisation of antibiotic resistance genes via integrons acts as evidence to how the biofilm enhances the ability to acquire antimicrobial resistance determinants through horizontal gene transfer.

### Mutation frequency

It is possible that cells grown in biofilms are inherently prone to spontaneous mutations due to the increased oxidative stress which contributes to DNA changes. Partial support of this hypothesis has been provided by the finding that *S. aureus* biofilms cultured with antioxidant compounds was shown to have a reduced mutation frequency that was comparable to the planktonic mutation frequency [67].

### Persister cell phenomenon

Persisters are the population of antimicrobial agent tolerant cells and are responsible for the severe chronic infectious disease. Bacterial biofilm contains resistant persister cells that exhibit multidrug and antimicrobial tolerance [68]. Late growing gram positive or gram-negative bacteria may exhibit multidrug resistance and antibiotic tolerance leading to tolerance or persistence.

Persister cells formation is controlled by various growth stages of bacterial communities, which are rapidly propagated and survive in the presence of lethal doses of antimicrobial agents [69]. High levels of persister cells were produced by stationary phase bacteria and correlated with the increasing resistance inside the biofilm. The immune system prevents the antibiotic action inside planktonic population, wherein there is survival of the persister cells [70]. Glycocalyx matrix helps the biofilm persisters to protect the immune system. The stages of bacterial growth decide the formation of persisters [71]. After termination of the antibiotics in sessile bacterial population, persister cells start re-inducing the growth of bacterial biofilm. The formation of persister cells also depend upon the metabolic activity rate of bacteria and was suggested that these cells are a dormant variant of the wild type and not mutant cells. Interestingly, persisters do not respond to exposure of bactericidal agent [72]. There is competition among the persisters for the antibiotic targets for the production of multidrug resistance (MDR) protein. It is noteworthy that antibiotics act as an antimicrobial by disrupting the function of target cells, rather than inhibition. Antibacterial compounds leads to cell damage. The tolerance phenomenon of persister cells has also been linked with programmed cell death (PCD) while the action of antimicrobial compounds leads the cell towards damage but not for complete cell death [73]. Within the biofilm, autolysis is the most common observation, which is performed by the peptidoglycan hydrolases called as autolysin. Kirby-Bauer disk diffusion test was studied in late growing bacteria which detect the bacterial resistance and evaluate the level of tolerance by replacing antibiotic discs impregnated by nutrients against certain antibiotics [74]. Persister cell formation in *S. aureus* stationary phase cultures has now been linked to ATP depletion [75].

### Multispecies interactions

Interactions between bacteria and fungi and their impact on biofilm susceptibility to antimicrobial agents have been studied in various experimental conditions of polymicrobial biofilms. In biofilms consisting of *C. albicans and S. aureus, S. aureus* associated with the fungal hyphae via the *C. albicans* Als3p adhesin and become coated in biofilm matrix probably derived from C. albicans [76]. Staphylococcal resistance to vancomycin was increased in polymicrobial biofilms formed by *C. albicans* and *S. aureus* which is due to the fungal matrix component, β-1,3-glucan, thought to act as a barrier to vancomycin diffusion in the biofilm [76]. There is an increasing need for the investigation of the complexities of polymicrobial biofilms and how species interact in biofilms to influence the antibiotic resistance of the members within the community.

### Target modification

Microbes have found ways to alter the molecular targets of antimicrobial agents. Altered targets may include, for example, DNA gyrase, a target of quinolone antimicrobial [77], RNA polymerase, a target of rifampin [78], the prokaryotic ribosome, a target of tetracycline and other protein synthesis inhibitors [79], and targets of antimetabolite drugs, such as the sulfonamides and related drugs [80]. An example of drug target modification is altering the penicillin binding protein (PBP) which is the target of β-lactam antibiotics in case of Staph. species. *Staphylococcus aureus,* the causative agent of serious infectious diseases, becomes resistant to these antibiotics by any one of the several mechanisms such as (a) mutation in PBP or (b) acquisition of new PBP with reduced affinity to penicillins, over expression of PBP, etc [81]. Methylation of drug binding targets on 16S rRNA by rRNA methyl transferases is responsible for aminoglycoside resistance in several bacterial species [82].

## Concluding Remarks

Systemic antimicrobial therapy can be used as an adjunct to mechanical therapy in patients with chronic periodontitis, patients who do not respond to mechanical treatment, and in patients with acute or severe periodontal infection. However, systemic antibiotics are to be used with caution. Antimicrobial resistance occurs naturally over time, as a result of genetic changes. Although drug resistance will likely persist and is to be expected, the overall level can be dramatically decreased with increased attention to the underlying molecular and cellular events related to antimicrobial resistance, the pharmacokinetic and pharmacodynamic properties of different drug formulations and evidences from microbiological analysis. The development of quick, effective molecular diagnostic techniques for identification of resistance genes of resistant pathogen can improve current strategies to combat antimicrobial resistance. Above all the things, measures to educate people about antibiotic resistance are an important strategy to prevent antimicrobial resistance.

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**Volume 3 Issue 4 April 2019**