

BIOFILM, DENTAL UNIT WATER LINE AND ITS CONTROL

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Abstract

Biofilms are well-organized communities of cooperating microorganisms that can include bacteria, algae, fungi and diatoms. Dental unit waterlines (DUWL) are an integral part of dental surgery equipment, supplying water as a coolant, primarily for air turbine and ultrasonic scalers. Surveys of dental unit waterlines (DUWLs) indicate that biofilm formation is a universal problem and great majority of bacteria that have been identified from DUWL are ubiquitous, although present in only low numbers in domestic water distribution systems, but can flourish as biofilms on the lumen surfaces of narrow-bore waterlines in dental units. DUWL contamination and its significance as a factor in nosocomial infection of patients and health care workers has stressed the risk to immunocompromized persons. Not only patients but also dentists and dental personnel are at risk of being infected with opportunistic pathogens such as *Pseudomonas* or *Legionella* species by means of cross-infection or after aerosol formation from water emanating from DUWL. Several methods of decreasing the level of contamination in DUWL have been proposed. At present, the goal of this review is to discuss various aspects of biofilm formation and effective standardized disinfecting methods to maintain low bacterial counts in dental water line. This will increase the awareness of potential health risks posed by biofilm formation and provide information on techniques and devices designed to control the microbial contamination of DUWLs.

Introduction

Bacteria exist in two forms i.e., planktonic (free swimming) and attached forms (in communities). Traditional studies of bacterial cells in planktonic (free-swimming) phase have focussed on pure culture physiology, a model for major microbiological studies today. However, the study of planktonic bacteria does not accurately reflect the growth of bacteria in nature because different microbial life style exists when bacteria live in association with different microorganisms and with different surfaces (1). Historically, Antonie van Leeuwenhoek was the first to examine bacteria from plaque on his teeth in the 17th Century followed by the observations of thus leading to the theory of biofilms (2).

A biofilm may be defined as a community of micro-organisms irreversibly attached to a surface, producing extracellular polymeric substances (EPS) (2). Bacteria in biofilm mode have an altered phenotype compared to their corresponding planktonic counterparts, particularly with regard to gene transcription, and in interaction with each other (2, 3). The conversion from a relatively simple planktonic cell to a complex, highly differentiated multi-cultural community is monitored by a close genetic regulation. In addition to bacteria, fungi, yeasts, algae, protozoa and viruses have also been isolated from biofilms in industrial and medical

settings but bacteria as microorganisms provide the best-studied model with regard to colonization of surfaces and subsequent biofilm formation (2). Furthermore, different biofilms are formed in different environments because of different hydrogeochemical properties. Depending upon the environment, in which biofilm formed, non living components also varies. Monocellular materials such as mineral crystals, corrosion particles, clay and silt particles, or blood components, from different environments may act as physical components of biofilms (2). Other important variables involved in cell-cell attachment and biofilm formation are: (i) properties of the substratum (texture or roughness, hydrophobicity, conditioning of film); (ii) properties of the bulk fluid (flow velocity, pH, temperature, cations, presence of antimicrobial agents); (iii) properties of the cell (cell surface hydrophobicity, fimbriae, flagella, EPS) (2).

BIOFILMS

Biofilms are heterogeneous and complex in structure, function and metabolism. The microbes in biofilm mode exhibit coordinate behaviour and live in cooperative consortia which is identical to higher multicellular organisms (3). There are number of reasons, due to which bacteria like to live in the form of biofilms; (1) Genetic material can be easily exchanged between microorganisms;

(2) Nutritive substances can be accessed much easily from the water phase; (3) Shelter can be provided by other organisms against excess of nutritive substances, toxic substances, drying and desiccation (2). Due to this joint relationship, biofilm bacteria are found to be more resistant to surfactants, biocides and antimicrobials. The various mechanisms conferring resistance to biofilms are: (1) Some of the cells of biofilm suffer from nutrient limitation and undergoes slow-growing or starved state. Thus, many antimicrobial agents are unable to target these slow-growing or starved cells (2) Another mechanism explores that 90% of dry weight mass of biofilm is comprised of exopolysaccharides (EPS). EPS protect the biofilms against deep penetration of antimicrobials in them. As a result, the cells present deeper in biofilms remain protected against bactericidal or bacteriostatic action of various antimicrobials. (3) Some of the cells have unique phenotypes in biofilms. Because they have anionic and hydrophobic nature, thus repel the biocides/disinfectants and protect the desiccation of biofilms. (4) Certain kinds of deposits are also present in the underlying surfaces of biofilms, acting as diffusion barriers. These diffusion barriers deactivate various antimicrobials and disinfectants and prevent their entry into biofilms (2). In one study, Xu *et al.* (4), using fluorescent probe and gene technology reported that only top one-fifth of the biofilm is metabolically active. Spatial heterogeneity due to physiological activity of biofilm is responsible for resistance against antimicrobial agents. Mechanisms like nutrient limitation and cell-cell signalling may switch cells into inactive non-growing protected phenotypes (4).

Biofilms are playing important roles in industries, medical settings, waste water treatments, terrestrial and aquatic ecosystems. One important aspect of biofilm is in detoxification of heavy metals. Bacteria present in biofilms either alone or in combined form with other microorganisms and in the presence of EPS components form an association which favours the detoxification and consequently removal of the heavy metals. EPS has negatively charged functional groups like pyruvate, phosphate, hydroxyl, succinyl and uronic acid (5).

Various genetics mechanisms play an important role in formation of biofilm. The planktonic bacteria which harbour plasmids, form healthy and thick biofilms as compared to the plasmid lacking strains. Strains without plasmids form only microcolonies without any further development and conversion into fully matured biofilm (6). If plasmids carry genes for resistance

to antimicrobial agents, then biofilm association will provide a mean of spreading bacterial resistance against various antimicrobial agents. One of the mechanisms responsible for transfer of resistance genes in bacterial biofilms is via natural horizontal gene transfer (conjugation). Conjugation occurs at greater rate between the cells present in biofilms than free swimming planktonic cells (6). This may be due to the reason that biofilm environment provides less shear force and better cell to cell contact resulting in greater conjugation ability. It has been reported previously that F conjugation pilus acts as a part in adhesion for both cell-surface and cell-cell interactions, resulting in development of a three-dimensional biofilm (7).

In humans, bacterial biofilms also play an important role with reference to both beneficial and harmful aspects. Among harmful aspects, one reported example is of caries, the result of a chronic undermining demineralisation of the teeth by organic acids that are produced by the bacteria of the dental biofilm while fermenting carbohydrates from the human diet (8). Another harmful aspect is catheter associated biofilm infections. The port of catheters is placed surgically or percutaneously in patients for long term effect. But it often leads to considerable morbidity, occasional mortality, and an increase in medical costs derived from its diagnosis, treatment, and mainly, prolongation of the patient's in-hospital stay due to development of biofilms in such devices (9). In contrast, in another study, human gut epithelial cells are a port for the development of mixed consortia of commensal bacteria. These mixed consortia of commensal bacteria provide a barrier against food borne pathogens. Other experimental studies and results from various repeated trials under controlled conditions have shown that certain gut bacteria, particularly species of *Lactobacillus* and *Bifidobacterium*, may exert beneficial effects in the oral cavity by inhibiting cariogenic *streptococci* and *Candida* sp. (10). Formation of dental plaque on teeth is also a good example of biofilm formation in both healthy and diseased mouths (11). Similar to plaque which is omnipresent, biofilm formation within the small bore plastic tubing in dental unit water lines (DUWL) is quite common. Dental units, in general, are equipped with different types of plastic tubings. The tubings are of different diameter and are most important surfaces for the development of biofilms. Biofilms develop within various tubing samples differ from one another in their size, texture and resistant to antimicrobials/biocides (12).

DUWL provide a particularly favourable environment for biofilm formation (13). Water at the tubing walls is almost stagnant, allowing bacteria to adhere and colonize the tubing surfaces. In DUWL, biofilm formation starts by presence of conditioned layer. Molecules of water may adhere to lumen surfaces by utilizing physical adsorption and chemisorption mechanisms. Once the conditioned substratum is formed, it can attract other molecules. The van der Waal's forces, electrostatic forces, hydrophobic forces, or chemisorption of bacterial fimbriae, pili or adhesions are few means which are helpful in attachment of different molecules (14). After adherence, the molecules enter the second phase i.e., quiet phase of surface associated lag time. In this phase bacteria prepare themselves for different types of adaptations. Some changes/changes in gene expression can be accomplished in this phase (15). After division and making phenotypic shifts, bacteria enter into the rapid phase of growth. During this phase, they secrete the cemented material (EPS), which binds the cells and protect them from shearing force of the fluid. Different microcolonies grow within the matrix, thus coaggregation of different microbes with each other and matrix increase the depth of the biofilms. Once bacteria adhered irreversibly, they increase their density enormously compared to the planktonic bacteria and it is at this stage that they secrete certain autoinducer signal molecule (16).

The risk of acquiring infections through DUWL supplies are known to be not very uncommon. Often potential pathogenesis can spread through surgical procedures, local mucosal contact, ingestion and inhalation (17). Different standards and strategies have been adapted to control DUWL transmitted infections. According to American Dental Association (ADA) (18), dental water should not have more than 200 colony-forming units per millilitre (CFU/ml) of aerobic, mesophilic, heterotrophic bacteria. Different methods like (1) antiretraction valves and retrograde aspiration of oral fluid; (2) filtration; (3) drying; (4) flushing of biocides; (5) Sterile water delivery systems (6) use of biocides/chemical disinfectants have been evaluated previously. Various authors have reported the use of biocides/disinfectants as effective decontamination methods to control DUWL contamination (13). Biocides are non-antibiotic, antiseptic, disinfecting chemical compounds, having both bactericidal and bacteriostatic properties (19). Other properties include that these should be effective at low concentrations, should be non toxic and biodegradable (19). The biocidal

action depends on (i) chemical properties (e.g. optimum pH and temperature of activity, reactivity), (ii) micro-organism (e.g. tolerance/resistance, metabolic status, number of organisms in the population), (iii) environment (e.g. surface type, water activity, presence of other reactive compounds). The biocide should therefore have a wide range of activity, both in terms of type of microorganisms susceptible and conditions of action (19).

Despite their extensive use and long history, the mode of action of a number of biocides has not been clearly established. Biocides affect a number of different target sites in microorganisms, which appears cumulatively to result in a loss of microbial viability. The effect of biocides on multiple target sites in microorganisms is probably the principal reason for the lack of development of bacterial resistance to biocides. Several biocides have been utilized as oral care antiseptics for decades without any adverse microbiological reports (20).

Different biocides are in use in DUWL including sodium hypochlorite, chlorhexidine gluconate, povidine iodine, peroxide (13), peracetic acid (21), ethanol, and glutaraldehyde. Integral automated flush systems in DUWL are commercially available. They employed glutaraldehyde flush systems in dentistry (22). Application of biocides to control DUWL biofilm contamination may be either as periodic shock treatments or by continuous treatment system (23). In these two treatments, different biocides not only act on biofilms differently but also effectively at varying concentrations. Furthermore, biocides behave differently against free planktonic forms and biofilms attached to various surfaces. For example, diluted solutions of sodium hypochlorite (NaOCl) effectively removes planktonic cells, but biofilm shows 150-3,000 times more tolerance against diluted solutions of NaOCl. Sims *et al.* (24) reported the effects of using varying concentrations (0.5%-5.25%) of bleach in dental settings. According to him, although bleach is effective in biofilms from tubing samples but it also causes (i) slow corrosion of metal fitting in dental units (ii) compliance problems in private practise dental settings (iii) reacts with matrix to create chlorinated by products (24).

CHLORINE DIOXIDE

Chlorine dioxide is another biocide which effectively removes biofilms, prevents metal corrosion and fouling of reverse osmosis membranes. In dental settings, 0.1% stabilized chlorine dioxide is also used as mouth rinse. It

reduced the bacterial counts in effluents of four stimulated DUWL to less than 200 CFU/ml. Stabilized chlorine dioxide used in private practice setting as well as its application as a lavage with ultrasonic scalers, result in significant ($p < 0.05$) reduction heterotrophic plate count (HPC) (25). Ethylene-diamine-tetra-acetic acid (EDTA), a divalent cation chelating agent, has been proved to be very effective agent against biofilms. It prevents catheter related infections by medically important microorganisms (26). Since bacteria from the biofilm are shed continuously while the film is in contact with water. Use of compounds like UV, hydrogen peroxide and ozone are advantageous in this situation. They can be continuously added into the water lines during patient treatment. Thus maintaining low levels of planktonic counts throughout the working day. Hydrogen peroxide has been used in dentistry as a bleaching agent, root canal irrigant, in dentrifices and mouth rinses. It has been used as a disinfectant (7% solution) for flexible endoscopes (27).

OTHER PRODUCTS

The other products, including dialox, sanosil, sporklenz, sterilex ultra would require evaluation in terms of materials compatibility before they could be recommended for routine use in DU waters (DUWS). A number of the other products, including alpron (a three-part component cleaner containing sodium hypochlorite, citric acid, and sodium- toluolsulfonechloramide), sterilex ultra (alkaline peroxide), and oxigenal (hydrogen peroxide), were reported to be effective in DUWS and resulted in a complete kill of planktonic cells as well as removal of biofilms (28).

Other oral antiseptics or chemical agents that have antimicrobial properties which are commonly used include: quaternary ammonium compounds, phenolic compounds, halogens, alcohols and heavy metals. These agents are chosen to be active ingredients of oral health care products because of their antimicrobial properties. They are safe to use in their normal working doses and stable over reasonably long shelf-life. Chlorhexidine and Bio2000 (active agents ethanol and chlorhexidine) achieved a complete kill of the total viable count (TVC) (13) but did not completely remove the biofilm. Likewise, the aldehyde-containing products tegodor and gigasept rapid eliminated the biofilm TVC (i.e., no viable cells were detected) but were unable to completely remove biofilm from the surface. However, the use of aldehyde-containing products may require occupational exposure monitoring for dental staff (28).

CHLORHEXIDINE

Chlorhexidine is among one of the most tested Compounds. At high concentration it is bactericidal and in regular concentration (0.12-0.2%) it is bacteriostatic. It also has good substantivity in the mouth. The chlorhexidine mouthrinse is also commonly used for symptomatic treatment of recurrent aphthous stomatitis/ulcers (29).

The emergence of bacterial resistance following biocidal exposure is not novel and has been described since the introduction of biocides in clinical practice. Bacterial isolates from clinical settings showing increased tolerance due to natural evolution, adaptations or lateral gene transfer and mutations have been documented in several studies (19). In addition there have been many reports highlighting the failure of disinfectants used for clinical applications (19). Biocides resistance in bacteria have been studied *in vitro*. Several mechanisms like efflux systems, intracellular traps, extrachromosomal precipitation at cell wall and degradation of biocides are important with reference to the biocides resistances in free planktonic microorganisms as well as in biofilms (19). The exact mechanisms of resistance in various strains are still being studied but it is clear that biocide resistance is an important clinical phenomenon (13).

Bacterial mechanisms are dependent upon the interaction of the bacterial cell wall, outer membrane or the spore outer layers with the biocides. They may act as permeability barriers to the intracellular uptake of antibiotics and biocides (30). Depending upon the type of biocide alongwith used concentration, it may damage DNA, proteins or enzymes, cell wall, cytoplasmic membrane resulting in death of microbes. Additionally, action of biocides on microorganisms also depend on the environmental conditions and the type of microorganism itself. The bacterial cell wall plays an integral role in relation to inactivation or insusceptibility to biocides (31).

The cell wall of Gram-positive bacteria has been recently studied (31). It consists essentially of highly cross-linked peptidoglycan, which can provide about 90% of the wall structure, together with 'secondary' wall polymers (teichoic acids, polysaccharides and proteins), which are covalently linked to peptidoglycan. The peptidoglycan is made up of amino sugars (N-acetylglucosamine and N-acetylmuramic acid) and various amino acids, some of which are in the unnatural D-form. The peptidoglycan and associated anionic polymers permit the entry of

large molecular weight polymers. The teichoic acids are major cell wall components of most Gram positive bacteria (31). Mostly, they are polymers of ribitol or glycerolphosphates attached to glycosyl and D-alanine ester residues.

The 20th century initially offered the use of antibiotics to fight against bacterial infections, but ended with the gloomy scenario of emerging multi-resistant bacteria. But 21st century emerges as a post-antibiotic era, highlighting the importance of novel strategies to control bacterial diseases. One of the novel strategies in use is to target the quorum sensing (QS) system of bacteria (16).

Quorum sensing (QS) is not only important for intraspecies survival and differentiation in bacterial communities, but also relates interspecies information between symbionts and competitors (16). It regulates gene expression by producing and responding to secreted autoinducers (AIs) whose concentrations reflect the population density, commonly exists in bacteria. Gram-negative bacteria use acylated homoserine lactones as autoinducers AIs, and gram-positive bacteria use oligopeptides (16). Among the gram-negative bacteria, two quorum-signaling mechanisms have been identified i.e., the *LuxI/LuxR* system and the *LuxS* system. In first system, bacteria use an acetylated homoserine lactone signal molecule. When the cell density is high, the binding of AIs to cell receptors regulates gene expression for a variety of phenotypes, such as production of specific virulence factors, protein production, bioluminescence and biofilm formation (16). Generally, each bacterial species uses its own signal; however, a common AI-2 signal has been discovered for interspecies communication (32).

Autoinducer-2 (AI-2) is the only species-nonspecific autoinducer known in bacteria and is produced by both Gram-negative and Gram-positive organisms. Consequently, it is proposed to function as a universal quorum-sensing signal for interaction between bacterial species possessing the characteristic *luxS* gene. The *luxS* gene is highly conserved among many species of gram-negative and gram-positive bacteria. AI-2 is able to regulate a range of genes and cellular processes. The extent to which AI-2-based signaling represents true quorum sensing or is dependent to some degree on the metabolic status of the bacterial cells remains to be determined. AI-2 is involved in mixed-species biofilm formation and interspecies gene regulation (16).

The chemical structure of the actual signal is still under investigation; however, crystallographic studies of the AI-2 receptor in *V. harveyi* seem to

suggest that AI-2 is a furanosyl borate diester formed from the metabolite 4,5-dihydroxy-2, 3-pentanedione (33). Unlike to *luxL* and *luxM* genes which are AIs for *V. harveyi* system 1 autoinducer (AI-1). These are important for the synthesis of hydroxybutanoyl-L-homoserine lactone, an important signalling molecules identified by purification of AI-1 QS system. Whereas, the ecological role of *luxS* in bacteria is still poorly understood, but it functions to allow bacteria to optimize gene expression in response to the density of all *luxS*-containing species occupying the same niche. *LuxS* converts S-ribosylhomocysteine to 4,5-dihydroxyl-2, 3-pentanedione, catalysing AI-2 formation (32). Whereas type II QS in the regulation of expression of virulence-related factors, motility, secretion systems, regulatory proteins, and polypeptides involved in the acquisition of hemin (32). Certain environmental conditions have been reported to regulate the AI-2 by bacteria. For example AI-2 activity in dental unit water line biofilm isolates is influenced by the presence of certain preferred carbon sources i.e., glucose (16).

Conventional methods used to control bacterial infection have been resulted in the development of resistant isolates (13). However, one novel method is to fight against bacteria by interfering with their command language and disrupting their virulence at non growth inhibitory concentration, thus without increasing their resistance profile (34). Number of studies have identified several molecules that function as QS inhibitors (16,34, 35). Identification of such inhibitors could present us with new opportunities for the development of novel nonantibiotic drugs for treating bacterial diseases in humans as well as in other animals and plants. As compared to conventional antibiotics, QS inhibitory compounds (QSIs) that do not kill or inhibit microbial growth are less likely to impose a selective pressure for the development of resistant bacteria (16). Furthermore, QSIs are not expected to cause harm to beneficial flora (34). Rasmussen *et al.* (35) identified two QSIs i.e., patulin and penicillic acid during screening a selection of *Penicillium* sp., Using DNA microarray-based transcriptomics, patulin (PAT) and penicillic acid (PA) were found to downregulate 45% and 60%, respectively, of the QS-regulated genes in *P. aeruginosa*, thus indicating specificity for QS-regulated gene expression. These approaches, also known as 'quorum quenching', 'anti-pathogenic', or 'signal interference', have been considered as feasible ways to prevent and combat bacterial infection (16, 34).

Conclusion

Microbial biofilms is proceeding on many fronts. One important aspect is the elucidation of the genes specifically expressed by biofilm-associated organisms, evaluation of various control strategies for either preventing or remediating biofilm colonization of medical devices. Role of biofilms in antimicrobial resistance including treatment of medical devices through the use of antimicrobial agents and antimicrobial locks as well as development of new methods for assessing the efficacy of these treatments are in progress. Biofilm in dental unit waterlines, once established, has proven hard to remove by applying disinfectants/biocides. There is a clear requirement for a reliable, relevant laboratory method to prevent microbial contamination within DUWLs, thereby permitting the objective evaluation of antimicrobial and antibiofilm products to control such contamination. The method must be economical and require minimal effort to use on the part of dental staff. There are currently no rational, evidence-based guidelines available to dentists for the control of DUWL contamination. The prevention strategies which are designed to reduce the impact of the biofilm in DUWLs are a real and continuing problem. Education should stress the need for improvement in the quality of water delivered to patients during treatment.

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