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Biofilms in Human Diseases: Treatment and Control

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Foreword

Biofilms are the most common mode of bacterial growth in nature. Highly resistant to antibiotics and antimicrobials, biofilms are the source of more than 65% of healthcare-associated infections, which, according to the World Health Organization (WHO), affect 1.4 million people annually. Biofilms are involved in 80% of all microbial infections in the body, including those associated with medical devices such as catheters, endotracheal tubes, joint prostheses and heart valves. Biofilms are also the principal causes of infections of the middle ear, dental caries, gingivitis, prostatitis and cystic fibrosis. Importantly, biofilms also significantly delay wound healing and reduce antimicrobial efficiency in at-risk or infected skin wounds.

Biofilms in Human Diseases: Treatment and Control outlines the scientific evidence and rationale for the prevention of biofilms infection; the role biofilms play in infection control and the issues concerning their resistance to antimicrobials. This book provides practical guidance for healthcare and infection control professionals, as well as students, for preventing and controlling infection.

The study of biofilms and the diagnosis of bacterial biofilm-associated human diseases have undergone change over the years. Formal testing for biofilm using quantitative qualitative tests is now only available in academic institutions where it serves as the standard methods of diagnosing bacterial biofilm infections. In practical terms, the establishment of diagnosis in bacterial biofilm infections has moved from quantitative and qualitative tests followed by scanning electron microscopic analysis and real-time polymerase chain reaction testing. These changes provide a new understanding of the diseases that cause biofilm infections, as well as necessitating changes in the way we approach the constellation of disorders that are caused by bacterial biofilm.

The tradition of expertise in the study of biofilms and preventions has been handed down. It is highly appropriate that this textbook is edited by Dr. Sunil Kumar and co-editors, who are one of the foremost researchers in this present generation and has continued the study of biofilm and human diseases associated with it. The book, through contributions by experts in the field, comprehensively

reviews the clinical features; etiology, histopathology, diagnosis and management of different biofilm-associated human diseases review some specific disorders that are of current significance. It will provide a valuable resource to all practicing clinicians and microbiologists.

Barabanki, India Dr. Sunil Kumar

Preface

A bacterial biofilm is a complex community of bacteria attached to each other, or associated with a surface or interface, and encased in extracellular polymeric substance (EPS). The composition of the EPS is complex and may contain polysaccharides, proteins, nucleic acid, lipids and metals. The EPS provides the 'house' of the biofilm, giving the residing microorganisms a safe haven from the effects of host immunity or administered antimicrobials. Bacteria within the bacterial biofilm can be responsible for causing and prolonging infection and human disease. Bacterial biofilm infections are, in general, healthcare-related, including those associated with the use of medical devices such as urinary and central venous catheters, endotracheal tubes and orthopedic prostheses. Other bacterial biofilm-related infections are prostatitis and those of chronic wounds. Many of the infections are of growing significance because they are related to the ever-increasing aging population. The Centers for Disease Control and Prevention report that $\sim 70\%$ of all human healthcare-associated infections originate from bacterial biofilms.

It is more important to know that the bacteria within the biofilm are significantly more tolerant of antimicrobials compared to their planktonic counterparts. This antimicrobial tolerance by biofilms can be 1000-fold higher than the susceptibility of free-floating or planktonic bacteria. Consequently, bacterial biofilms pose a significant challenge to patients in both hospital and community healthcare settings. Addressing the prevention and control of biofilms will dramatically help in decreasing infection rates, patient morbidity and mortality. This in turn will reduce the escalating costs of bacterial biofilm-related infections faced by the healthcare profession. Biofilms in Human Diseases: Treatment and Control is the first book that deals specifically with the fundamentals of infection control and biofilms in healthcare. The book is divided into 19 chapters; it begins by describing the biofilms and its type in Chap. 1. This chapter introduces the reader to biofilm formation and its role in human diseases and discusses the basic principles of biofilm. Rest of the other chapters address the challenges facing healthcare providers—infection prevention, hand hygiene, decontamination and the significance of changing practices in healthcare and introduce readers to infections associated with invasive devices and wounds.

This book focuses clearly on the area of biofilms and the problems they pose to humans. Subsequent chapters offer extensive reviews on biofilms health diseases, biofilms of medical devices, antimicrobials, microbial resistance and biofilms and their association with urethral and central venous catheters. The final chapter covers biofilms and its control using probiotics.

Biofilms in Human Diseases: Treatment and Control provides biologists, medical personnel, healthcare workers, infection control professionals, microbiologists, as well as students and academics, with a practical text to support clinical practice. It will help healthcare workers understand the evidence base and rationale for biofilm infection prevention in an easy-to-follow format. This book is multi-formatted, with some chapters providing healthcare practice to combat biofilms and others presenting information on specific biofilm-related infections. Overall, this book will provide its readers with a comprehensive, concise and informative text that highlights the significance of biofilms in infection control and the urgent need to prevent their formation. This is an area that is frequently overlooked and neglected in modern medical and healthcare education.

Barabanki, India Dr. Sunil Kumar

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Abbreviations

ABU	Asymptomatic bacteriuria
AD	Atopic dermatitis
AGNB	Aerobic gram-negative bacilli
AHL	Acyl homoserine lactones
AI-1	Autoinducer-1
AI-2	Autoinducer-2
AIP	Autoinducer peptides
ampC	Ampicillin C
AMPs	Antimicrobial peptides
APTMS	Amine functionalization
ASEM	Atmospheric scanning electron microscope
BCE	Before Common Era or Before Current Era or Before Christian Era
BFM	Bifidobacteria-fermented milk
BIND	Biofilm-integrated nanofiber display
BLIS	Bacteriocin-like inhibitory substance
BV	Bacterial vaginosis
C. albicans	*Candida albicans*
C10:Δ2	*cis*-2-decenoic acid
C2DA	*cis*-2 decenoic acid
C4-HSL	N-butanoyl-1-homoserine lactone
CAUTI	Catheter-associated urinary tract infection
CF	Cystic fibrosis
CFSM	Cell-free spent media
CFU	Colony-forming units
cis-DA	cis-2-Decenoic acid
CLABSI	Central line-associated bloodstream infection
CLSM	Confocal laser scanning microscope
CoNS	Coagulase-negative staphylococci
COPD	Chronic obstructive pulmonary disease

CRC	Colorectal cancer
CVC	Central venous catheter
3D	Three dimensional
DEBS	Dry eye blepharitis syndrome
DNA	Deoxyribonucleic Acid
dPNAG	Deacetylated PNAG
DSF	Diffusible signal factor
E.coli	*Escherichia coli*
EAC	Esophageal Adenocarcinoma
EAEC	Enteroaggregative *Escherichia Coli*
Ebp	Endocarditis- and biofilm-associated pilus
eDNA	Extracellular DNA
ENT	Ears, nose and throat
EPS	Extracellular polymeric substance
ESEM	Environmental scanning electron microscope
ETT	Endotracheal Tubes
FA	Fatty acids
FAI	Fatty acid inhibitor
FAO	Food and Agriculture Organization of United Nations
Fg	Fibrinogen
FISH	Fluorescent in situ hybridization
Fn	Fibronectin
FruA	Exo-β-D-fructosidase
FTF	Fructosyltransferase
GDA	Glutaraldehyde
GERD	Gastroesophageal Reflux Disease
GI	Gastrointestinal
GT	Glucosyltransferase
HAAs	3-(3-hydroxyalkanoyloxy) alkanoic acids
HCAI	Healthcare-associated infections
HCB	Hydrocarbonclastic bacteria
HICA	2-Hydroxyisocaproic acid
IBD	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
ica	Intercellular adhesion
ICK	Infectious crystalline keratopathy
IL-18	Interleukin 18
IOL	Intraocular lens
IUPAC	International Union of Pure and Applied Chemistry
K. pneumonia	*Klebsiella pneumoniae*
LA	Lyngbyoic acid
LAB	Lactic acid bacteria
MBC	Minimum bactericidal concentration
MDR	Multidrug resistant
MDRB	Multidrug-resistant bacterium

MIC	Microbial influenced corrosion
MIC	Minimum inhibitory concentration
MPC	Methacryloyloxyethyl phosphorylcholine
MRS	Man–Rogosa–Sharpe
MRSE	Methicillin-resistant *Staphylococcus epidermidis*
MRSHa	Methicillin-resistant *Staphylococcus haemolyticus*
NAC	N-acetyl cysteine
N-AHL	*N*-acyl-homoserine lactone production
Nd:YAG	Neodymium-doped yttrium aluminum garnet
P.aeruginosa	*Pseudomonas aeruginosa*
PAH	Polycyclic hydrocarbons
PAS	Periodic Acid–Schiff
PDMS	Polydimethylsiloxane
PIA	Polysaccharide intercellular adhesion
PMMA	Polymethyl methacrylate
PNAG	Poly-β(1,6)-*N*-acetyl-D-glucosamine
PS	Polysaccharide
PslG	Psl-specific glycoside hydrolase
QCM	Quartz crystal microbalance
QS	Quorum sensing
QSI	Quorum sensing inhibitors
16S rRNA	16S ribosomal ribonucleic acid
S. aureus	Staphylococcus aureus
S. haemolyticus	Staphylococcus haemolyticus
SAMs	Self-assembled monolayers
SCFA	Short-chain fatty acids
SCVs	Small-colony variants
SEM	Scanning electron microscope
SIBO	Small intestinal bacterial overgrowth
Spp.	Species
SRBs	Sulfate-reducing bacteria
SSI	Surgical-site infections
UC	Ulcerative colitis
UCAs	Ultrasound contrast agents
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection
UV	Ultraviolet
VAP	Ventilator-associated pneumonia
Vn	Vitronectin
VVC	Vulvovaginal candidiasis
WHO	World Health Organization

Chapter 1
An Introduction to Microbial Biofilm

Sunil Kumar, Ankita Srivastava and Saumya Rastogi

Abstract Bacterial biofilms are a major cause of human and animal disease. They are associated with antibiotic resistance among pathogenic bacteria. Bacterial biofilms can be harmful as well as useful. In this chapter we explain bacterial biofilm formation and composition and consider many of the harmful and beneficial effects of bacterial biofilms. Additionally, this chapter contemplates control strategies and the future of biofilm studies.

Keywords Microbe · Biofilm · Bacteria · Control · Human

1.1 Introduction

From the early work of bacteriologists—the fathers of current biological sciences—to the 1970s, bacteria were more or less thought of as single, free-floating microorganisms. Utilizing this pure microorganism culture model, scientists were able to study several harmful bacteria and develop biocides to kill them (Donlan and Costerton 2002; Wang et al. 2017). Huge numbers of drug-resistant bacteria and the subsequent issue to kill bound bacterium crystal rectifier required a re-evaluation of microorganisms and a reconsideration of the way bacteria aggregate among self-generated matrices, known as biofilms, endowing them with mechanisms for resisting biocides (Marcinkiewicz et al. 2013; Song et al. 2016). Biofilms were determined a couple of centuries before their connection to ill health (Brandwein et al. 2016). In 1684, Antonie van Leeuwenhoek, some microscopical observations, about animals in the scurf of the teeth, the substance called worms in the nose, the cuticula consisting of scales called biofilm (Lane 2015). Throughout the earlier part of the twentieth century several scientists reported that the majority of bacteria were not free-floating but were hooked up to numerous surfaces, like rocks sitting at the bottom of lakes (Dang and Lovell 2016). Scientists began to comprehend that some sessile bacteria

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were directly associated with ill health—in 1977 a *Pseudomonas aeruginosa* aggregation was found in bodily fluid samples from the lungs of patients infected with fibrocystic disease of the pancreas (Bjarnsholt et al. 2009; Hauser et al. 2011). In 1978, Clark reported that a crucial element of mutant bacterial biofilm sugar, glycocalyx, appears on teeth (Donlan 2002; Joo and Otto 2012). Castleton formally introduced the term "biofilm" in 1978. Biofilms have different types of morphologies, based on the constituent bacterium in addition because the conditions beneath that biofilm was fashioned (Bogino et al. 2013; Chandki et al. 2011). Model human pathogens that type biofilms necessary for virulence embody *Escherichia*, *P. aeruginosa*, *Enterobacteria enterica*, *Staphylococcous aureus*, *Vibrio cholera*, etc., through the model is maybe *P. aeruginosa*, Associate in Nursing timeserving microorganism of the human tract and a key think about fibrocystic disease of the pancreas patient mortality (Kostakioti et al. 2013; Mulcahy et al. 2014). As our understanding of microorganism biofilm formations developed, vital points within the organic process started to be scrutinized as potential targets for anti-biofilm medicines (Miquel et al. 2016; Moshiri et al. 2018). Biofilms are also recognized as having importance in different natural and artificial environments, having an impact on crop productivity, food technology, metal corrosion, specific medical processes, and microbial mats—a term usually utilized by applied and environmental microbiologists as a sheet of microorganisms found on rock surfaces, in caves, wetlands, sediments, salt marshes, lakes and seas, thermal springs, hypersaline ponds and lagoons, gas and petroleum seeps, oil wells, etc. (Abdullahi et al. 2016).

1.1.1 Biofilm Growth and Development

A necessity of biofilm formation is that bacteria get close enough to a surface. As bacteria approach a surface they encounter both attractive and repulsive forces (Donlan 2002; Petrova and Sauer 2012). At nearly 10–20 nm from a surface, negative charges on a microorganism's surface are repelled by the negative charges on most natural environmental surfaces (Buck and Andrews 1999; Pembrey et al. 1999). This repulsion may be overcome by attractive van der Waals forces acting between surfaces and microorganisms as well as by utilization of fimbriae and flagella that supply mechanical attachment (Renner and Weibel 2011; Tuson and Weibel 2013). Biofilm formation takes place in three stages: attachment, maturation, and dispersion (Kostakioti et al. 2013). The attachment stage is classified as a two-stage process: initial reversible attachment and irreversible attachment (Crouzet et al. 2014). An irreversible connected biofilm can tolerate stronger physical or chemical shear forces within the initial reversible attachment stage, flagella and type-IV pili-mediated motilities being necessary. Flagella are necessary for the initial interactions between cells and surfaces (Karimi et al. 2015; Petrova and Sauer 2012) sort IV pili-mediated vellication motilities alter connected cells to return along and mixture to make micro-colonies (Higashi et al. 2007). O'Toole and Kolter showed that *P. aeruginosa* flagella-deficient mutants could not land on surfaces and type-IV pili-deficient mutants were not ready

to develop into micro-colonies (O'Toole and Kolter 1998; Watnick and Kolter 1999). For human pathogens like *Staphylococcus epidermidis* and *S. aureus* the initial step of biofilm formation is the attachment to human matrix proteins, such as fibronectin, clotting factor, vitronectin, etc. Microorganism surface elements recognizing adhesive matrix molecules connect covalently to the peptidoglycan on cyto-membranes (Buttner et al. 2015; Speziale et al. 2014). *S. aureus* has over 20 microorganism surface elements acknowledging its adhesive matrix molecule genes—*S. epidermidis* RP62A has only 12 (Otto 2009, 2012). Non-covalent adhesions, like those mediate by autolysins, additionally contribute to the initial attachment of biofilms (Otto 2008). The production of an extracellular polymeric substance (EPS) matrix signifies the irreversible part of microorganism attachment to a surface. The EPS matrix of *P. aeruginosa* has been well studied and in all probability is attributable to the role that *P. aeruginos*a biofilm plays in the progression of monogenic diseases (Kostakioti et al. 2013). Alginate, a significant saccharide element of *P. aeruginosa* EPS matrix, is made by bacteria that are connected to a surface (recently connected bacteria) in quantities many times that of the host organism's cells. The gene AlgT, additionally needed for alginate production, down-regulates flagella genes (Orgad et al. 2011; Wozniak et al. 2003). Once the primary layer of the biofilm is made, cells of identical or different species are recruited to the biofilm from the majority fluid. Biofilm grows from a thin layer and forms a "mushroom" or "tower" structure (Sauer 2003) of very thick biofilm (>100 layers). The bacteria within such structures are organized according to their metabolism and tolerance for air, for example, anaerobic bacteria favor deeper layers thereby avoiding exposure to certain chemical elements. Bacteria inside biofilm communities "communicate" in different ways and take on different specialized functions. As a biofilm matures, additional biofilm scaffolds, such as proteins, DNA, and polysaccharides, are secreted into it by bacteria, becoming entrapped (Singh et al. 2017). After biofilm maturation the dissemination step takes place—equally essential to the life cycle of biofilm. Biofilm dispersal is attributable to a myriad of things, such as a lack of nutrients, intense competition, and large populations. Dissemination can come about within the whole biofilm or only a section of it. The initiation of biofilms at the opposite sites is promoted by the discharge of organism bacterium (Roy et al. 2018).

1.1.2 Beneficial and Naturally Occurring Biofilms

1.1.2.1 The Beneficial Effects of Biofilm Formation

Natural Environments

Biofilms are omnipresent. Not all biofilms are harmful and several conjointly play very important roles within the ecology of our planet and therefore life. The report, "Global Environmental Change: Microbic Contributions, Microbic Solutions," found that "the basic chemistry of Earth's surface is set by biological activity, particularly

that of the numerous trillions of microbes residing in soil and water. Major part of the living biomass on Earth has such types of bulk sharing of microbes and therefore, have major roles within the employment of parts that are necessary for life" (Gougoulias et al. 2014). With relation to earlier studies it is well known that microorganism are early colonizers of clean surfaces submerged in water. Scientists are close to realizing a pattern to how biofilms locate and attach to clean surfaces under water (Donlan 2002), whether the surface in question is the hull of a boat or a brand new sea vent at significant ocean depths. The development of biofilms quickly commences in locations where bacterial populations are found. It is widely acknowledged that microorganisms colonizing biofilms have evolved alongside other organisms. The majority of such microorganisms are considered beneficial (Donlan 2002).

Water and Wastewater Treatment

One of the simplest examples of the useful application of biofilms is within the treatment of waste material. Decay happens partially because microorganisms work on the tissue of dead organisms. Exploitation of this involves the right microorganisms within the right sort of biofilm, enabling the treatment of waste material and sewage: the microorganisms within such biofilms process and thereby remove harmful organic material from the water (Peterson et al. 2010).

Even before biofilms were recognized and became the topic of intense analysis, engineers were exploiting natural biofilm activity within the environment (without having knowledge of biofilms) via water-cleaning systems. Biofilms have been used effectively in water and waste material treatment for over a century. English engineers developed the first sand filter treatment strategies for water and waste material. In such filtration systems the filter medium—the sand—provides a surface for microbes to connect to and process organic material within the water. Such biofilm formations decompose "bad" components within the water, effectively filtering it. Usually, such biofilms stay connected with the filtration system and might be cleaned only once the system is flushed.

Scientists and water treatment engineers have conjointly discovered that water and waste material processed using biofilm systems produces a "biologically stable" product compared with water filtered using alternative approaches to treatment. This suggests there is seemingly less organism contamination in water that has experienced biofilm-based filtering than water processed via other treatment systems. This goes on to suggest that water treated with biofilms generally has a lower disinfectant demand (e.g., the demand for use of chlorine) and contains fewer medical by-products than water treated via alternative treatment systems.

Remediation of Contaminated Soil and Groundwater

One of the less well-known helpful applications of biofilms is in the clean-up of oil and petrol spills—with bound bacteria having the ability to consume oil and petrol.

Oil is formed by the decomposition of vegetation. Bioremediation is a term that refers to the engineering of a biofilm which can be introduced into the world of oil or petrol spills to assist with clean-ups—in a natural, non-harmful manner in terms of the environment.

Bioremediation biofilms have emerged as a technology providing an alternative treatment for the clean-up of groundwater and soil at several sites contaminated with hazardous chemical waste (Ojuederie and Babalola 2017). Bioremediation leads to a reduction in the concentration and mass of several subsurface contaminants (e.g., fossil fuel hydrocarbons and chlorinated organics) and provides helpful evolution within the bacteria of biofilms that enables them to tackle other contaminants like significant metals (e.g., mercury).

Microbial Leaching

The extraction method of metals from ores is named "leaching." For years, copper was reacted with, for example, acid—a process harmful to the environment. In fact, most technological activities of this sort result in cyanogenic products. Nowadays roughly 10–20% of deep-mined copper in the United States is extracted from inferior metal ore assisted by the use of biofilms. Mining firms are investing significantly to develop this method of extraction for different precious metals. How does a biofilm accomplish this task? In this case bacteria consume the ore, that is, that material encasing the copper particles, thus facilitating its removal. This has clear application to the most common biofilm supported process, referred to as "heap activity". Inferior ore is placed in an exceedingly large "heap," and sprayed with an acidified water—a process that encourages the expansion of specific bacteria that oxidize the ore, enabling soluble metal particles (copper) to be recovered from the water.

1.1.3 The Harmful Effects of Biofilm Formation

Of course, not all biofilms are helpful. We have already considered the beneficial effects of biofilms in waste product treatment plants and bioremediation, however, there is a negative side to biofilms. Phosphoric compounds and organic waste, from agriculture, and inadequately treated municipal waste often finds its way into rivers, seas, and oceans. These nutrients cause an excessive enrichment of water, a process known as eutrophication, that successively causes explosive growth, or blooms, of protoctists. When these protoctists die they cause a widespread depletion of oxygen and therefore create hypoxic "dead zones" where anything requiring oxygen will be unable to survive. In a similar manner to microorganism like eubacteria that harvest copper from inferior quality ores, there are other microorganism that act on metals causing what is known as bio-corrosion or MIC (microbial influenced corrosion). Chief among these are the salt-reducing microorganisms (SRMs)—when attaching to the wall of water distribution pipes, conduits, or oil pipelines bacterial biofilms appear

in anaerobic zones having a suitable surface roughness, leading to the corrosion of metal surfaces.

1.1.3.1 Legionnaires' Disease

In microbiological terms, the bug associated with this disease is claimed to be fastidious, that is, within the laboratory its growth is arduous. This is a little surprising since in nature it grows in locations where levels of nutrition are very low. *Legionella pneumophila* is widely found in nature. It grows individually in soil, water, and warm area and as a biofilm on wooden slats or the organic batting of condensers of air conditioning units. *L. pneumophila* can survive over a large range of temperatures, 0–63 °C or (145 °F), and remained unidentified until a virulent disease of a antecedent respiratory disease affected attendees at an American Legion convention in Philadelphia in 1976. The convention that year was held in the Belleview-Stratford Hotel with many Legionnaires being World War II veterans. On the second day of the convention, several delegates fell sick with an acute pneumonia-like illness. Before the illness had run its course, over 200 Legionnaires had become sick and 30 had died. Scientists named the microorganism responsible *L. pneumophila* after the victims and the disease's primary point of attack, that is, the lungs. The organism was found to be widely distributed demonstrating some uncommon nutritional requirements (organic compounds, amino acids, and iron). The primary supply of the infection was found to be the cooling towers of the air-conditioning units within the hotel, facilitating circulation throughout the building. The Centers for Disease Control and Prevention showed that those veterans who had spent the most time outside the hotel were at greatest risk of obtaining Legionnaires' Disease. Although this was the first officially recognized case, samples kept in freezers, from several antecedent and unexplained cases of respiratory disease, suggested it was not the primary incidence. Legionnaires is currently recognized as a type of respiratory disease. The organisms liable for its occurrence are found in hot-water tanks, shower heads, taps, hot tubs, indoor and outdoor water features like fountains, and soil.

1.1.3.2 Black Band Coral Disease (from NOAA's Coral Health and Monitoring Program)

In this disease the withering of corals starts with a white spot encompassed by a ruddy or dark band on generally solid coral development. Over a short period of time bands develop in all directions, creating a generally round area where just the dead white coral skeleton remains. Developing between 1 mm and 1 cm per day Black Band Disease can totally wreck a coral outcrop within a couple of months.

It was first noticed in the 1970s as dark bands moving over the surface of star and brain corals in regions including the Caribbean and Florida Keys.

The associated pathogens are a consortium of microscopic organisms comprising photosynthetic cyanobacteria (e.g., *Phormidium coallyticum*), sulfide-oxidizing microscopic organisms (*Biggiatoa* spp.), and sulfate-decreasing microorganisms

(*Desulfovibrio* spp.) in addition to a vast number (maybe upwards of 500) of other bacterial species not found in healthily coral tissues or in neighboring waters. The dark (or red) bands are a direct result of patches (phycoerythrins) produced by cyanobacteria.

The consequence is development of an oxygen-denied zone, wealthy in hydrogen sulfide, created by sulfate-reducing and sulfide-oxidizing microscopic organisms. Sulfate-reducing microscopic organisms develop rapidly inside the dark bands, decreasing sulfate levels in seawater and delivering significant amounts of sulfide that is oxidized by *Biggiatoa*—a procedure that uses all accessible oxygen and thus creates dangerous hydrogen sulfide. Corals or polyps die from the consolidated effect of oxygen stress and impacts of hydrogen sulfide.

1.1.4 Naturally Occurring Biofilms

Biofilms are ubiquitous in organic life. Virtually all species have mechanisms by which they adhere to surfaces. Biofilms can develop on all non-shedding surfaces in non-sterile liquid or wet environments. Biofilms also grow within the most extreme environments, from extraordinarily hot springs to terribly acidic environments and frozen glaciers. Biofilms may also be found on rocks and pebbles at the bottom of streams or rivers and less frequently on the surfaces of stagnant pools of water. Biofilms represent important elements in food chains in rivers and streams, consumed by aquatic invertebrates, subsequently consumed by fish. Biofilms are found on the surfaces of plants. They may either contribute to crop diseases or, like nitrogen-fixing bacteria on the roots of plants, exist symbiotically with plants. Crop diseases associated with biofilms include citrus canker, Pierce's Disease (in grapes), and bacterial spot (e.g., in peppers and tomatoes). Studies have discovered that biofilm development occurs within the intestines. This was primarily supported with fact that most commonly made molecules by the system conjointly support biofilm production and were related to the biofilms development in gut. The appendix holds a mass of microorganism associated with biofilms and helps re-inoculate the gut with sensible gut flora. In the United States, biofilms have been shown to grow in showers since they supply a wet and warm environment. Biofilm formation also occurs within water and sewage pipes—leading to corrosion. Biofilms on floors and counters create issues with sanitation, of significant concern in areas associated with food preparation. The presence of biofilms in soil causes bioclogging. Biofilms in cooling systems or water-heating systems may lead to disease. Biofilms found in marine engineering systems, like pipelines used by the offshore oil and gas industries, result in substantial corrosion. Bacterial adhesion to the hulls of boats is the platform for biofouling oceangoing vessels. Once a collection of microorganisms forms on a surface, it becomes easier for different marine organisms like barnacles to connect. Such fouling will in turn reduce vessel speeds by up to 20%, extending voyage times and increasing fuel consumption. Additionally, time spent in dry dock refitting and repainting reduces vessel productivity and therefore ship lifetimes are

reduced. Stromatolites are stratified, growing structures shaped in shallow water by sedimentation, binding, and cementation of assorted grains of matter by microbial biofilms, particularly blue–green algae. Stromatolites have been identified in ancient records of life on Earth and are of course still forming today.

1.1.5 Biofilms in Health and Medicine

Biofilms have surface-appended networks of microorganisms that are inserted and develop within a self-delivered lattice of EPSs. Biofilms can be found in various fields, for example, restorative medicine and human health. Biofilm formed during the time of transmission causes human sickness basically for illnesses related with inactive surfaces including restorative gadgets for inner just as outer use. Biofilm diseases on inserted medical gadgets are hard to prevent on account of their vastly improved defenses against macrophages and anti-infection agents, in contrast with free-living cells. This prompts extreme clinical difficulty—often resulting in patient death (Srivastava and Bhargava 2016). The "sacred goal" of biofilm contamination is an "early warning" strategy, taking into consideration the non-obtrusive discovery of biofilm initiation on biomedical implants and practical means of reacting to associated diseases. Such diagnostic abilities are currently under development (Bauer et al. 2006).

1.1.6 Control of Biofilms

There are a few procedures currently utilized for the control of biofilms, some of which are outlined in Table 1.1 (Subhadra et al. 2018). Biofilms are profoundly

Table 1.1 Strategies for the control of biofilms

S.no.	Strategy	Method/agents	Examples	References
1.	Inhibition of initial biofilm attachment	Altering the chemical properties of biomaterials	Antibiotics, biocides, iron coatings	de la Fuente-Nunez et al. 2014; Dror et al. 2009; Ramos et al. 2011; Yamanaka et al. 2005
		Changing the physical properties of biomaterials	Use of hydrophilic polymers, hydrogel coatings, heparin coatings	Appelgren et al. 1996; John et al. 2007; Li and Lee 2017
2.	Biofilm removal	Use of matrix-degrading enzymes	Polysaccharides degrading enzymes, nucleases	Darouiche et al. 2009; Li and Lee 2017

impervious to many ordinary antimicrobial treatments and furthermore ensure contaminations persevere. Past investigations have demonstrated an extreme interest for novel methodologies to control biofilm-based contaminations as opposed to customary antimicrobial treatments. Of these, two methodologies are, for the most part, used to control biofilm arrangements in medicinal services. The first incorporates the improvement of biofilm inhibitors based on the understanding of sub-atomic instruments of biofilm development. The other involves altering biomaterials utilized in therapeutic gadgets to counteract biofilm development (Subhadra et al. 2018).

1.1.7 Biofilm and Antibiotic Resistance

Past investigations have shown that biofilms are related to the development of anti-infection, safe microscopic organisms. Perfect exchange leads to advances in development and hereditary decent of common microbial networks. Bacterial biofilms cause endless contaminations due to their increased resilience to anti-toxins and disinfectant synthetic compounds as well as opposing phagocytosis and other immune systems (Hoiby et al. 2010). Microscopic organisms that append to surfaces and develop as biofilms are shielded from anti-infection agents—leading to biofilm contaminations, for example, those related with implanted gadgets (Stewart 2002). In biofilms the following factors like poor anti-toxin entrance, supplement restriction and moderate development, versatile pressure reactions and the arrangement of persisted cells are responsible to establish a multi-layered cover. The microbes in a biofilm are 1000 times more impervious to anti-infection treatments than similar lifeforms that develop planktonically.

1.1.8 The Future of Studying Biofilms

Biofilm research has progressed significantly since its inception when attachment and the colloid hypothesis shaped its examination. The utilization of confocal microscopy to image living, hydrated biofilms prompted different biofilm inquiries. This procedure was followed by a range of atomic strategies, including fluorescence in situ hybridization (FISH), restriction of columnist quality articulation, proteomics, and transcriptomic-based examinations to comprehend the sub-atomic reason for biofilm arrangements and advancements. As a result, a few examinations have accounted for some of the explicit qualities of, and proteins required for, biofilm arrangements and advancements, biofilm stage-specific quality articulation, the division of work during biofilm improvement, and the spatial and transient restriction of quality articulation (Donlan 2002; Rice et al. 2016).

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Chapter 2
Biofilms: The Good and the Bad

Suresh K. Yadav and Somali Sanyal

Abstract Biofilms are well-structured, cooperating microbial communities adhered to various types of surfaces. Microbes forming biofilms secrete slimy extracellular polymeric substances (EPSs) which provide biofilms with their resistance against antibiotics. Biofilms have several advantages and disadvantages. Exploring the negative side of biofilms first—biofilm formation interferes in crucial processes like heat and mass transfer, fluid dynamics, and also causes bio-corrosion thereby increasing maintenance costs and decreasing the overall yields from plants. Bio-corrosion also increases the chances of bacterial adhesion and contamination of processed food products, dairy products, and brewing products. Biofilms affect the sea food and aquaculture industries by clogging cages and interfering with nutrient inflows. Biofilms have numerous harmful effects that are associated with the medical industry, such as infections associated with the insertion of tubes, catheters, and valves, as well as surgery. Considering the positive aspects of biofilms we note that the judicious use of biofilms can provide solutions to modern day problems. They can be effectively used for the bioremediation of soil and groundwater as well as being used to treat oil spills. They provide cost-effective alternatives in the mining industry in the form of bioleaching and biofilm-based bioreactors for municipal/industrial waste disposal. Biofilms can be used as biosensors for the reliable and quick detection of chemicals as well as in the treatment of contaminated water.

Keywords Biofilm · EPS · Bio-corrosion · Remediation · Oil spillage

2.1 Introduction

Biofilms are not new to science. They have existed for many years. According to *Nature Reviews Microbiology*, the existence of biofilms dates to about 3.25 billion

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years. For example, biofilms are known to exist in the hydrothermal rocks of Pilbara Craton in Australia and in hot springs and deep-sea vents (Hall-Stoodley et al. 2004). The "Animalcules" described by A.V. Leuwenhoek in 1674—when he used his microscope to look at what he had scraped from the surface of his teeth—was a biofilm (Garrett et al. 2008). Bill Costerton first introduced the term "biofilm" in 1978. Biofilms refer to heterogeneous clusters comprising of different populations of microbes enclosed in a self-produced matrix (mainly of exo-polysaccharides) often attached to inert (plastic, glass, rocks, etc.) or organic (mucosa, cuticle, skin, etc.) surfaces (Costerton et al. 1995). Biofilms represent well-structured, organized, and co-operating communities of microbes. Biofilms develop from naturally occurring sessile prokaryotes (Hobley et al. 2015; Kolter 2010; Sachs and Hollowed 2012). The International Union of Pure and Applied Chemistry (IUPAC) defines biofilms as "aggregates of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substances (EPSs) adhere to each other and/or to a surface." Biofilms have the ability to adapt to environmental conditions (Vert et al. 2012). EPSs, also referred to as slimes, are comprised of a mixture of extracellular biopolymers of lipids, proteins, and polysaccharides (Aggarwal et al. 2016; López et al. 2010). Most naturally occurring biofilms are heterogeneous, highly diverse in nature, and are comprised of several microbial communities. However, studies of biofilms are conducted using single species (Vlamakis et al. 2013).

Several microbes have been reported to form biofilms, including prokaryotes like bacteria (both Gram-positive and Gram-negative; Abee et al. 2011), cyanobacteria (Rossi and De Philippis 2015), and archaea (Orell et al. 2017), and eukaryotes such as fungi (Joubert et al. 2006; Fanning and Mitchell 2012) and microalgae.

Depending on species, a micro-colony is comprised of cells (10–25%) and EPSs (75–90%) (Costerton et al. 1987). Cells in the matrix have a mushroom shape and lack Brownian movement. Water is responsible for the transportation of nutrients inside and toxins outside the matrix as well as maintaining the osmotic pressure and motility of the microbe (Costerton 1999). EPSs have varying compositions including proteins and nucleic acids. However, they are primarily composed of polysaccharides. These polysaccharides may be neutral or polyanionic. It is the presence of uronic acids (D-glucuronic acid, D-galatouronic acid, and mannuronic acid) or ketal-linked pyruvate that provides the anionic properties that help in the adherence with calcium and magnesium ions cross linking the polymer, thereby providing biofilms with greater strength. Biofilms have 1,3- or 1,4-β linked hexose residues forming their backbones. The production of EPSs increases with biofilm age and an excess of carbon, and the limitation of nitrogen, potassium, and phosphate promotes their synthesis (Kokare et al. 2009).

The EPSs of biofilms provide shelter and help maintain homeostasis for bacteria residing in such biofilms. Bacterial biofilms usually remain unaffected by antibiotics and the human immune system. It is EPSs that provide this protection against antibiotics and the human immune system by preventing the diffusion of compounds from the surrounding environment into the matrix as well as acting like anionic exchangers. This protection is most prominent against hydrophilic and positively charged antibiotics containing aminoglycosides. EPSs also sequester metals and toxins and

by up and down regulation of gene expression they not only provide protection against various environmental stressors, such as pH, UV radiation, osmotic shock, and dehydration, but also help growth in nutrient-deficient conditions (Koch et al. 2001).

Biofilm formation is a complex process in which a free-floating planktonic form is transformed into a sessile form. This process involves the expression of several genes which lead to the establishment of biofilms (Masahiro et al. 2005; Sauer et al. 2004). This multi-step process, allows microbes to adapt to diverse environmental and nutritional conditions (Hentzer et al. 2005). Biofilm formation has following distinct phases: (1) adherence to a surface; (2) micro-colony formation; (3) the three-dimensional growth of a micro-colony; (4) biofilm formation; and (5) maturation and dispersal.

2.2 Mechanism of Bacterial Biofilm Formation

The formation of a biofilm is regulated by various physical, chemical, and biological processes. The attachment of cells to a substrate (adhesion), followed by cell-to-cell attachment (cohesion) determines the strength and properties of a biofilm. According to Fletcher, attachment of cells to a surface occurs in 3 stages: (1) adsorption/accumulation of microbes in a substrate; (2) attachment/consolidation of microbes and the substrate by the formation of polymer bridges; and (3) the growth/colonization of microbes on the surface of a substrate (Fletcher 1980). However, a more detailed eight-step mechanism was proposed by Characklis and Marshal, including the formation of an initial conditioning layer, reversible and irreversible adhesion, and the eventual detachment of cells from a matured biofilm for subsequent colonization (Characklis and Marshal 1990).

2.2.1 The Conditioning Layer

This is the foundation on which biofilms grow and can be organic or inorganic in nature. Anything present in the bulk of the material, brought by gravitational forces or flows, that settles on the substrate becomes a constituent of the conditioning layer. Surface charge and potential can be altered by the interaction between the conditioning layer and the substrate and this alteration facilitates the accessibility of bacteria. The substrate provides the harbor and nutrient supply that helps bacteria grow.

2.2.2 Reversible Adhesion

Planktonic cells are transported from the bulk liquid to the conditioned substrate by the physical forces of bacterial appendages. Of all cells reaching the substrate, only a fraction reversibly adsorb to the surface. Several factors, such as available energy, bacterial orientation, surface properties, and temperature and pressure conditions, play important roles in bacterial adhesion. The physical forces involved in bacterial adhesion include van der Waals forces, steric interactions, and electrostatic interactions, collectively known as Derjaguin, Verwey, Landau, and Overbeek (DVLO) forces (Rutter and Vincent 1980). These long-range physical interactions are also known as physisorption.

2.2.3 Irreversible Adhesion

Many of the reversibly adsorbed cells become irreversibly adsorbed to surfaces of conditioned substrates. The physical appendages of bacteria and several chemical interactions such as oxidation and hydration also facilitate irreversible adhesion and help with the association of bacteria over a surface (Ganesh and Anand 1998). The bacterial association over a substrate surface depends on the hydrophobic and hydrophilic properties of the two (De Weger et al. 1987).

2.2.4 Micro-colony Formation and Three-Dimensional Growth

After the initial attachment of bacteria to a substrate, an exponential or rapid growth is observed in the cellular population of a biofilm. The adhered cells divide and newly formed daughter cells move upward, forming a mushroom-like structure. This structure also facilitates the continuous supply of nutrients to the basal layer, that continues to divide. Exponential growth depends upon the presence of nutrients in the bulk medium. During this stage, the process of adhesion is subsided by biological processes that increase biomass. Secretion of EPSs also takes place, with polysaccharide intercellular adhesion (PIA) being an integral component that increases the bonding between cells in the presence of bivalent cations (Dunne 2002).

2.2.5 Biofilm Formation

At high cell concentrations, when the rate of cell division becomes equal to the rate of cell death, a series of signaling mechanisms, also known as quorum sensing, is

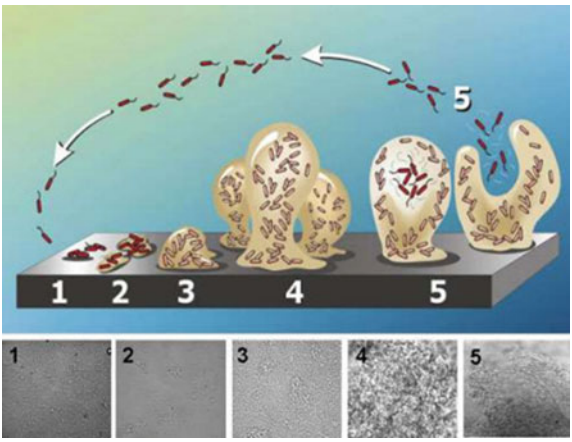

Fig. 2.1 Stages in biofilm development (Cogan and Keener 2004)

triggered in biofilms. The quorum-sensing mechanism stimulates the synthesis of alginate—an integral part of EPSs (Bassler 1999).

2.2.6 Maturation and Dispersal

Once the cells of a biofilm are mature, they start secreting enzymes, such as alginate lyase, N-acetyl-heparson lyase, and hyaluronidase, that breakdown the polysaccharides holding the biofilm together. This breaking of polysaccharides releases surface bacteria, allowing them to colonize a fresh substrate (Sutherland 1999) (Fig. 2.1).

2.3 Applications of Biofilms

Biofilms have advantageous applications in several fields, such as bioremediation, the clearance of oil spills, bioleaching, waste water treatment, and municipal/industrial waste treatment. Negative influences of biofilms are felt in the food, dairy, and brewing industries, as well as industries connected to sea food, aquaculture, and medicine.

2.3.1 Biofilm Uses

2.3.1.1 Water Treatment

Nowadays biofilms are utilized in sewage water treatment plants as a secondary treatment to water that has already been treated via a primary process—focusing on

the removal of dissolved organic matter. There are three ways biofilms can be used in secondary treatment, that is, fixed film, floating film, and lagoon system (Mancl 2009).

2.3.1.2 Fixed Film

In this system, biofilm remains attached to a substrate with water flowing over it. The fixed-film method can use trickling filters, rotating biological contractors, and sand filters (Mancl 2009).

2.3.1.3 Suspended Film

In this system, biofilms remain suspended in the water and grow by absorbing nutrients and organic matter. They grow into micro-colonies, which eventually settle as sludge and can be reused by resuspending them in water. Examples of the use of suspended films include activated sludge, extended aeration, and batch reactor systems.

2.3.1.4 Lagoon System

Lagoons are settling ponds in which treated water is kept for longer periods of time. Here, natural resources such as algae, sunlight, and water are exploited. After secondary treatment, almost all pathogens and solids are removed. In addition, 10–20% of the nitrogen is removed—utilized by decomposer bacterial for their growth and development. Water treated in this manner can be used for non-potable purposes, such as agriculture and irrigation.

2.3.2 Bioremediation

This process includes the use of biological systems to reduce pollution levels in water bodies or the soil. The process of bioremediation depends on the enzymatic activity of microbes converting toxic environmental waste to less toxic or harmless products such as water and carbon dioxide (Das and Dash 2014). Usually, the process of bioremediation involves a transfer of electrons from donors to acceptors via aerobic or anaerobic processes. Generally, aerobic processes are much faster than their anaerobic counterparts. Electron donors serve as food reservoirs for microbes and are limited in non-contaminated sites. However, in contaminated sites, the release of electron donors creates competition between available acceptors trying to restore balance. Several redox reactions utilize trace elements and a change in oxidation number is associated with the toxicity and solubility of metals found at contaminated sites (Joutey et al. 2015). For example, heavy metal sulphates are converted

into sulphide forms, thereby facilitating their immobilization and removal from contaminated sites (Beyenal et al. 2004). Biofilms are employed for bioremediation as negatively charged EPSs that capture harmful cations from the bulk medium. The application of biofilms for bioremediation does not need any specialized instrumentation, as is the case for ozonization and chlorination. Compared with physiochemical methods, biofilms provide a cost-effective approach (Singh et al. 2006). Bioremediation is mainly categorized depending on the location of pollutant treatment. In the case of in situ *bioremediation,* pollutants are treated on-site; during ex situ *bioremediation* pollutants are treated off-site. The binding capacity of EPSs can be further improvised using synthetic biology and genetically engineered bacteria that may increase the natural chelating ability of the environment. A class of cysteine-rich, heavy metal–binding proteins, mostly found in plants (Cobbett, and Goldsbrough 2002), also called phytochelatins, have shown promising results in heavy-metal remediation. A cadmium-chelating bacteria (10:1) was constructed by Bae et al. in 2000. A similar strain could be developed to absorb arsenic from water bodies—and issue that is currently causing huge problems (Bae et al. 2000). Besides bacteria, fungi also help in the bioremediation process (Mishra and Malik 2014). The addition of sources of carbon and phosphorous, along with an oxygen supply—a process known as bio-stimulation—speeds up the bioremediation process. The inoculation of specific competent microbes to a contaminated site to facilitate faster bioremediation is known as bio-enhancement or bio-augmentation (Tyagi et al. 2011). Bio-enhancement enhances biofilm formation which increases bioremediation efficiency. This method is utilized in sites that have been freshly contaminated where endogenous degrading microbes are comparatively rare.

2.4 Oil Spills and Contaminated Groundwater

Oil spills represent a major threat to the marine environment, they not only affect marine ecosystems but also human health through the transfer of polycyclic hydrocarbons (PAHs) into the food chain (Dasgupta et al. 2013). Many PAHs are carcinogenic (Deziel et al. 1996). Methods like volatilization, photo-oxidation, chemical oxidation, and bioaccumulation are rarely successful, therefore cost-effective and safe methods for the rapid removal of PAHs are required (Prince 1997). Several species of bacteria of marine origin have been documented to degrade hydrocarbon, and bacteria belonging to subphyla α-, β-, and δ-proteobacteria are well known for this ability (Engelhardt et al. 2001). Biofilm provides a safe, effective, and rapid method for cleaning oil spills. Several new species offering this potential have been discovered and are being genetically engineered to perform this task (Dasgupta et al. 2013). Petroleum oil–degrading bacteria are also known as hydrocarbonclastic bacteria (HCB). These bacteria not only clear oil spills but also contaminated groundwater in the same manner that biofilms absorb arsenic from contaminated water bodies.

2.5 Microbial Leaching

The extraction of metal from ore is a cumbersome and tedious process. The majority of precious metals are present in the Earth's crust in very minute quantities. Currently, such metals are isolated using chemical methods that are not ecofriendly and have serious environmental impacts. Leaching is not a new concept—chemicals have been traditionally used for metal leaching, for example, acids are used in copper leaching. Such leaching processes leave toxic products. To reduce the environmental impact, the concept of microbial leaching came into existence. Today approximately 10–20% of copper is mined from low-grade copper ore with the help of biofilms. A biofilm-assisted leaching process is usually a "heap leaching" process. In this process, low-grade ore is kept in a heap with mildly acidified water sprayed over it to promote the growth of bacteria like *Thiobacillus*, oxidizing the ore and releasing water soluble forms of cupric ions.

2.6 Biofilm Reactors

Contaminated sites having no, or very low, microbial populations or lacking optimum conditions for the degradation of pollutants require ex situ bioremediation processes in engineering bioreactors with biofilm linings that entrap or immobilize an inert material that supports its growth. Biofilm reactors are used for biochemical conversion and the sorption of pollutants, particularly heavy metals and hydrocarbons from municipal and industrial wastewater (Boon et al. 2002). Biofilm bioreactors have been commercially used for treating industrial wastewater for over two decades (Qureshi et al. 2005). They are used when free-floating microbes in suspension do not produce adequate biomass or the biomass is not sufficient for volumetric conversion. Biofilm bioreactors have numerous advantages over conventional treatment processes, such as enhanced metabolic activity, increased flow rates, larger mass transfer areas, and optimum physicochemical control.

2.7 Biofilms in Biosensors

Apart from leaching metals, biofilms can also be used for the development of biosensors. A biosensor is a device that can detect a substrate even in extremely minute quantities with great accuracy, sensitivity, specificity, and reproducibility. Biosensors have three major components: a substrate-detecting part, transducers, and an output device. Biofilms are used for the substrate detection of toxic metals such as arsenic and mercury in groundwater. In this manner biofilms can perform a dual function, that is, detecting as well as absorbing heavy metals from groundwater.

2.8 Biofilm Integrated Nanofiber Display

Biofilm Integrated Nanofiber Display (BIND) is a novel protein-engineering system that might, in the future, form living foundries for the large-scale production of biomaterials that can be programed to provide unique properties not possible with existing materials (Nguyen et al. 2014).

2.9 The Harmful Effects of Biofilms

Bacteria have the unique ability to grow on almost any substance where nutrition is available. This property eventually leads to the formation of biofilms that become problematic, especially in industrial processing plants (Tarver 2016). Such biofilms interfere in several crucial processes like heat and mass transfer and fluid dynamics and lead to the corrosion of any surface they are attached to, thereby increasing maintenance costs and decreasing overall yield and profit (Srey et al. 2013).

2.9.1 The Food and Dairy Industry

As biofilms are resistant to sanitizers, there is always the probability of some form of contamination in processed food plants. Hydrophilic and abraded surfaces increase the chance of bacterial entrapment and thus the chances of food contamination in any manufacturing process. However, the formation of biofilms are nowadays avoided by improved structural design of processing plants, temperature controls, and better cleaning agents like alkalis, in combination with sequesters or chelators along with anionic wetting agents. Sanitizers used include halogens, acids, peroxygens, hydrogen peroxide, and quaternary ammonium compounds (Trakoo 2003). *Pseudomonas* spp., thermophilic *Geobacillus stearothermophilus*, and *Listeria monocytogenes* are common contaminants in food processing plants, especially dairy plants. Food-borne diseases caused by biofilms on food matrixes or processing equipment may cause intoxication or infection. Almost everything found in processing plants, such as water supplies, pipelines, membranes, gloves, tubes, and packaging materials, has the potential to cause infection (Camargo et al. 2017).

2.9.2 Aquaculture and the Sea Food Industry

In the aquaculture industry, biofilms not only compete with cultured species for food, nutrients, and space but also clog nets and cages. In the case of freshwater aquaculture, apart from blocking and clogging nets, biofilms may also harbor several potential

pathogenic bacteria (Cai et al. 2013), including *Aeromonas hydrophila*, *L. monocytogenes, Salmonella enterica*, or *Vibrio* spp. *Salmonella* contaminates seafood and is one of the major contaminants of poultry products (Mizan et al. 2015).

2.9.3 The Brewing Industry

Brewing is slightly safer in terms of bacterial contamination because of its low pH (3.8–4.7) and ethanol concentration (0–8%)—few genera are reported to form biofilms under such extreme conditions (Jespersen and Jakobsen 1996). The most important common contamination being from *Lactobacillus brevis* while *Lactobacillus lindneri* and *Pediococcus damnosus* also have high contamination proportions. Other than these, yeast and *Pectinatus* cells are also major contaminants found in breweries.

2.9.4 Bio-corrosion

Bio-corrosion or microbial influenced corrosion (MIC) is caused by *Thiobacillus*-like bacteria which act upon metals—their main class being sulphate-reducing bacteria (SRB). These bacteria form biofilms in water distribution pipes, heat exchanger pipes, and oil supply pipes leading to corrosion and thus their early periodic replacement. Bio-corrosion is an issue of concern in fluid-related equipment and machinery such as propeller blades in fermenters or the propellers of ships—the hydrodynamic properties of such equipment being impacted by bio-corrosion.

2.9.5 The Medical Industry

Biofilms have the unique ability to tolerate antibiotics and immune systems (Bryers 2008). Owing to this property, biofilms can develop in all medical inserts (Auler et al. 2010) such as catheters, intra uterine tubes, and cardiac valves. Several diseases such as cystic fibrosis, native valve endocarditis, otitis media, periodontitis, and chronic prostatitis appear to be caused by biofilm-associated microorganisms. In potable water systems, biofilms have the potential to harbor pathogens like *Legionella pneumophila*, nontuberculous mycobacteria, and *Helicobacter pylori* (Donlan 2002).

Initial contamination starts from microbes transferred from either a patient or healthcare worker or other external source. *Staphylococcus aureus* and *Staphylococcus epidermidis* are commonly associated with biofilms formed on medical devices that cause healthcare-associated infections (von Eiff et al. 2005). In long-term care facilities *Acinetobacter baumannii*, *Escherichia coli, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* are more prevalent (Niveditha et al. 2012). Devices like

central venous catheters develop extraluminal biofilms within a week of catherization—a major cause of catheter-associated blood stream infections (Donlan 2008). In the case of urinary catheters, the risk of catheter-associated infection increases by 10% each day when a catheter is in place (Donlan 2001). Biofilms grow very easily and very rapidly (within 24 h) on endotracheal tubes (ETTs), representing a major cause of ventilator-associated pneumonia (Bauer et al. 2002; Amin 2009). Microbes that form biofilms on ETTs include the multidrug-resistant bacterium MRSA and Gram-negative bacilli such as *K. pneumoniae*, *E. coli*, *P. aeruginosa*, and *Acinetobacter* spp. (Ramirez et al. 2007). Surgical site infections occur following surgical procedures and are caused primarily by patients' skin. Bacilli and cocci are common biofilm-forming microbes with *S. aureus* being the most common (Kathju et al. 2009). Infected and non-infected sutures both showed the presence of biofilms. Even non-infected sutures showed 66.6% positive results for biofilms (Edmiston et al. 2015).

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Chapter 3
Biofilms in Human Health

Surojeet Das, Shivani Singh, Monica Steffi Matchado, Aashna Srivastava and Akash Bajpai

Abstract Biofilm is a surface-attached cluster of microorganisms rooted and proliferating in a self-fabricated matrix of polymeric materials. Bacteria existing in biofilms can be more resilient in comparison with their free-floating counterparts to antimicrobials. Biofilms play a substantial role in human disease transmission and perseverance, especially for inert surface-related disease, like cases of infections related to medical devices for internal or external use. Due to their better resistance against macrophages and antibiotics in comparison to free living cells, biofilm-triggered infections on implants are difficult to eradicate. While the formation of biofilms is largely understood, the means of eliminating and controlling them once they have been formed are still the subject of research. Biofilms associated in medicine are particularly difficult to handle due to the sensitivity of the human tissue and medical devices. The chapter aims at discussing biofilm development, their influence on human health and difficulties related to biofilm control.

Keywords Biofilms · Staphylococci · Candida · Catheter · Medical devices

3.1 Introduction

The first-reported evidence of biofilm production by microbes was made in the seventeenth-century era, by Antonie van Leeuwenhoek who witnessed 'animalcules' flocking on dead and living matter. Leeuwenhook was curious and inventive; he found the presence of these microscopic animals on teeth where tarter was diagnosed. Now as known, the deposits which contained various forms of animalcules were bacteria of tooth plaque. After Leeuwenhoek's early work in 1940, the presence of 'bottle effect' was reported in marine microorganisms. It was observed that bacterial development was significantly augmented when they were provided with a surface to

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attach. Zobeli reported that the count of bacteria is greater on surface as compared to surrounding seawater (Heukelekian and Heller 1940).

Despite all the above reports of biofilms existence, the first physicochemical investigations of bacterial biofilms were only made possible after the 1970s. There were reports which reported the utilization of electron microscopy to identify the biofilm on a filtering filter on wastewater management plants. After these efforts, it was found that biofilms were formed by different types of microbes and it unveiled that polysaccharides were the initial component to form the matrix material. The study was carried forward with the help of electron microscopy, which provided the information for the structure of biofilm. A study also depicted that various bacteria spent maximum of their presence within a surface-attached community (Costerton et al. 1978).

Further studies on the structure of biofilms depicted that glycocalyx or polymer-formed matrix of biofilms acted as the protective mechanisms for the digestive enzymes produced by bacteria. These digestive enzymes helped in aggregating the metabolic competence of the cell. Glycocalyx is a polyanionic polysaccharide which is a hydrated component generated by different types of polymerases attached to a lipopolysaccharide material of bacterial cell wall. In addition to this, the glycocalyx offers a physical and chemical barrier for fractional defence against antibacterial agents (Costerton et al. 1995). Biofilms can be formed by both multiple or single species and these biofilms have different physiochemical properties. A new concept of "Biofilm Model" came into consideration to study biophysical, chemical and structural features of biofilms.

3.2 Biofilm Structure

Biofilm shows major variation in their structure and composition in different environments which make the characterization of biofilm very difficult. Biofilms are very complex type of systems in which microorganisms attach on a layer of surface and are entrapped in a matrix constructed by organic polymer derived from the microbe. Biofilm matrix may also contain non-cellular material, microbial components, corrosion particles, blood components, clay or slit particles. Biofilms can be more complex in water systems as compared to medical devices where they are represented as general or rod-shaped, single or coccoid microorganisms (Percival et al. 2000). So, in general, biofilms can be defined as microbial cell-inactivated extracellular polymer matrix which acts as functional ecosystem which is homeostatically and independently regulated.

3.3 Biofilm Development

The formation of biofilm is not a very simple process; generally, it consists of five steps (Palmer and White 1997)

(1) Surface growth by conditioning films.
(2) Shift of microorganism into surface of nearness and closeness.
(3) Adherence (surface adherence of microorganism to the surface by irreversible and reversible mode).
(4) Surface colonization that helps in development and division of microbe, formation of microcolony and biofilms, change in genotype and phenotype.
(5) Biofilm cell dispassion and removal.

3.3.1 Growth of Conditioning Film on Surface

Microbes do not attach rapidly to a substratum within a natural environment but attach to most substrata which is known to form by conditioning films (Mittelman 1996). Chemical alteration of the unique surface is a result of the complex composition of the conditioning film, which in turn, influences the frequency and magnitude of adhesion towards microbes.

Primary evidence given by, suggested that a "conditioning" film existed in most biofilms. However, this is still a topic of discussion within the published literatures that whether a conditioning (Rittle et al. 1990) film represents a pre-requisite for bacterial adherence or not (Chamberlain 1992). Glycoproteins, humic compounds (Baier 1984) and complex polysaccharides are present in the conditioning layer which is in the aquatic or terrestrial environs (Marshall et al. 1971). In evaluation, the conditioning films are complex and resolute by the spot being conditioned in human host. In this context, the proteinaceous "pellicle" conditioned to the enamel of teeth is composed of glycoproteins, albumin, lipids, lysozyme, phosphoproteins and added components of saliva and gingival crevicular fluids. Few added categories of conditioning films have been made known, predominantly on biomaterials which are operational for humans. The physicochemical properties of substratum get changed on the basis of importance of the conditioning film used for the biofilm growth. The nutrient source which is concentrated and the noteworthy trace elements are provided generously by the conditioning film. It has been observed that the conditioning films might essentially prevent rather than endorse the connexion of specific bacteria. The external landscape to which a microbial cell connects is also crucial to biofilm establishment. As the unevenness of a surface upsurges, the increase of bacterial adhesion will occur (Characklis and McFetes 1990).

It has been observed that metallic surfaces are more energetically attracted to the attachment of the pioneer colonisers (Beech and Gaylarde 1989). For these metallic surfaces, different compositions may vary like cellular metabolism, adhesion and

production of exopolymers. Vieira et al. (1992) reported the role of metal surfaces after the adhesion of any bacterial colonies. In further studies, it was also observed that aluminium surfaces were fouled within few hours by *Pseudomonas fluorescens*, trailed by brass and copper (Pringle and Fletcher 1983). In continuation to this, it was also reported that Teflon or other components of plastics, other than metals or glass which included surface toughness and unevenness attracts bacterial attachment and altered the physicochemical properties of the surface. Despite all these reports and documents (Bendinger et al. 1993), these outputs are incomplete and require validation, as many other studies have reported contrary results. This may be due to standardized approaches of non-availability of hydrophobicity surface calculations (Percival and Thomas 2009).

3.3.2 Movement of Microorganisms Towards Surface

Usually to initiate movement of microbial cells and nutrients towards the surface, a well-reported fluid dynamics method has been utilized (Fletcher and Marshall 1982). These comprise effects of gravitation, sedimentation, transport of mass, thermal diffusion, Brownian motion and molecular diffusion. Laminar and turbulent flow includes two main flow conditions which exist inside (Characklis 1981). The property of urinary flow system and bloodstream are obvious and reported as laminar flow which is also characterized by rapid flow in the centre and no border mixing (Characklis and McFetes 1990; Characklis and Turakhia 1990) with smooth flow movements and patterns. The main property of laminar flow includes the straight path of microbes and nutrients, which also persist in a steadied location dictated by a specific movement level. Apart from this, flow of turbulence is arbitrary and disorganized, which leads to increased mixing of nutrients and bacteria for microbial adherence (Lappin-Scott et al. 1993). Eddy currents (unchangeable flow and random) are obvious in turbulent flow and these relate to different varied sweeping forces (Percival and Walker 1999), which initiates the bacteria to move in the minimum distances of the surface which further helps in supporting the chance of bond formation (Lappin-Scott et al. 1993). Motility by microbes, gravitational attractions and Brownian diffusion process are some few factors which are taken into consideration for adhesion (Bryers 1987) when being in quiescent or static atmosphere (Walt et al. 1985). Adhesion is always initiated by movement and process of bacterium. This is because of the presence of abundant potential energy to overcome any repulsive forces known to activate the substratum and the bacterial surface in question (Marmur and Ruckenstein 1986). It is usually found that decrease in adhesion is because of the reduction in motility. Gravitational cell sedimentation is another factor that is responsible to affect surface colonization, often only of significance in moving systems when there is an occurrence of co-aggregation (Fletcher 1977).

3.3.3 Adherence

The adherence of microorganism generally takes place after conditioning of the surface and transport of bacteria to the area in proximity to the substratum. The phenomenon of adhesion and introduction of biofilms was first reported in 1943 (Zobell 1943). It was proposed to be a two-step process: Reversible and Irreversible processes. The microbes are first attached weakly to the surface by reversible adhesion (Whittaker et al. 1996), followed by a more strong and permanent irreversible adhesion. The intrusion of bacteria is observed in this process, by the help of explicit bacterial adhesins, which attach to relapse on the matrix of extracellular polymeric components and substratum (Marshall et al. 1971). There is a direct correlation of bacterial adhesion with the length of gap between the surface and the microorganism (Busscher and Weerkamp 1987). It is observed that for distances in the range of 10–20 nm, Van der Waal's and electrostatic interactions are predominant, whereas, for distances greater than 50 nm only Van der Waal's forces are active. When the gap is lower than 1.5 nm from the surface, Van der Waal's electrostatic and specific interactions play a major role between the cell and the surface. The premiere influence on adhesion to a surface is visible by the surface of the microorganism's cell. The factors affecting the rate and degree of microbial attachment include the hydrophobicity of cell surface (Danielsson et al. 1977), the occurrence of fimbriae and flagella, and the extent and configuration of generated EPS. Detachment of bacteria from the substrate was reported when it was treated with proteolytic enzymes (Bashan and Levanony 1998); this, in turn, hinted the possible role of proteins for bacterial adhesion. It is the presence of relatively higher proportions of residues which are hydrophobic in nature, which affects the cell surface connections and hydrophobicity (Rosenberg and Kjelleberg 1986). It is due to the presence of such fimbriae (Bullitt and Makowski 1995) that microorganisms are able to resist the early electrostatic repulsion obstacle which lies between cell and substrate (Corpe 1980).

3.3.4 Colonization for Development and Division of Microbe, Formation of Microcolony and Biofilms, Change in Genotype and Phenotype

Extracellular cementing substances produce irrevocable adhesion if cell exists at a surface for a specific period of time (Costerton et al. 1978). This extracellular material which is connected with the cell has also been called glycocalyx. It is a slime layer, sheath or a capsule, and this substance of biofilms may be around ninety-to-fifty percept part (Flemming et al. 2000) of the complete organic carbon. This organic matrix also consists of proteins, glycoproteins and nucleic acids. Gram-negative bacteria mainly consist of neutral or polyanionic polysaccharides, however, extracellular polymeric substance (EPS) has a disparity in chemical and

physical properties. EPS matrix is also known to comprise of Uronic acids (such as D-galacturonic, D-glucuronic and mannuronic acids) (Sutherland 2001) or ketal-linked 50 S.L pyruvates (Percival et al.). Therefore, biofilm has anionic properties which allow cross-linking of divalent cations (Flemming et al. 2000) such as magnesium and calcium. Biofilms made up of gram-positive bacteria yield an EPS which is predominantly cationic (Marshall et al. 1971). Fresh and marine water bacteria both have been recorded association (Corpe 1970) of extracellular polymers in bacterial attachment. Bacteria isolated from these environments were analysed and were discovered to have been made up largely of acidic polysaccharides. However, the limit to which polysaccharides contributed in the adhesion process was debatable (Fletcher 1980). Excess polymer production may sometimes avert adhesion, however, little amount of polysaccharides may be required at the beginning for adhesion. Varying degrees of solubility can result in both hydrophilic and hydrophobic nature of EPS (Brown et al. 1977), which is highly hydrated. The composition and the structure mainly establish the primary confirmation as the polysaccharide content of EPS which has a significant impact on the biofilm. The backbone structures of 1,3- or 1,4-b-linked hexose residues (Sutherland 2001), which are firm and usually insoluble or poorly soluble are a part of bacterial EPS, although the other EPS molecules are easily soluble in water.

EPS presents several benefits to a biofilm (Bryers 1984; Marshall 1992): Increased absorption of heavy metals and nutrients, greater cohesive forces, protection of immobilized cells from environmental changes (Uhlinger and White 1983), stipulation of a medium for intercellular communication, the appropriation of microbial products and other microbes and transfer of genetic material. Polymer bridging helps in interaction of extending polymers on cell surfaces which interacts with vacant bonding sites. Polymer mechanisms for polymer bridging have been put forward strongly; however, it is yet to be made completely clarified (Characklis and Cooksey 1983). Exopolymer–substratum interactions can mediate connection of bacteria to the substratum which are primarily covalent bonds (Corpe 1970).

Major research has been done into the ecology of sessile microbial populations, and the focus has primarily been on the extracellular polymers (Costerton et al. 1981) which are produced by the cells. The occurrence of microbial exopolymers mainly takes place in aquatic habitats in the form of distinct capsules firmly fixed to the cell surface or as slime fibres slackly linked with or disconnected from the cells. It is now believed that several capsular polymers serve as holdfasts, which hold the cells to the inert surfaces and to each other, yet the extent to which they aid in other interactions between sessile bacteria and their bacteria is still a matter of understanding.

EPS affects the physical properties of biofilms including thermal conductivity, diffusivity and rheological properties. Irrespective of charge density or its iconic state, EPS has few properties of molecular sieves, diffusion barriers and adsorbents resulting into an effect on physiochemical processes like fluid fractional resistance and diffusion phenomenon (Costerton and Lashen 1984). EPS can also act as an ion exchange matrix due to it's predominantly polyanionic highly hydrated nature. It serves to increase local concentrations of iconic species like ammonium, potassium and heavy metals, whereas opposite effects are produced on anionic groups. Effect of EPS on uncharged molecules including potential nutrients such as sugars has

also been witnessed (Hamilton and Characklis 1989). However, EPS can act as a nutrient trap, particularly under oligotrophic conditions which give clear indication of biofilm bacteria to be concentrate in nature and use cationic nutrients such as amines (Costerton et al. 1981). On the contrary, there is a partial restriction of penetration of charged molecules such as biocides and antibiotics by this (Wahl 1989).

3.3.5 Interaction of Microorganisms inside Biofilm

Cellular interactions and competitive behaviour (Connell and Slatyer 1977) are the different parameters implied in biofilms used for heart and lungs. (James et al. 1995). Microbial succession representing a common feature is consistently under a state of fluctuation (Fredrickson 1977) as a result of competition strategies by microbes (Characklis 1981). Conditioning films required during the adhesion process in hearts and lungs have specific requirements (Connell and Slatyer 1977) as pioneer colonisers. These primitive colonisers are progressed by different events followed by different numbers of biological and physiological events. Reports revealed that there are lung infections (Baier 1984) caused by streptococcus mutants and its mutualistic association with *Candida albicans* (Stewart et al. 1997). These microorganisms which are associated in the antagonistic component (Baier 1984), perpetually interact in close proximity of the microorganisms in a biofilm. The mechanism of synergism is vital for varied classes of biofilm development.

3.4 Antibiotics and Biofilms

Treatment of bacterial infections today is limited to antibiotics amidst the Armour of therapeutic agents which are particularly built up to kill or cease the growth of a specific bacterium. The distinct biology of bacterial groups, i.e. formation of biofilms was not considered during the development of these agents. The symptoms caused by planktonic cells released from the biofilm are reversed characteristically by antibiotic therapy; however, it fails to terminate the biofilms (Carmona-Torre et al. 2017). This is the reason of persistent occurrence of symptoms despite cycles of antibiotic therapy until surgical removal of sessile population. Biofilms release planktonic bacteria cell which is a natural arrangement of programmed detachment making it niduses of acute infection. Biofilms probably elude antimicrobial challenges by various mechanisms (Parsek and Singh 2003).

1. The agents are unable to go through the full depth of the biofilm.
2. Nutrient limitation is experienced by some cells in a biofilm leading to a ravenous state and hence these cells have limited sensitivity to several antimicrobial agents.
3. Unique and protected biofilm phenotype is adopted by few cells which is a biologically programmed retort to augmentation on a surface.

Bacterial communities have the ability to adapt to different environmental stresses such as temperature, nutrient availability, osmolarity, UV radiation, oxygen tension and desiccation. These stresses may act as obstacles to carry out bacteria's normal functions which result in the formation of a complex matrix of biopolymers, Biofilms (Costerton et al. 1978a, b). Biofilms are formed either by single or multiple and complex microbial niches along with other organic substances including secreted extracellular polymeric substances (EPS). EPS matrix is the major constituent of biofilms involved in many vital roles including biofilm tolerance to antibiotics and disinfectants. Being a protective matrix, it prevents biocides by limiting the transportation through it and also by the sacrificial reaction. It also helps in forming the complex phenotypes of cells by involved in the development of a nutrient gradient.

Development of biofilms composed of various stages including forming a strong connection to the surface, forming of microcolonies attachment and transformation of tender biofilms into well-structured mature biofilms (Hall-Stoodley et al. 2004). Biofilms are either associated with abiotic or biotic surfaces. Community-level tolerance to different environmental stresses shows a higher level of resistance compared to single cells' tolerance and results in 10–1000 times more antibiotic resistance than single planktonic cells. Tolerance of biofilms is directly correlated with the rate of maturation.

Biofilm matrix plays an imperative role in antibiotic resistance. It follows various molecular mechanisms to develop resistance towards antibiotics. Glycocalyx layer is an integral part of the biofilm which serves as one of the resistance mechanisms. It provides strength and acts as adherent to withstand the unfavourable host environments and also it provides resistance to antibiotics. It helps exoenzymes to screen the antibacterial agents and mediates metabolite degradation of biocides. Genetic adaptation is another vital molecular mechanism in-term of antibiotic resistance in biofilms. Multiple antibiotic resistance operons reported in *Escherichia coli* regulates the expression of various genes responsible for multi-drug resistant phenotypes. Similarly, the expression of *ampC* gene present in *Pseudomonas aeruginosa* biofilms is upregulated in the presence of antibiotics.

To prevent or control the infections caused by bacterial biofilms, many antimicrobial treatments are brought into the field. Even though many effective antibiotic treatments are currently in practice to prevent the microbial infections, the probabilities of eradicating the biofilms infections are very less. Treatments have become more challenging and many clinical investigations have been carried out to find the effective control of biofilms infections.

Betalactam, colistin, ciprofloxacin and tobramycin are few antibiotics which have been tested for their pharmacokinetics and kinetics properties against biofilms in animal model. Ciprofloxacin and colistin have shown the ability to destroy the surface bacteria and on the depth, respectively. However, reports have shown that due to the low penetration level of ciprofloxacin through the biofilms of *P. aeruginosa,* ciprofloxacin lost its ability to kill the bacteria. In this case, the development of antibiotic tolerance of *P. aeruginosa* towards ciprofloxacin may be due to the oxygen limitation.

3.5 Pathogenic Mechanisms

Various mechanisms for pathogenesis have been proposed for biofilms. These mechanisms include the following steps:

1. Adherence to a solid substrate.
2. Increasing of the metabolic efficiency by implementing "division of labour".
3. Performing phagocytosis for evading host defences.
4. Obtaining a high-density population of microorganisms.
5. Generation of relatively higher virulent strains of microorganisms by exchange genes.
6. Production of higher concentration of toxins.
7. Protection against antimicrobial agents.
8. Transmission of microorganisms to different sites by detachment of microbial colonies.

Biofilms form preferably on passive or dead tissue and are generally found on medical instruments and remains of dead tissue such as fragments of dead bones; like endocarditis, they can also develop on active tissues (Ward et al. 1992). Antigens are released by sessile bacterial cells which help accelerate the making of antibodies; however, the bacteria within the biofilms remain active due to ineffectiveness of the antibodies. This may lead to immune complex mutilation to nearby tissues. Biofilm infections are hardly ever detected by the host defence system despite tremendous cellular and humoral immune reactions in individuals (Cochrane 1988).

Bacterial species common in our environment or are commensal with the human body form a major portion of the infectious diseases effecting mildly compromised humans. Electron microscopy has brought out the fact that the surfaces of medical devices are home to a large number of slime encased bacteria. Biofilm formation has been discovered in tissues singled out from non-device-related chronic infections. The infections can be due to a single or an assortment of bacteria or fungi species.

3.6 Biofilm and Human Diseases

In case of lack of a foreign body, biofilm disease is a very distinct body. Chronic airway infection are usually witnessed in cases of cystic fibrosis, tuberculosis, chronic wound infections, endocarditis, dental caries, osteomyelitis, periodonititis and biliary tract infection. Observations have also been made that in the soft tissues (e.g. the intestines or lungs), exposure of biofilm microbiota may be only to sub-minimum inhibitory antibiotic concentrations. Bacterial physiology will be impacted by such exposure, including the development of genetic and phenotypic in biofilm. It will also impact the capability of antibiotics to function as signalling molecules. In union, these outcomes would speed up the surfacing and proliferation of antibiotic resilient bacteria from the biofilm (Andersson and Hughes 2014).

3.6.1 Oral Cavity

Structurally and functionally organized biofilm on their oral surfaces have diverse multispecies microbial communities known as oral microbiome (Marsh and Zaura 2014). The facts seem to denote that microbes and disease-causing species which are of therapeutic significance work together within the periodontal microbiota in different ways that can direct circumstances that affect periodontal health or diseases. The ability of these pathogens to inhabit the oral microbiota provides latent source for dispersal to distant body sites and results in the possibility of developing systematic infections, mostly in those with immunodeficiency (Colombo et al. 2009).

One of the most generic oral infectious disease is periodontal disease like periodontitis and gingivitis, which are related with an enterprise of an extremely pathogenic biofilm that instigates an immune/inflammatory host response that shows the way to destruction of the underneath periodontal tissue and finally tooth loss (Lafaurie et al. 2017). Substantial biodiversity in the supragingival and subgingival plaque in fit oral cavity has been brought out by several studies. There is marked difference in the bacterial composition of supragingival and subgingival plaque. Supragingival bacterial microbiota in healthy adults is primarily composed of gram-positive cocci and anaerobic gram-positive rods with a preponderance of streptococci and *Actinomyces naeslundi,* respectively, however, in the subgingival plaque anaerobic gram-negative rods and facultative anaerobic gram-positive cocci are the prevalent microorganisms (Palmer 2010). Periodontal biofilm has anatomical propinquity to the gingival bloodstream and due to these periodontal pockets can act as a pool of microbial pathogens and their products and also inflammatory mediators and immune complexes that can spread to other sites of human body (Han and Wang 2013). The calcium flux is modified due to colonization and also helps in invasion of mucosal cells and release of toxins (Lamont and Jenkinson 1998). Microbial interdependencies and resilience to biofilms are generated by set-up of multiple synergistic to slight environmental perturbations (Marsh and Zaura 2014). In oral biofilms, there is a likelihood that a relationship between yeasts and other bacteria may persist. Increased candida stack favoured the existence of oral streptococci in a metagenomic investigation of elderly patients (Bamford et al. 2009).

3.6.2 Upper Airways

Sub-acute and acute exacerbations of chronic diseases have been depicted, however, sinusitis (or rhinosinusitis) is usually acute or chronic. Due to similarity of symptoms, it is usually problematic to clinically differentiate among them. There is a rising opinion that chronic rhinosinusitis is characterized by biofilm growth (Foreman et al. 2011).

3.6.3 Lower Airways

Biofilm infection may lead to lower respiratory tract infection, the prime example of which is Pseudomonas aeruginosa in cystic fibrosis patients (Singh et al. 2000). Upsurge of thick, sticky pulmonary secretions and chronic colonization and/or infection with a constrained group of organisms (*P. aeruginosa*, *Stenotrophomonas maltophilia*, and *Burkholderia cepacia*) are distinguished by cystic fibrosis which is an inherited disease. As per some studies, exchanges in mixed eukaryotic-prokaryotic biofilms (polymicrobial infections) in cystic fibrosis lungs may direct towards unfavourable clinical outcomes (Leclair and Hogan 2010). It has also been established that fungal biofilms found in the lung may also be a factor to infection.

3.6.4 Gastrointestinal and Urinary Tracts

Bacterial microbiota growing as healthy biofilm population is profoundly found on the mucosa of the gastrointestinal tract (Macfarlane 2008). Interactions between bacteria and yeasts possibly exist and play a part in health and disease in the gastrointestinal tract which is hugely a polymicrobial biofilm. The urinary tract has a diverse metagenome and a polymicrobial environment capable of preventing several diseases like urinary tract infections, bacterial vaginosis, sexually transmitted diseases and yeast infections. A selective inhibition of other species is mainly mediated by high acidity from bacterial metabolism (Gajer et al. 2012). Several studies brought to light that *Escherichia coli* and *Staphylococcus aureus* detach and cause genitourinary tract infection demonstrated in biofilm formation (Yousefi et al. 2016). Biofilm production is assumed to mediate the enteroaggregative phenotype which is characterized in *E. coli* in diarrhoea syndrome.

3.6.5 Wounds

Presence of bacterial biofilm chronic wounds has been written about in recent literature, and it has been specified that it leads to their persistence (Cooper et al. 2014). Microbial biofilms are found in diabetic foot ulcer which is characterized as a non-healing wound and is supposedly a clinical trial burden to patients. The presence of biofilm appears to cause delay in healing and is potentially a risk for causing infection (Seth et al. 2012). Biofilms can be prevented and once caused can be restricted using wound debridement and dislodging (Percival 2017). Non-random association within the wound site has been discovered between *S. aureus* and *P. aeruginosa* which are frequently isolated together in these patients (Fazli et al. 2009). *S. aureus* can bind to fibrinogen present in the bone matrix as it has fibrin receptors and hence may start biofilm formation. It is easier for the pathogen to colonize soft tissues, skin

and even bone (i.e. osteomyelitis) due to the ability of *S. aureus* to bind collagen, fibronecin and laminin with affinity by forming a biofilm (Ciampolini and Harding 2000). Further studies are required to figure out ways of identifying and monitoring colonization of biofilm to that quick response towards treatment can be initiated (Vyas and Wong 2016). Studies also show that pathogenic fungal species also have a role to play in these infections (Branski et al. 2009).

3.7 Main Characteristics of Biofilm Mediated Diseases

Biofilm behaves differently in comparison with their planktonic counterparts associated with respect to both their growth rates and their propensity to resist antimicrobial treatment and host defences (Fux et al. 2005). Production of antibodies is well stimulated by the bacterial antigens at the biofilm surface. These antibodies form immune complexes at the biofilm surface, however, these antibodies cannot seep into the biofilm to put an end to the infection. The immune complexes thus formed often damage the colonized tissue. Bacterial biofilm when formed on vascular surfaces like endothelium of heart valves accrete blood components such as fibrin and platelets (Gilbert et al. 2002). Due to this, the tissues close to the biofilm may encounter collateral damage by invading neutrophils and immune complexes (Hoiby et al. 2011). In vitro studies using bacterial isolates are the primary sources of evidence which supports the theory that recalcitrance of infection is caused by biofilm. These bacterial isolates may not appropriately reflect in vivo conditions or wildlife pathogens. The unique characteristics of the infections related to biofilms are as following:

1. Indolent pathogenic patterns with acute periods and alternating quiescent.
2. Response to antibiotic therapy may be an initial, however, due to biofilm being protected from antimicrobials the relapses are very frequent.
3. These infections may be polymicrobial in nature as the predominant bacteria are wither bowel flora or common members of autochthonous skin or perhaps very common environmental organisms.
4. During the placement and removal of a biomedical device, it may be difficult to recover bacteria from adjacent fluids and tissues.

To reduce the risk, mortality and morbidity associated with biofilm infections four strategies are majorly employed:

1. Aseptic technique and sterile precautions during device placement.
2. Antibiotic or antimicrobial eluting devices to be used.
3. Agents may be infused to eliminate the organism within it or to disrupt an established biofilm.
4. A definitive treatment strategy is the removal of infected device, however, it may cause physical and psychological burden to the patient in case of complex implanted devices such as mechanical heart valves or fracture fixation hardware (Del Pozo and Patel 2013).

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Chapter 4
The Role of Biofilm in Originating, Mediating, and Proliferating Infectious Diseases

Amresh Kumar Singh, Vivek Gaur and Anand Kumar Maurya

Abstract Homogenous or heterogeneous networks of bacteria, responsible for the synthesis of biofilms, can attach themselves to lattices of natural polymers or extracellular polymeric substances (EPSs). These polymers include glycopeptides, lipids, and lipopolysaccharides holding the biofilm together via a structured framework. Microbiota found within biofilms often play a part in the decomposition of organic matter, the restoration of various ecological recalcitrant pollutants, and the fixation of gas, sulfur, and metals. Aside from these helpful effects, biofilms can be extremely pathogenic. A significant number of biofilms associated with different diseases are comprised of solitary bacterial categories. The exception to this generalization are biofilms associated with catheters and voice prostheses—quite similar to oral cavity biofilms in that they are regularly comprised of diversified pathogenic and non-pathogenic organisms. The etiology of such diseases are known to be related to bacterial aggregation and biofilm development on indwelling devices or tissues. Biofilms likewise encourage gene transfers among bacteria, which can favor the incorporation of several virulent strains. Another possible component mediated by biofilm cells is differential gene expression. An optimal example of species complexity is found in oral biofilms—caused by many different microorganisms. To date more than 350 bacterial species have been found to be responsible for dental plaques.

4.1 Introduction

Biofilms play a very important role in all human microbial infections. It is difficult to provide the precise prevalence and incidence of biofilms associated with acute/chronic infectious diseases. Favorable conditions for biofilm production in infectious diseases may depend on contact with oxygen and nutrients in the periphery. However, tissue necrotic debris, low oxygen supply, lack of immune response,

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depletion of growth factors, and acidic conditions occur in the case of deep biofilm layers. This chapter will describe our current understanding of the role biofilms play in originating, mediating, and proliferating different kinds of infections in human beings.

The pathologic process of biofilm formation in different infectious diseases follows four distinct stages (Hollmann et al.).

(a) **Surface attachment**

Adherence of planktonic prokaryotic cells takes place either as a consequence of physical forces or via a locomotive organ used by bacteria, such as pilli or flagella. Many favorable conditions like surface functionality, temperature, and pressure may significantly regulate bacterial attachment. The different physical forces associated with bacterial adhesion to surfaces are van der Waals forces, steric interactions, and electrostatic (bi-layered) interactions. Surface-to-cell and cell-to-cell attachments are defined as adhesion and cohesion. Transfer of information among microorganisms occurs due to the formation of auto-inducer signals that appear via the assertion of biofilm-specific genes. At this point, bacteria start secreting a matrix of extracellular polymeric substances (EPSs) to fix the aggregation of a biofilm (Gupta et al. 2015).

(b) **Micro-colony formation**

In this stage the thickness of a micro-colony reaches around 100 μm. Micro-colonies in biofilms are composed of different microbial communities and because of their close proximity to one another there is an enhanced substrate exchange, an appropriation of anabolic and catabolic products, and an ejection of toxic (nitrogenous) by-products. Fermentative or anaerobic bacteria start catabolizing the complex forms of organic substances into acids and alcohols. These end products are further utilized as substrate by acetogenic bacteria (Fig. 4.1).

(c) **Biofilm maturation**

This stage includes the collection and accumulation of cells, leading to the formation of micro-colonies, followed by growth, development, and maturation of adhered cells. This ripening/maturation phase includes the adaptation of biofilms to external environmental conditions by changing their physiological and metabolic structures.

(d) **Dispersal and detachment**

In this stage the microbial network in the biofilm scatters, marking shedding that produces distinctive saccharolytic enzymes that cause biofilm lysis. Fixing of polysaccharides occours due to subsequently detachment of surface bacteria dwelling at the superficial structure of biofilm for aggregation to another surface. *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* release alginate lyase, *Escherichia coli* secretes *N*-acetyl-heparosan lyase, and *Streptococcus* spp. produce hyaluronidase enzymes responsible for the lysis of a biofilm's matrix. This is the phase where bacteria upregulate the expression of flagella proteins and consequently become motile,

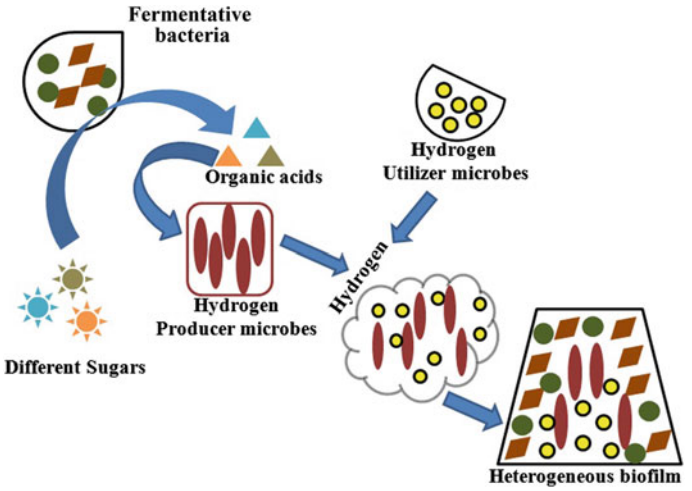

Fig. 4.1 Fermenters utilize different sugars to produce organic acids

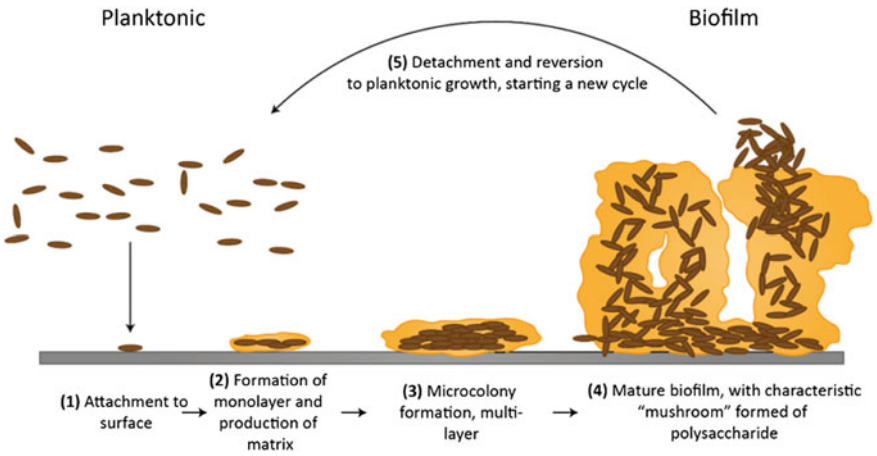

Fig. 4.2 Diagrammatic representation of biofilm formation (Hollmann et al.)

translocating to other sites (Gupta et al. 2015; Chen and Wen 2011; Stoodley and Stoodley 2005) (Fig. 4.2).

4.1.1 Biofilm Origination and Mediation

Planktonic growth to biofilm represents a transition that first happens because of an environmental change and involves different regulatory networks. This results in the

translation of messages (signals) into a coordinated genetic expression, thereby moderating the spatial and temporal reconstruction of a microbial cell. This cellular reconstruction helps in the modification of the expression of different surface molecules, the utilization of nutrients, and expressed virulence factors. Inside the biofilm, bacteria are enclosed in a self-produced extracellular matrix. Nutrients become trapped inside the matrix and are utilized in metabolic pathways by the bacterial community and water molecules are effectively retained through hydrogen bond interactions with polysaccharides (hydrophilic interactions) (Kostakioti et al. 2013).

Thus, the structural composition of the matrix forms turgid bacteria and vigorous structures with strong tensile strengths, bringing them into close proximity, allowing intimate cell-to-cell interactions and nuclear material exchange. Interactions between bacteria can enhance the dissemination of different drug resistance markers and other virulence factors that result in biofilm-forming pathogens and the establishment of several chronic infections (Table 4.1).

Table 4.1 Biofilm-forming pathogens and associated infections (Wilson 2001)

Disease	Associated pathogens
Dental caries	*Streptococcus mutans, Actinomyces* and *Lactobacillus* spp.
Gingivitis	*Veillonella parvula, Fusobacterium nucleatum*, *Campylobacter* spp., *Treponema* spp., some unknown aetiology
Periodontitis	*Bacteroides forsythus, Porphyromonas gingivalis, Prevotella intermedia, Aggregatibacter actinomycetemcomitans*
Prosthetic heart valves	CoNS, *Staphylococcus aureus*, oral streptococci
Prosthetic hip/knee joints	CoNS, *Peptococcus* spp., *Staphylococcus aureus,* AGNB
Central venous catheters	AGNB, CoNS, *Staphylococcus aureus, Candida* spp.
Hydrocephalus shunts	CoNS, *Staphylococcus aureus,* AGNB, *Corynaebacterium* spp.
Voice prostheses	*Staphylococcus aureus, Candida* spp.
Urinary tract infections	AGNB, *Candida* spp. *Enterococci*, CoNS
Lung infections driven by cystic fibrosis	*Staphylococcus aureus, Psedomonas* spp., *Burkholderia cepacia, Haemophilus influenza*
Contact lens infections	*Pseudomonas* spp., CoNS
Staphylococcal infections of the skin	*Staphyloccous aureus*
Chronic ulcers	*Pseudomonas aeruginosa*
Other skin diseases	*Streptococcus pyogenes*, *Klebsiella* spp., *Clostridium* spp., *Propionibacterium acnes, Staphylococcus aureus, Pseudomonas aeruginosa*

AGNB aerobic gram-negative bacilli, *CoNS* coagulase-negative *Staphylococcus*

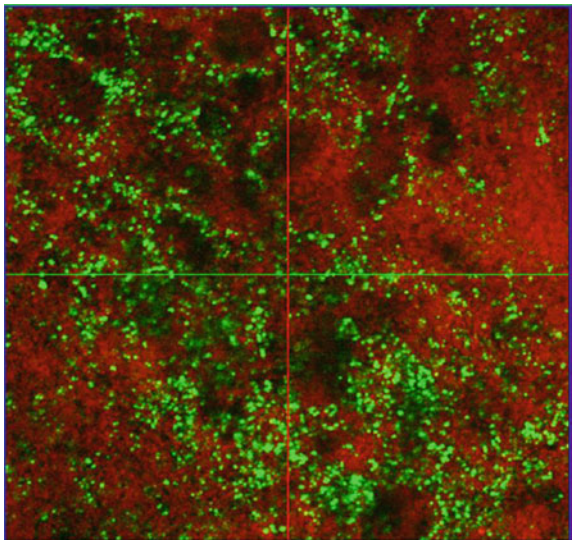

Fig. 4.3 Polymicrobial biofilm formed by *P. aeruginosa* (*red*) and *S. epidermidis* (*green*)

The case of a biofilm-mediated infection developing from *P. aeruginosa* experiences a characteristic transition from acute virulent pathogen to a cystic fibrosis (CF)-adapted pathogen, allowing it to persist in the lungs for years or even decades. This is due to the overproduction of the polysaccharide matrix alginate, leading to the formation of a mucoid biofilm that tolerates antibiotics, resists phagocytosis, and contains components of natural/native and acquired immune responses. These mucoid biofilms persisting within the CF lung lead to the development of a distinct antibody response. This prompts a chronic inflammation that is mediated by granulocytes and that results in severe damage to the lung tissue of CF patients (Hollmann et al.) (Fig. 4.3).

4.2 Indwelling Devices Where Microbes Frequently Cause Biofilms

The majority of these species are from typical microflora and cause biofilms at naturally occurring sites in human beings. Most of the pathogenic forms of human diseases and plant infections are influenced by biofilms. Some common examples are linked to dental care, lower and upper respiratory tract infections, infections related to surgical implants, ventilators, cystic fibrosis, and urogenital and other medical devices (Wilson 2001; Hollmann et al.).

The role of biofilms in implant infections has been established in numerous systems, however, their role in non-implant diseases is not well established. Table 4.2 provides some examples of indwelling devices where microbes have been found to frequently cause biofilms (Kokare et al. 2009).

Table 4.2 Sites where microbes frequently cause biofilms (Wilson 2001; Kokare et al. 2009)

Organism	Site of infection
Staphylococcus aureus	Implantable devices
Staphylococcus epidermidis and other CoNS	Implantable devices
Pseudomonas aeruginosa	Lungs of cystic fibrosis
Escherichia coli and other *Enterobacteriaceae*	Urinary catheters
E. coli	Gastrointestinal tract
Streptococcus spp.	Teeth
Actinomyces spp.	Teeth
Lactobacillus spp.	Vagina, teeth
Enterococcus spp.	Hip arthroplasty, prosthetic heart valves
Staphylococcus aureus and CoNS	Central lines, intrauterine contraceptive devices, prosthetic heart valves
Candida albicans	Artificial plural prostheses
Pseudomonas aeruginosa, Escherichia coli, Proteus spp., *Candida albicans, Serratia* spp. *Staphylococcus aureus*	Contact lenses

CoNS coagulase-negative *Staphylococcus*

4.3 Biofilm-Mediated Infectious Diseases

For centuries, mankind has suffered from various kinds of acute bacterial infections and fatal disease due to pathogenic microorganisms. Meanwhile, microbial ecologists have established that surface-associated bacterial biofilms are widely spread in various natural environments, where they usually present as distinct from individual planktonic bacteria. Whereas biofilms show specific biological properties compared with planktonic bacteria. Moreover, it has been identified that the universal use of various types of indwelling implanted medical devices in humans may lead to the adhesion of microorganisms and cause colonization and infections. This topic helps with the understanding of the mechanism involved in the capacity of bacterial biofilms to survive and develop into biofilm-related infections (Lebeaux et al. 2014).

4.3.1 Barrett's Esophagus and Gastric Cancer

In the human gastrointestinal (GI) tract, the large intestine is the site where microorganisms are heavily colonized. The independent occurrence of bacteria in the large intestine, as individual cells, was initially observed by microscope, however, many of them exist in micro-colonies or live in symbiotic relationships with different species on the surfaces of particulate materials. Biofilms associated with the esophagus, stomach, and intestinal parts of the GI tract are usually multiflora consortia

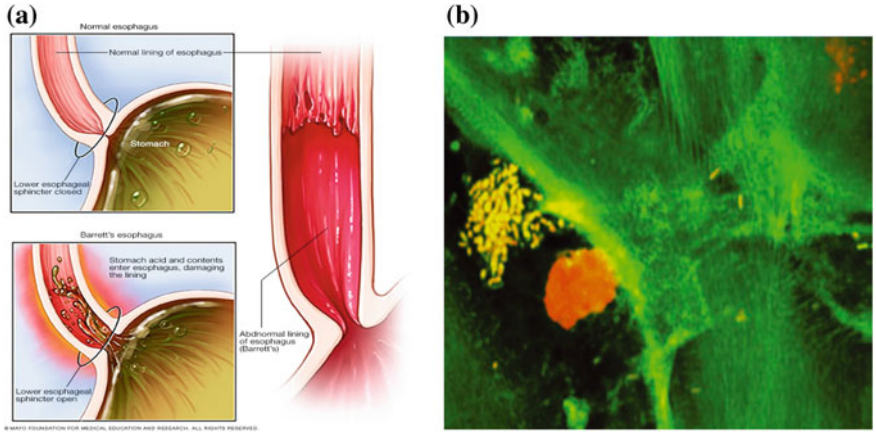

Fig. 4.4 **a** In Barrett's esophagus normal esophageal cells are replaced with abnormal cells (Mayo clinic 2017). **b** Barrett's esophagus showing bacterial growth in aggregates

whose growth is influenced by factors associated with the environment, nutrition, and chemical composition (Macfarlane and Dillon 2006).

Barrett's esophagus is the term used to describe a metaplasia of the lower end of the esophagus in which generally the squamous epithelium changes into a columnar epithelium mucosa. Barrett's esophagus is triggered by acid reflux in patients—a condition for which 10% of sufferers go on to develop Barrett's. Adenocarcinoma has demonstrated an increased rate of incidence over the last 20 years in patients with Barrett's esophagus—it is the commonest cause of death due to cancer in the United Kingdom (Fig. 4.4).

The etiology of gastric cancer is associated with *Helicobacter pylori*; it appears it may cause duodenal and gastric cancer due to an indirect mechanism of chronic colonization of the superficial gastric mucosa (Macfarlane and Dillon 2006).

4.3.2 Endotracheal Tube Colonization and Ventilator-Associated Pneumonia

The growth of biofilm on the surface of endotracheal tubes and the development of bacterial colonization occurs within very short periods of time after endotracheal intubation. Ventilator-associated pneumonia (VAP) is caused by aerosolization of biofilms during mechanical ventilation or disruption during tracheal suctioning. It is a common nosocomial infection in intensive care units—often associated with significant morbidity and mortality. Endotracheal tube biofilms are usually polymicrobial in nature and composed of many organisms found within oropharyngeal (*Streptococcus* and *Prevotella* species) and enteric flora ESKAPE organisms (*Enterococcus*

faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.)

According to Boisvert et al. (2016) around 50% of VAP cases are associated with the same causative agents found in bronchoalveolar lavage and endotracheal tube biofilms, leading to treatment failure and mortality.

4.3.3 Cystic Fibrosis

In CF there is abnormal mucociliary and other host defense mechanisms caused by mutations in the CF transmembrane conductance regulator gene (CFTMCRG). CF leads to chronic lung disease caused by persistent bacterial infections of the airways, leading to lung infections. The main pathogen in adult patients with CF is *Pseudomonas aeruginosa,* enhancing lifelong chronic airway infection. Contrary to catheter-associated biofilms, in this case *Pseudomonas aeruginosa* forms untethered biofilms that accumulate within the sputum of minor airways. When bacterial motility is restricted, bacteria form similar biofilms that accumulate within high-density gels that include the presence of neutrophil elastase, DNA, and amino acids in sufficient quantities to promote biofilm aggregate formation as a result of chronic inflammation. As a matter of significance this non-attached biofilm formation is also responsible for resistance—magnifying the effect of antibiotics (Boisvert et al. 2016) (Fig. 4.5).

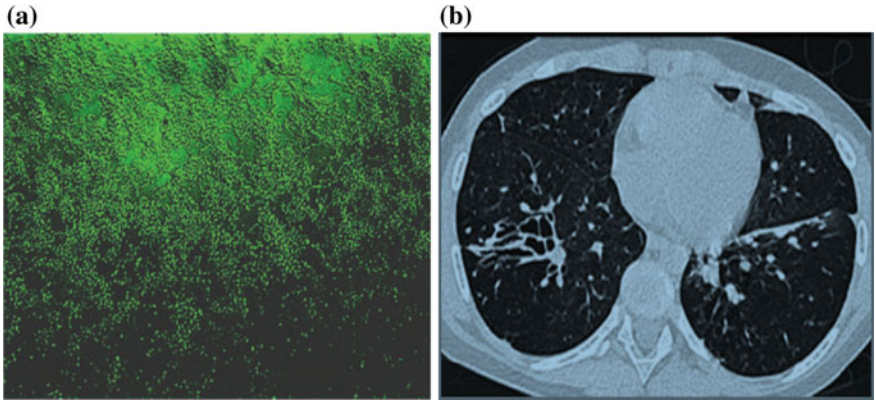

Fig. 4.5 **a** *Pseudomonas aeruginosa* biofilms (Walker et al. 2005). **b** Severe bronchiectasis in end-stage cystic fibrosis (Davies et al. 2007)

4.3.4 Chronic Otitis Media

The upper respiratory tract, from the nose, paranasal sinuses, middle ear, and up to the throat, is commonly associated with inflammatory infections in children. Children are more prone to such infections—they have short eustachian tubes that are less functional compared with those in adults. They may complain of complicated upper airway illnesses due to infections caused by bacteria that may develop as otitis media with fluid effusion, a chronic suppurative bacterial infection with mastoiditis, and even cholesteatomas—even after optimal antibiotic treatment has been initiated. It is difficult to confirm the cause of chronic infections in the middle ear due to negative culture clinical samples. As bacteria are often not isolated it is difficult to explain such occurrences, although recurrences or exacerbations are intriguing. It has been postulated that a local inflammatory response without bacterial intermixing might cause infections. However, various experiments using animal models demonstrated that bacterial biofilms could cause such infections.

To elucidate the presence of bacteria in biofilm aggregates peptide nucleic acid fluorescence in situ hybridization (PNA FISH) was used by Bjarnsholt. The discharged pus, taken from the ears of 5/6 (83%) children with chronic suppurative otitis media (CSOM) (Fig. 4.6) was observed and evidence was recorded of biofilms in biopsies from the middle ear of 8/10 (80%) adults (those who were initially treated for CSOM). Hence it is accepted that bacterial biofilms play a role in several chronic infectious diseases of the middle ear (Bjarnsholt 2013; Bagtzoglou 2008).

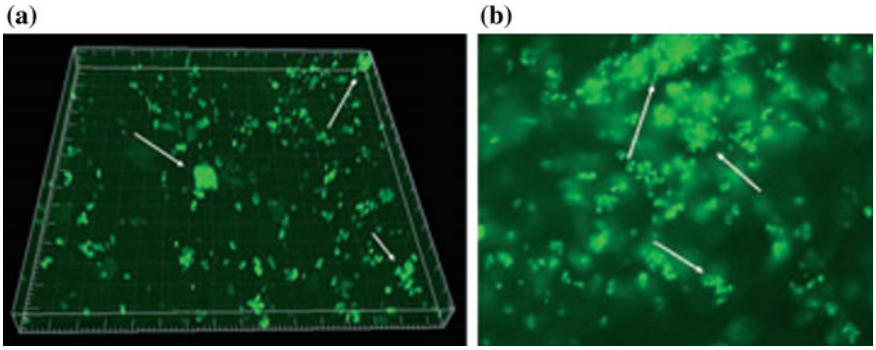

Fig. 4.6 *Staphylococcus aureus* biofilms (*arrows*) in otorrhea from a patient with chronic suppurative otitis media as determined by species-specific peptide nucleic acid fluorescence in situ hybridization

4.3.5 Dental Plaque

An example of synergism between mammalian defense mechanisms and bacterial pathogenesis is dental plaque. The surface of teeth are colonized by oral bacteria as single cells or pairs of cells. Bacteria dominate the initial stages, observed through various stages of amitotic cell division and micro-colonies formed as monolayers. Synthesis of multilayered biofilms is the result of continuous cell division. *Streptococci* dominate early colonization and compose 60–90% of the initial flora. The remaining 10–40% of bacteria are mainly Gram-positive bacilli, predominantly *Actinomyces*. The microbiota of the oral cavity provides an optimum environment during healthy conditions, but ecological shifts may happen within the microbial community that result in two major oral diseases—dental caries and periodontal disease (Bagtzoglou 2008).

4.3.6 Urinary Tract and Catheter-Associated Infections

Urology is one of the main areas where biofilm-forming bacteria can become a serious health problem. The main areas where the presence of biofilms have been identified are: the urothelium, prostatic calculi, and implants. Renal tissue can be invaded by biofilm formations that are adhered, by bacteria, to the uroepithelium, causing pyelonephritis that is responsible for chronic bacterial prostatitis.

Biofilms develop into urethral stents as well as forming on the intraluminal part of catheters, thereby inflicting blockages. Therefore, catheter-associated urinary tract infections (CAUTIs) are among the foremost common healthcare-associated infections within intensive care units, caused by commensalism skin flora (e.g. coagulase-negative *Staphylococcus* species and *S. aureus*), although enteric Gram-negative bacilli might also harbor infections (Boisvert et al. 2016). In the majority of cases these infections are caused due to single microbes like *E. coli*, *Pseudomonas aeruginosa*, *Enterococcus* spp., and *Candida*, *Klebsiella,* or *Enterobacter* spp. In this type of medical device, microorganisms producing urease, an enzyme that hydrolyzes urea into ammonium ions, can cause encrustation, infected bladder calculi, and urinary obstruction. The formation of ammonium ions increases the pH of the urine and makes it alkaline in nature, finally causing the precipitation of magnesium and calcium phosphate crystals. A layer of calcium phosphate crystals protects bacteria from the antimicrobial effects of compounds used to impregnate catheters. The main source of urinary infections is *Proteus mirabilis*, in addition to *Proteus vulgaris* and *Providencia rettgeri*. Such organisms have several virulence factors that allow for the manufacture of biofilms, such as mannose-resistant fimbriae, capsulated structures, and urease (Soto 2014). To prevent or reduced catheter-associated infections surfaces coated with minocycline–rifampicin or chlorhexidine–silver–sulfadiazine are used to prevent bacterial colonization, microbial adhesion, biofilm formation, and bloodstream infections (Boisvert et al. 2016).

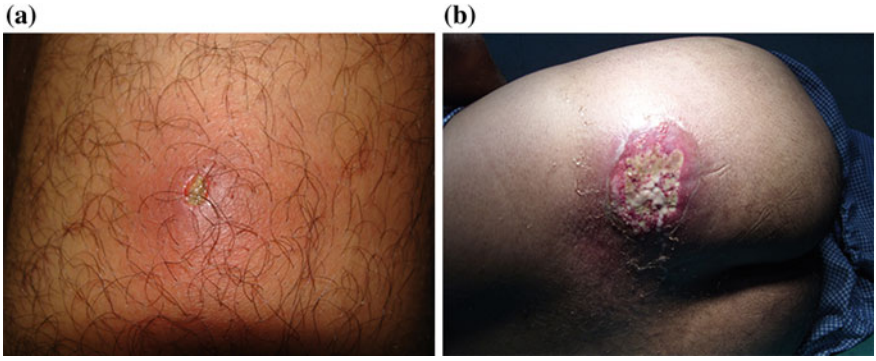

Fig. 4.7 *Staphylococcus* infection: **a** furuncles and **b** carbuncles

4.3.7 Skin Infections by Staphylococcus

Different species of *Staphylococcus* are commonly found in skin. In the normal flora of the surface of skin the most prevalent species are *S. epidermidis* and *S. hominis.* The nasal cavity and healthy skin may contain *S. aureus*. A wide range of skin infections are due to pathogenic strains of *S. aureus*—it is quite contagious. Skin-to-skin contact may lead to a rapid spread of infection. Most people are asymptomatic carriers of chronic nasal infections—carrying *S. aureus* infections in their nostrils. This means that it is easy to transfer bacteria from nasal regions to the hand and then to other individuals. If a hospital patient at a healthcare facility is colonized with antibiotic-resistant strains of *S. aureus*, then such strains can easily be passed on to healthcare providers and other patients.

Infections are caused by *Staphylococcus;* an infection is often enhanced by the secretion of chemicals by some virulent strains. The virulence factors of *Staphylococcus* include hemolysins called staphylolysins that are cytotoxic for many somatic cells including skin and leucocytes. Virulent strains of *S. aureus* are coagulase-positive because they produce coagulase—a plasma-clotting protein that plays a role in the formation of skin abscesses. *S. aureus*, when associated with pyoderma, causes a purulent-type of skin infection. Many strains of *S. aureus* produce leukocidins, causing pus formation that kills WBCs. These purulent skin infections may initially manifest as folliculitis but lead to furuncles or deeper abscesses called carbuncles when the infection spreads (OpenStax Microbiology 2018) (Fig. 4.7).

4.3.8 Chronic Ulcers

Chronic wounds may affect only the epidermal and dermal layers of the skin or may affect tissues all the way to the fascia. Chronic ulcers (non-recuperating ulcers) are

characterized as unconstrained injuries that typically occur in the lower extremities including the hip, knee, ankle joints, femur, leg, and foot. Anaerobes such as *Bacteroides*, *Clostridium*, and *Streptococcus* invade up to the endodermis region, insulated from the healing influence of oxygen. Facultative anaerobic bacteria are responsible for many devastating infections resulting in non-functioning limbs or gangrene. Aerobic bacteria including *Staphylococcus epidermis*, *Corynebacteria*, and *Propionibacteria* are more clearly identified with the superficial epidermal layers of the skin but might also be involved in the infective process (Iqbal et al. 2017; Suthar et al. 2017).

Gram-negative mixed flora is often found in older ulcers, but *Staphylococci* and *Streptococci* bacteria are normally found in new ulcers. In addition, different types of ulcers are affected by the numbers of bacteria, for example, a clinical infection will develop in 60% of diabetic foot ulcers but only 20% of venous leg ulcers colonized by *Staphylococcus aureus* (Iqbal et al. 2017).

4.3.9 Prosthetic Graft Infection

Biomaterials such as pyrolytic carbon or polyester are widely used in many prosthetic devices while performing cardiac and vascular surgery. Formation of biofilms on these prosthetic materials is the result of aggregation of microorganism, where microbial cells start embedding within the surface of an aqueous matrix of extracellular polymers. This particular growth pattern characterizes the feasibility of microorganisms to survive in unfavorable conditions. The mechanisms responsible for microbial adhesion are complex and are not completely understood to date. Several conditions, such as roughness, surface chemistry, hydrophobicity, and surface free energy, all appear to play a role in the process of adhesion. *S. aureus*, *S. epidermidis*, and *P. aeruginosa* are the most common and frequent microorganisms found to be involved in prosthetic infections and biofilm formation on cardiovascular prostheses (Litzler et al. 2007) (Fig. 4.8).

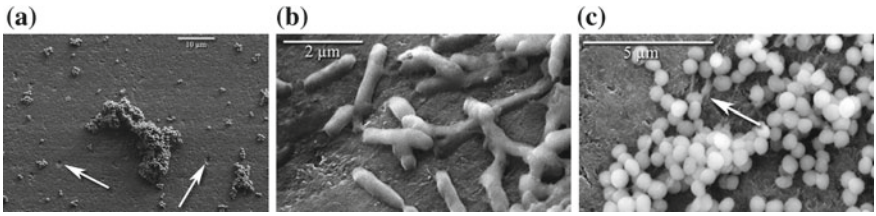

Fig. 4.8 Biofilms of **a** *Staphylococcus aureus*; **b** *Pseudomonas aeruginosa*; and **c** *Staphylococcus epidermidis* on heart valve prostheses

Table 4.3 Microorganisms found in biofilm-related HCAIs

Healthcare-associated infections	Biofilm-causing microorganism
Central line–associated septicemia	CoNS, *C. albicans, K. pneumoniae, P. aeruginosa, S. aureus, S. epidermidis*
Ventilator-associated pneumonia	*Candida* spp., *K. pneumoniae, P. aeruginosa, S. aureus, S. epidermidis*
Catheter-associated UTIs	CoNS, *C. albicans, A. baumannii, P. mirabilis, E. coli, K. pneumoniae, P. aeruginosa, S. aureus, S. epidermidis*
Surgical wound, prosthesis-related infection	*Candida* spp., *E. coli, Staphylococcus* spp, MRSA, *P. aeruginosa, S. aureus, S. epidermidis*

UTIs urinary tract infections, *CoNS* coagulase-negative *Staphylococcus*

4.3.10 Healthcare-Associated Infections

Healthcare-associated infections (HCAIs) are caused by a number of agents, most commonly bacteria, but also fungi, parasites, viruses, and prions. It is important to understand the routes of transmission of such microorganisms that are associated with the risk of developing HCAIs. Several reservoirs, such as water, food, and human body surfaces, might represent sources of HCAIs. The most widely known microorganism linked to HCAIs is the hospital "superbug"—methicillin-resistant *S. aureus* (MRSA) (Table 4.3)—which can lead to septicemia or bacteremia in intensive care units. The direct or indirect transmission of microorganisms from one host to another is often due to airborne contamination, close contact with infected surfaces, or use of contaminated food. New hosts can be infected by inhalation, ingestion, breaks in the skin barrier following surgery or insertion of intravenous lines, or through mucous membranes, including the nose, eyes, and mouth. It is possible to avoid HCAIs by the application of strict hygiene procedures and the proper use of disinfecting agents in healthcare settings (Percival et al. 2015).

4.4 Other Biofilm-Mediated Infections

The role of biofilms in originating, mediating, and proliferating infectious diseases is becoming more dominant due to an increasing variety and form of infections related to biofilms. There are several reports on biofilm formation by different microorganisms that mediate infections in humans in several ways. Waterborne, airborne, food contamination, and household biofilms also play a role in the spread of hospital-acquired infections due to renovations being made to old hospitals. The increased possibility of airborne fungal and bacterial pathogens, present as biofilms in the infrastructure of old buildings (molds, Legionella) puts patients at higher risk due

to the increasingly aggressive action of microbes in medical and surgical interventions (implants, organ transplants, use of devices) (Jordan 2004). A few reports have shown that micro-colony development and the creation of an extracellular grid in skin lesions occurs both in vivo and in vitro. In vitro biofilm arrangement has been exhibited for different individuals from the common flora of human skin, including *Staphylococcus epidermidis* and different *Corynaebacteria* spp. (Coenye et al. 2008).

4.5 Conclusion

The development of biofilms can be considered a double-stage biological procedure, constrained through surface attachment and cell-to-cell communication. Accumulated microorganisms, protected and lined by an extracellular framework, are important to biological processes, such as nutritional stimulation and the threats associated with microbial or chemical assault. In the human body, biofilms might trigger diligent chronic infectious diseases. Once the identification of a biofilm has been made and has been related to a medical condition, clinicians should suggest surgical removal or replacement and therapeutic treatment. Antibiotics, anti-inflammatory substances, and anti-biofilm activities should be used to treat biofilm-associated infections. Nowadays it is very promising that paths involve molecular mechanisms to convert biofilm arrangements into anti-biofilm products, however, this is a delayed process requiring significant time to complete. Non-intrusive and potentially negligibly intrusive detection techniques and standard guidelines for biofilm procedures may improve the possibility of new biofilm-oriented solutions. The vast number of biofilm inhibitors are still to be comprehensively investigated. If clinical practitioners are promptly made aware of the importance of bacterial biofilm origination, its development, and its relation to serious infections, then more translational research, new diagnostics, and therapeutic approaches may be developed (Chen and Wen 2011).

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Chapter 5
Modern Methods in Microscopy for the Assessment of Biofilms

Manodeep Sen and Pushpa Yadav

Abstract Scientific imaging technique is important for the analysis and understanding of complex natural systems. A biofilm comprises any group of microorganisms which cells stick to each other and often also to a surface. It is pandemic integrated important scenario executed by microorganisms to sustain in occasionally coarse environmental conditions. It is bacterial colonies adhered to a surface and fixed in an outer polymeric substance which provides for the protection, strength, and nutrients of the various bacterial species inherited. A few techniques have been currently used for biofilm studies that have committed to broad knowledge on biofilm structure and composition. Another microscopic technique such as light and electron microscopy and new latest techniques have been enclosed using confocal laser scanning microscopy (CLSM), focused ion beam SEM, Fluorescent microscopy, high-frequency acoustic microscopy, and atomic force microscopy. In this chapter, immersed by discriminating aspects emphasizes the advantages and obstructions of several methods. Other imaging methods have been used to identify biofilm biomass and cell viability. That is why we explain different microscopy methods, including their advantages and disadvantages. This chapter summarized the more novel applications with the purpose to encourage research and new microscopic techniques in microbiology.

Keywords Biofilm · Confocal laser scanner microscopy (CLSM) · Focused ion beam (SEM) · Fluorescent microscopy · High-frequency acoustic microscopy · Atomic force microscopy

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5.1 Introduction

A structured group attached on a living or inert surface formed by microbial cells sticked to each other and surrounded by the self-produced extracellular polymeric matrix is known as biofilm (Wu et al. 2015). Biofilm is the formation of surface-attached cellular agglomerations; contributing significantly to increasing bacterial resistance against antibiotics and innate host defenses. Biofilm formation of pathogenic bacteria has an enormous impact on the outcome of many bacterial infections. Around 65% of clinical infections treated by physicians in the developed world are characterized by the involvement of biofilms (Mengi et al. 2013).

Biofilms cause recurrent invasive infections that are difficult to eradicate because of their high resistance to antimicrobials and host defense mechanisms. The use of combinations of antifungal agents may improve the management of biofilm-related fungal infections and prevent the emergence of resistance associated with monotherapy (Íñigo and Del Pozo 2018). The microbial biofilms develop on or within native medical devices (e.g., contact lenses, central venous catheters and needleless connectors, endotracheal tubes, intrauterine devices, mechanical heart valves, pacemakers, peritoneal dialysis catheters, prosthetic joints, tympanostomy tubes, urinary catheters, and voice prostheses) (Donlan 2001). Bacterial biofilms are three-dimensional extracellular matrices composed of carbohydrates, proteins, and extra polysaccharides that develop on solid–liquid or solid–air interfaces in the body (Anastasiadis et al. 2014).

Demanding and advancing antibiotic treatment is usually helpful to control the worsening of chronic biofilm infections induced by dispersed bacteria and reduce the biofilms, but cannot extinguish the biofilm infections, because the minimal concentration of antibiotic for extermination of mature biofilm is difficult to reach in vivo. Biofilm consists of different stages, i.e., formation of a conditioning film, nonpermanent attachment, permanent attachment, growth and maturation, and dispersal of mature biofilm.

Bacterial biofilms are characterized as greatly resistant to antibiotic treatment and immune responses. Although it is well-known that antibiotic treatment is currently most important and effective measure for the control of microbial infections, antibiotic treatments are almost impossible to extinguish biofilm infections. In vitro and in vivo experiments demonstrated that the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for biofilm bacterial cells were usually much higher (approximately 10–1000 times) than the planktonic bacterial cells. The efficient antibiotic MBC in vivo for biofilm destruction is therefore impossible to reach by conventional antibiotic administrations due to the toxicities and the side effects of antibiotics and the limitation of renal and hepatic functions (Wu et al. 2015).

5.2 Diagnosis of Biofilm Infections

Biofilms are especially difficult to diagnose since the small nidus of infection is often missed when tissue is extracted for culture. It is important to discriminate whether the infection is a localized biofilm or a free-floating planktonic infection since the treatment modalities for these two forms of infections are significantly different. In particular, biofilm infections, in reverse to the planktonic modes of infection, are resistant to clearance by antimicrobial agents.

In order to better diagnose biofilm infections, we have advanced a rapid biofilm lateral flow immunoassay that detects host antibodies generated against biofilm specific proteins in the sera as well as an in vivo diagnostic agent that is based upon labeled antibodies against biofilm specific antigens that can be injected into the host and localize at areas of biofilm infection.

Generally, biofilm infection could be imagined if a patient has one of the clinical signs as shown in Table 5.1.

5.2.1 Routine Microbiological Examination

Conventional microbiological method includes sample collection, microbial cultivation, identification and tests of antibiotic susceptibilities, in which appropriate sample collection is essential according to our clinical experiences. For example, in patients suspected for foreign body-associated biofilm infections, at least 4–5 pieces of tissue biopsy from different sites next to the prosthesis suspected infection are needed to avoid a false negative result. The prostheses, catheters, or stents and other foreign bodies taken out from patients due to suspicion of biofilm infections should be sent for microbiological examinations. For the microscopy and culture-negative samples, if the patients are highly suspected for biofilm infections clinically, additional microbiological techniques might be helpful for the diagnosis of biofilm infections (Wu et al. 2015).

5.2.2 Different Microscopic Methods

Laser Scanning Confocal Microscopy: It is very descriptive analysis, especially to determine the time course of the biofilm formation and the detection of characteristic stages from the adhesion step to the development of a mature biofilm. It is also particularly adapted to visualize the biofilm structure and matrix or to quantify the biofilm size. Although this method is suitable to study biofilms on abiotic or thin biotic surfaces, studying bacterial biofilm on a fungal filamentous colony is still very challenging. Indeed, most filamentous fungi build thick, complex, tridimensional networks in culture.

Table 5.1 Clinical signs of suspected biofilm infections

Possible biofilm infections	Clinical manifestations and paraclinical changes	The common pathogens
Endocarditis	Patients equipping with or without prosthetic heart valves or pacemaker, who have intermittent fever and bacteremia with an identical pathogen and without an obvious focus, but higher C-reaction proteins and/or erythrocyte sedimentation rate with or without leukocytosis	*S. aureus*, *Streptococcus* species, coagulase-negative staphylococci, *Enterococcus* species
P. aeruginosa biofilm in CF/COPD	Patients with CF or COPD, who have been detected mucoid *P. aeruginosa* in sputum	*P. aeruginosa*
Intravenous catheter biofilm	Patients with central venous catheter or hemodialysis catheter, who have recurrent bacteraemia with an identical pathogen	Coagulase-negative staphylococci
Urinary catheter biofilm	Patients with urinary catheter, who have recurrent urinary tract infections with the same pathogen	Gram-negative rods, *Candida* species, *Enterococcus* species
Biofilm infections of orthopaedics	Patients with joint prostheses or orthopedic fixation devices, who have chronic pain locally and sign of prostheses loosening	*S. aureus*, coagulase-negative staphylococci
Wound biofilm	Patients with chronic wound and recurrent wound infections	*S. aureus*, *P. aeruginosa*

CF cystic fibrosis, *COPD* chronic obstructive pulmonary diseases

Even if thick objects can be imaged by confocal microscopy, the attenuation of the laser penetration and the fluorescence emission often decreases the quality of the final images over a depth of 50 μm. Moreover, because fungal colonies are not rigid, it is difficult to handle the microorganisms without disturbing the biofilms. Due to the thickness of the samples, the few microscopic analyses of bacterial biofilms on fungal hyphae are usually only performed on a small part of the fungal colony, therefore containing only few hyphae. All this limits our ability to describe biofilm distribution on the fungal colony and thus can bring biases into the analysis in case of the heterogenic distribution of the biofilm within the fungal colony (Guennoc et al. 2017).

Scanning Electron Microscopy It is a powerful tool for structural analysis, but it requires biological samples to undergo lengthy, chemically complex multistep preparation procedures, arguably altering some features in the sample. The essential goal of this study was to maintain the sample as pristine as possible to allow for the creation of authentic images. Several aspects of the method we propose here were designed with this goal in mind. First, the support disks enable lifting of the biofilms with almost no perturbation as can be verified visually, as compared to lifting it with tweezers which inevitably alters the native structure. Second, sample is only dried using a low-power pump to keep it just below the dew point where water vapor pressure is gradually decreased due to absorption by the hygroscopic silica gel. This prevents harsh sample desiccation and breaks or deformations caused by it, as is the case when the biofilm is exposed to various solvents and materials. Above all, an effort was made in designing this method to shorten as much as possible the time interval from culture to microscope (Raab and Bachelet 2017).

The method describes here offers various advantages discussed below. Table 5.2 compares native SEM imaging to other SEM techniques used for biofilm imaging.

Table 5.2 Comparison between native SEM and other SEM methods used for biofilm imaging

	Sample preparation	Equipment	Time	Resolution
Native SEM	Primary fixation vapor phase (GA) drying	Desiccator	>1 h	Medium to high (up to 15 nm/pixel)
Conventional SEM	Primary fixation (GA) secondary fixation OsO_4 dehydration CPD sputter coating	CPD device sputter coating apparatus	Hours to days	High
Cryo SEM	Plunge frozen in slushed liquid nitrogen at − 210 °C temperature raise to −95 °C temperature reduce to − 125 °C sputter coating	Liquid nitrogen In vacuo transfer container cryo preparation chamber sputter coating apparatus	Minutes	Lower than conventional (good for matrix imaging)
ESEM	None	None	Minutes	Low (good for matrix imaging)
ASEM	None	None	Minutes	High only bottom view without sample manipulation

ESEM environmental scanning electron microscope, *ASEM* atmospheric scanning electron microscope

Fluorescent Microscopy Fluorescent staining is a common tool for both quantitative and qualitative assessment of pro- and eukaryotic cells subpopulation fractions by using microscopy and flow cytometry.

Fluorescent microscopy is largely free of above limitations and provides a reasonable alternative to the cytometric measurements. However, in the presence of adherent and/or spore-like cells, they largely overlap leading to the limitations of direct cell selection and counting algorithms in the microscopic images. The situation gets even more complicated when the cells are not equidistantly stained; image quality and color balance vary in different fields of view.

Automatic or semiautomatic analysis of cells seems to be a fast and easy approach to the microscopic data quantification. In the last two decades, a number of methods and computer-assisted algorithms have been developed to resolve the cell counting issue implemented in a number of both commercial and free software tools. Existing software solutions include cell counting and classification algorithms, estimation of their parameters from microscopic imaging, 3D reconstructions from confocal microscopy data, and several other more specific applications. However, automatic microscopic image analysis remains challenging in the presence of adherent and/or spore-like cells that are common conditions in biofilm studies. Automatic counting methods are usually based either (i) on detection, selection, and counting of discrete objects or (ii) on the statistical analysis of the image properties that avoid direct counting approach and estimate some effective characteristics from the statistical properties of the entire image (Bogachev et al. 2018).

High-Frequency Acoustic Microscopy: High-frequency Acoustic Microscopy is the combination of optical and acoustic imaging method of infectious biofilm matrices. Ligand-targeted ultrasound contrast agents (UCAs) were used as a novel method for preclinical noninvasive molecular imaging of early to late-stage biofilms. Early diagnosis of biofilm matrix formation is a challenge in immunocompromised patient such as those undergoing chemotherapy (cancer patients) with infection-associated biofilms. The combination of ultrasound and targeted UCAs is a unique molecular imaging technique for the detection of biofilms (Anastasiadis et al. 2014).

Several imaging modalities have been used to detect biofilm biomass and cell viability. Here, we discuss several microscopy approaches, highlighting their advantages and disadvantages (Table 5.3) (Azeredoa et al. 2017).

5.3 Conclusion

Few methods are aligned with the increasing robustness of image processing and analysis algorithms, which in combination could reveal even more details than before. Improvements and adjustments of this technique could adapt it to a variety of other biological systems.

Table 5.3 Microscopy techniques applied to the study of biofilms

Microscopy technique	Application	Advantages	Limitations
Light microscopy	Visual identification of biofilm formation quantitative assessment of biofilm biomass useful combination with transmission electron microscopy or scanning electron microscopy	Simple sample preparation cheap and easy to perform imaging of larger parts of a sample compared to electron microscopy	Minimal quantitative assessment of biofilm biomass useful combination with transmission electron microscopy or scanning electron microscopy Simple sample preparation cheap and easy to perform imaging of larger parts of a sample compared to electron microscopy Limited magnification and resolution sample staining necessary morphotypic differentiation relatively gross lacking discriminatory detail
Confocal laser scanning microscopy	Biofilm visualization and quantification of structural parameters biofilm spatial structure spatial distribution of viable bacteria, localized cell death antimicrobials effect on cell viability	Resolution compatible with single-cell visualization reconstruction of 3D images of a sample no need for extensive computer processing	Cation of structural parameters biofilm spatial structure spatial distribution of viable bacteria, localized cell death antimicrobials effect on cell viability Resolution compatible with single cell visualization reconstruction of 3d images of a sample no need for extensive computer processing Use of fluorophores is required limited number of reporter molecules (e.g., no universal matrix probes exist) interference of local properties of the biofilm with the fluorescence probes natural auto-fluorescence may hide signal of interest

(continued)

Table 5.3 (continued)

Microscopy technique	Application	Advantages	Limitations
Scanning electron microscopy	Study of the biofilm spatial structure evaluation of the effects of exposure to antibiofilm drugs biofilm formation kinetics assessment qualitative support for findings from quantification methods (high correlation) possible quantitative analysis using dedicated imaging software	Resolution higher than other imaging techniques (resolves surface details) good depth of field ability to image complex shapes wide range of magnifications (20–30,000)	Tedious and time-consuming sample preparation lacks vertical resolution preparation processes (fixation, dehydration, and coating with a conductive material) can destroy sample structure or cause artifacts
Cryo SEM	Topography/structure of the glycocalyx structural detail of the internal structure of the biofilm (freeze fracture) good for liquid, semiliquid and beam sensitive samples	High-resolution capability when compared to low-vacuum techniques sample viewed in fully hydrated state simpler and faster sample preparation than traditional SEM, allowing less sample destruction and artifacts	Lower resolution than conventional SEM melting and cracking of the frozen surface of the sample at high magnifications due to the heat generated by the focused electron beam highly expensive and specialized equipment
Environmental SEM	Imaging of samples in their natural state dynamic study of gas/liquid/solid interactions in situ and in real time (e.g., in situ observation of the highly hydrated glycocalyx)	Preservation of the biofilm's integrity in its natural state no pretreatment required visualization of images at high magnification of hydrated and nonconductive living bacterial biofilms	Reduced resolution due to lack of conductivity in wet samples sample damage caused by the focused electron beam at high magnification due to absence of metal coating
Focused ion beam SEM	Exploitation of the subsurface structure of biofilms 3D reconstructions mainly used to study environmental biofilms	Not prone to relevant artifacts highly precise cross-section of the sample exploration the subsurface structure of the biofilm	A vacuum is generally required possible decrease in resolution caused by ion beam damage

(continued)

Table 5.3 (continued)

Microscopy technique	Application	Advantages	Limitations
Atomic force microscopy	Quantitative biofilm analysis used to confirm findings obtained with other quantitative or imaging techniques determination of adhesion forces between biofilm and substratum, as well as cohesive strength biofilm topography in situ imaging	Nondestructive technique works under ambient conditions, which minimizes pretreatments and artifacts even on liquid surfaces (enables in situ imaging) same resolution along and perpendicular to the surface 3D reconstruction qualitative and quantitative assessment of living biofilms under physiological-like conditions	Inability to obtain a large area survey scan sample damage or artifacts caused by tip shape and size (although generally considered negligible)

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Chapter 6
Molecular Methods for the Assessment of Microbial Biofilms

Amresh Kumar Singh and Vivek Gaur

Abstract In a vast majority of metabolically active strains results in negative culture even if the patient is infectious, the usage of molecular methods is one of the important tools for analyzing approximately 99% of strains causing microbial colonies directly on biofilms and also permits the information to be assembled on a protocol of treatment. This technique not only provides an authenticated source to understand the information of bacterial biofilm but also does the assessment of targeted biofilm interference strategies in vivo and marks in identification of mono or multispecies population of bacteria. Different molecular techniques like next-generation sequencing (NGS), polymerase chain reaction (PCR), hybridization and microarray technology are highly specific and sensitive, which enables the discovery of new concepts regarding the role of molecular methods for diagnosis of microorganisms in biofilms. This advancement is designed in order to detect the presence of the etiologic agents as well as used to design the protocol for antibiotics.

Keywords Molecular methods · Microbial biofilms · Next generation sequencing · Polymerase chain reaction

6.1 Introduction

Over a period of time, the only method to detect and identify the microorganism causing biofilm was culture method. Approaches based on culture are a captivating tool for understanding the potential of physiological and biochemical impact of organism those are isolated; however, they lack in providing information on the diversity of complex microbial colonies.

Among the microbial diversity, only 0.1–1% can be evaluated by conventional methods, i.e., culture-dependent, which enable the isolation of only feasible and cultivable organism that is under quality laboratory conditions. Majority of active strains occur in the environment in the state of anabiosis, being viable but non-culturable (VBNC), which falls into one of the three categories:

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1. Obligate parasitic and symbiotic organisms unable to isolate on microbiological culture media.
2. Well-known species in the identification of which culture-dependent methods prove to be scanty.
3. Unknown species that have never been isolated before due to the inappropriate methods.

It is very necessary to detect or identify microorganism by using advance techniques which cover approximately 99% of strains causing biofilm. Molecular methods are important tools for analyzing microbial colonies directly on biofilms and it also allows the assembly of information for the treatment efficiency (Otlewska et al. 2014).

6.2 Why Molecular Methods?

- Molecular techniques are powerful and appropriate tools that are proficient to quantify and identify in a single assay (Suzuki et al. 2004).
- Application of molecular (PCR) techniques to water and biofilm samples provided promising results which help in the detection of potential pathogens (Miguel)
- Molecular techniques are more specific, sensitive, and rapid detection methods.
- Molecular methods are promising replacement to microbiological culture-dependent techniques and make it reliable to assess the biodiversity of microorganisms (Otlewska et al. 2014).
- It also helps in the collection of data on the treatment efficiency, the adequacy of pipe and reservoir materials and to assess the success of preventive measurements (Miguel).

6.3 Different Methods Used to Assess Biofilm: Ergin (2017)

1. **Classical and conventional methods**

- Microtiter plate assay
- Tube adherence method
- Congo red agar method
- Biofilm ring test
- Biofilm bioreactor

2. **New radio imaging methods**

- Ultrasound
- Photobioreactor
- Confocal laser scanning microscopy (CSLM)

- Fluorescent in situ hybridization (FISCH)
- Spectrometry
- Nuclear magnetic resonance imaging

3. **Serological methods**

- Enzyme-linked immunosorbent assay (ELISA)

4. **Molecular methods**

- Next-generation sequencing technologies
- PCR techniques
- DNA–DNA hybridization technique (Li et al. 2004)
- Microarray technology.

6.4 Next-Generation Sequencing Technology

Next-generation sequencing (NGS) has the potential as well as power to transfigure the diagnostic microbiology laboratory by reducing current time-consumption and labor-intensive techniques with a single, all-inclusive diagnostic tests.

The term NGS is a comprehensive one which collectively alludes to very high-quality DNA sequencing approaches which result in mass production of genomic information in a single reaction by various methodologies. The introduction of NGS into the literature is as "deep" "high throughput" or "massively parallel" sequencing.

In 1977, Frederick Sanger and his colleagues introduced Sanger sequencing. It has been recognized as the reference standard technique for DNA sequencing for four decades. Although Sanger sequencing was a success to produce early information or maps of the human genome, its passive active demanded more vigorous or robust DNA sequencing technologies that could speed up genomic data in a smooth, rapid, and more affordable manner. Later, the GS-20 sequencing platform from MRD Life Sciences, as this was the first non-Sanger-based sequencing system, was launched in 2005. A massively parallel pyrosequencing technique was executed that laid a solid foundation of a new-wave of high-throughput analysis of genomic information known as **next-generation sequencing** (Swanson et al. 2016).

One of the next-generations DNA sequencing techniques is pyrosequencing method which determines the order of nucleotide that helps in detection of a pyrophosphate molecule (PPi) released during the synthesis of DNA. PPi is released due to polymerization of nucleic acid which is later converted to ATP as enzyme sulfurylase acts on it. As a result, the energy released which is to be used in the in next step, i.e., oxidation of luciferin in a reaction catalyzed by the luciferase enzyme, which is simultaneously accompanied by light emission. Tracking which of the dNTPs was added allows determination of the template sequence (Otlewska et al. 2014).

6.4.1 Advantages of NGS

- It has the capability to diagnose a broad range of the pathogenic organism.
- Increased availability, decreased cost per base.
- Information generated by using NGS could assist with the development of new diagnostic tools.
- It is potential to eliminate the multitude of microbiological and serological tests that are currently conducted on biofilm.
- It has also been successfully applied in the field of human genetics and precision medicine.
- It is useful in sequencing of multigene panels, noninvasive prenatal testing for the diagnosis of fetal chromosomal disorders and exome and whole-genome sequencing (WGS) for the precise assessment of common and rare genetic disorders, disease-specific variants and cancer-specific alleles.

6.4.2 Utility of NGS in Clinical Microbiology: Deurenberg et al. (2016)

1. Management of outbreak.
2. Finding molecular cases.
3. Pathogens are characterized and surveillance.
4. Targeted NGS of the 16S-23S rRNA (Table 6.1) cluster region for early pathogenic bacteria identification in a clinical specimen.
5. WGS and taxonomy.
6. Metagenomics in clinical microbiology.
7. The transmission of zoonotic pathogenic microorganisms from animals to humans is determined.

Table 6.1 16s rRNA primers used in gene sequencing (Miguel)

27F	Forward	AGAGTTTGATCMTGGC TCAG	20
357F	Forward	CTCCTACGGGAGGCA GCAG	19
1492R	Reverse	TACGGYTACCTTGTTAC GACTT	22

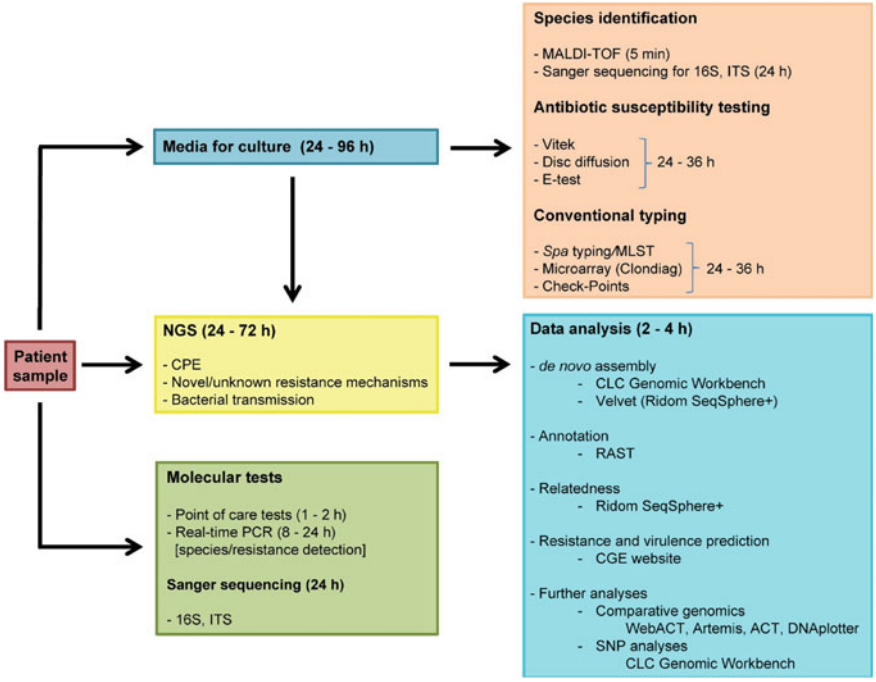

Fig. 6.1 Laboratory workflow of NGS

6.4.3 Workflow of NGS

There are several steps that are common among the majority of NGS techniques, with the exception of single-molecule real-time NGS. A typical NGS workflow in a clinical laboratory is as given in Fig. 6.1 (Deurenberg et al. 2016).

6.4.4 Clinical Sample/Specimen

Ideally, samples are collected and transported in a triple-layered packaging to the clinical microbiology laboratory during disease progression. The type of specimen depends on the patient's clinical diseases for example urine, cerebrospinal fluid (CSF), brain tissue, stool, sputum, blood including serum and plasma, oropharyngeal/nasopharyngeal swab, peritoneal drainage, synovial and ascitic fluid, bile, purulent specimen, or tissue, that contains the genetic material (DNA/RNA) of interest.

6.4.5 Nucleic Acids Sequencing

Many types of NGS platforms are used for nucleic acid sequencing but two main NGS platform methods are currently in use, including Illumina and Ion Torrent (Table 6.2).

The Illumina
Direct combination reaction involving repetitive cycles of single base inclusion, imaging, and dye chemistry termination is done by reversible dye terminator sequencing. The most widely accepted NGS technology is sequencing by union (Table 6.2). The option of paired-end sequencing that allows reading to be provoked from each end of a single clonal fragment is offered by Illumina sequencer. Read pairs are the arrangement of forward and reverse reads that allows read alignment precisely and detection of insertion or deletion of bases in the genome of an organism. The gap between the forward and reverse reads is familiar with paired ends reads and enables assembly algorithms to reconstruct the sequencing for repetitive sections of DNA, such as homopolymeric or AT/GC-rich regions of the genome. The sequencing market for both microorganisms and larger organisms has been influenced by Illumina in recent years, attributable to the platform's high sequence throughput, low error rate, and low sequencing value per base. Illumina features a line of machines serving a multitude of purposes and sequencing power on all scales. The HiSeq XTM, HiSeq, NextSeq, and MiSeq are included in Illumina sequencing platforms (Swanson et al. 2016) (Fig. 6.2).

The Benchtop Ion Torrent
The benchtop Ion Torrent platform by life technologies is the other popular components of the NGS portfolio (Table 6.2), which includes the Personal Genome Machine (PGM) and the Ion Proton. It is similar with other alternative platforms in their sequencing by synthesis methodology and amplification by emulsion PCR. Ion Torrent platforms dissent from different technologies within the detection step. H^+ ions are released throughout the incorporation of bases and are measured rather than of using fluorescence or chemiluminescence detection strategies making the Ion Torrent sequencers very sensitive pH-scale meters. Flexible reagent chips are present in both the PGM and the Ion Proton which generate variable scales of sequencing output (10 Mb, 100 Mb, 1 Gb, 10–100 Gb) according to the user's desired sequencing coverage. The speed of sequencing has popularized these platforms. From DNA extraction to generate comment, i.e., the entire workflow can be completed within a day, which makes the Ion Torrent sequencers most feasible for targeted sequencing or minor genome sequencing projects (Swanson et al. 2016).

6.4.6 Sequence Data Analysis

Data analysis is the biggest challenge which leads in the introduction of NGS in the clinical microbiology laboratory, with the usage of the various application software

Table 6.2 List of different gene sequencing platforms (Swanson et al. 2016)

NGS platform	Sequencing chemistry	Instrument	Maximum read length	Uses
Roche-454	Pyrosequencing; sequencing by synthesis	GS FLX+	1000 bp	It is not in used after 2016; long reads made the platform well suited for de novo assembly and pathogen discovery
Illumina	Reversible terminator chemistry	NextSeq	300 bp	Current ball of fire of NGS platforms; supplies bidirectional reads; platform can be used for discovery of pathogen, exome sequencing, targeted sequencing; also overcomes homopolymeric regions
ABI SOLID	Sequencing by ligation, oligonucleotide probe ligation	5500 SOLID	75 bp	High sequence accuracy quality makes this platform equipped for genome resequencing and polymorphism analysis
Ion torrent	H^+ ion-sensitive transistor	Ion proton I	200 bp	This platform is acceptable for small genome sequencing, exome sequencing, and targeted sequencing

(continued)

Table 6.2 (continued)

NGS platform	Sequencing chemistry	Instrument	Maximum read length	Uses
HeliScope	Reversible terminator chemistry	HeliScope single-molecule sequencer	35 bp	It requires the addition of a poly(A) tail; the platform is effective at sequencing native viral genomes and immune-precipitated methylated DNA; It has the ability of sequencing small sample quantities; high platform costs and poor sales lead caused production to cease
PacBio	Real-time sequencing; phospholinked fluorescent nucleotides	PacBio RSII	50 kb	This platform provides long-read sequencing and a low degree of bias; best in industry for de novo assembly, targeted sequencing, and base modification detection
Oxford nanopore	Real-time sequencing; electronic sensing or nanopore sequencing	MinION	>50 kb	A portable, USB-powered sequencing device that is under development and has been used by participants in MAP for de novo assembly and resequencing

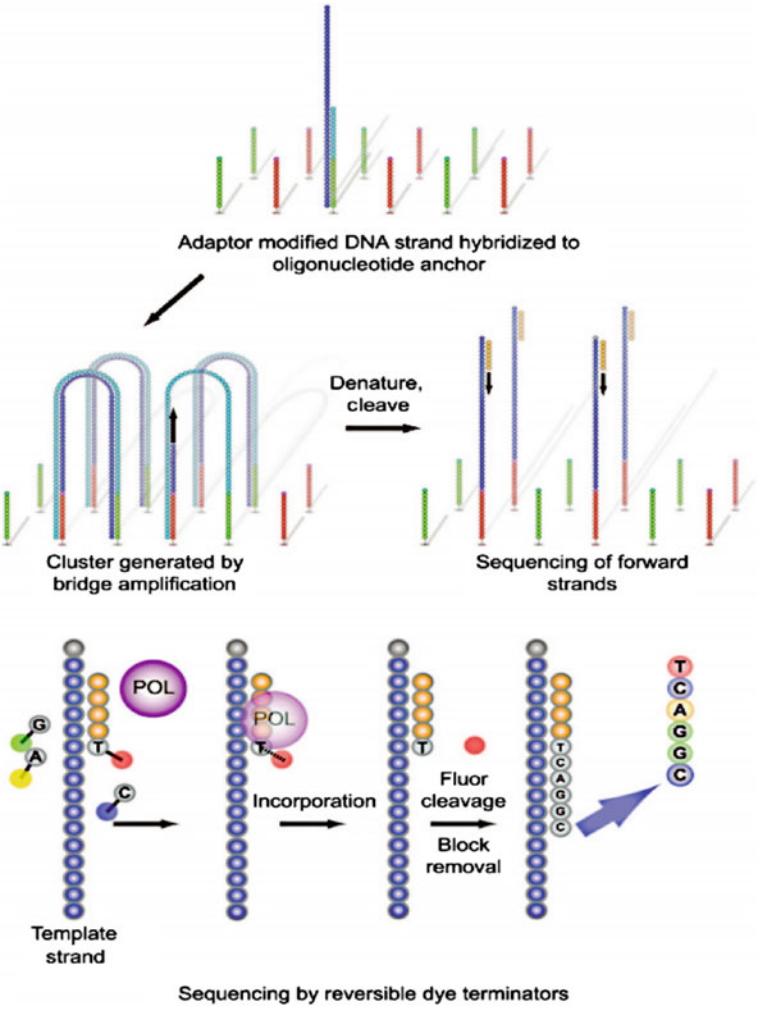

Fig. 6.2 Illumina genome analyzer sequencing

packages available. Therefore, scientific comprehension is required for more in deep analysis, on the genomic property and the biological evolution of the microorganism under investigation (Deurenberg et al. 2016).

- CLC Genomic Workbench (Qiagen), Spades and Velvet are used to characterize the genomes.
- A gene-by-gene comparison using a multilocus sequencing typing (MLST) approach to investigate the genetic relationship is done either by studying the conserved core genome (cgMLST) or the whole genome (wgMLST), which includes a set of variable accessory genes.

- SeqSphere (Ridom) and BioNumerics (Applied Math, Biomérieux), some of the multiple software package or online applications, such as EnteroBase and BIGSdb (Bacterial Isolate Genome Sequence Database) Jolley and Maiden can be used for this purpose.
- The establishment of a common terminology for genetically related strains is allowed with the use of an established cgMLST.

6.4.7 Application of NGS

The whole spectrum of disease-causing organisms in a given sample is tested by metagenomic NGS, without requiring specific primers or probes. The sequencing using NGS widely identifies even unusual pathogens unexpectedly in biofilm sample. The limitation of the current diagnostic scheme is bypassed by the invention of NGS and thus highlights the efficiency of NGS. The initial diagnostic evaluation for multiple pathogens in biofilm is assessed by the physicians with this scheme of NGS (Swanson et al. 2016).

NGS resulted in very high-quality reads in analysis of biofilms from postoperative surgical site infection (SSI) and associated predictive factors. Extraction and sequencing of DNA from formalin-fixed paraffin-embedded (FFPE) material by NGS was successfully used to identify bacterial species in chronic or nonhealing wounds (König et al. 2014).

According to Vierheilig et al. (2016), from the NGS results, the derivation of microbial community from the taxonomic composition can be done directly. A crucial insight of the constitution and standing is given of the investigated sample with the assistance of NGS by identifying signature taxa or observation of quantitative shifts between dominant taxa with known characters. A deep amplicon sequencing database is marked beyond and independent of taxonomic identification that enables the investigation of relatedness of colonies in various clinical samples based on the phylogenetic history of their members.

6.5 Polymerase Chain Reaction (PCR)

It is necessary to use more specific, sensitive, and rapid detection methods arising from molecular techniques to assess several harmful biofilm pathogens. Direct examination of the existing bacterial DNA in the sample is done by molecular method which helps in identification of the bacteria present in the sample. Microbial DNA can be analyzed by this very rapid technique which could be assessed by fluid progression of molecular technology. The main in the application of molecular methods for identifying and quantifying microorganisms in human infections is to prevent the

whole process from contaminants to obtain quality microbial DNA from the sample (i.e., the process of DNA extraction) (Wolcott and Cox 2014).

PCR is the widely used method of processing DNA, which has a comparatively long history of use in the diagnosis of pathogens than the other techniques. Through a polymerase reaction, PCR utilizes primers (Table 6.3) that create copies of the area which attaches to the complementary regions of bases in the DNA fragments of microorganism and multiply twice of the target sequence after the completion of every cycle of PCR. Quantity or copies of microbial DNA are in the original template which quantitates only by real-time PCR as it has the capability to find ct ratio; in complete sense, the number of cycles required before the real-time signal reaches a detection threshold that can be correlated to an absolute copy of microbes available in the original sample which makes PCR unique and is used to quantitate "bacterial load."

Table 6.3 Primers (forward and reverse) and target genes used to detect bacterial DNA in biofilm (Wolcott and Cox 2014; Dalwai et al. 2007)

Species	Sequence (5′ to 3′)	Length (in bp)
E. coli	ATCATGGAAGTAAGACTGC TTGCTGTGCCAGGCAGTTT	356
E. faecalis	CATGAGCAATTAATCGG CATAGCCTGTCGCAAAAC	444
Enterobacteriaceae	TGAATCACAAAGTGGTAAGCG TGGGGATGACGTCAAGTCAT	300
Streptococcus spp.	AGATGGACCTGCGTTGT GCTGCCTCCCGTAGGA GTCT	17 20
P. gingivalis	CTTGACTTCAGTGGCG GCAG AGGGAAGACGGTTTTC ACCA	20 20
Mycobacterium spp.	ACCAACGATGGTGTGTCCAT CTTGTCGAACCGCATACCCT	20 20
Campylobacter jejuni	GCTCAAAGTGGTTCTTATGCNATG GCTGCGGAGTTCATTCTAAGACC	24 23
Universal (multiple species)	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTAATCCTGTT	19 26
Human cells	GGCTTCCTAGAGACCAATCA CAGAGAGCTGAACAAAGAGATT	295

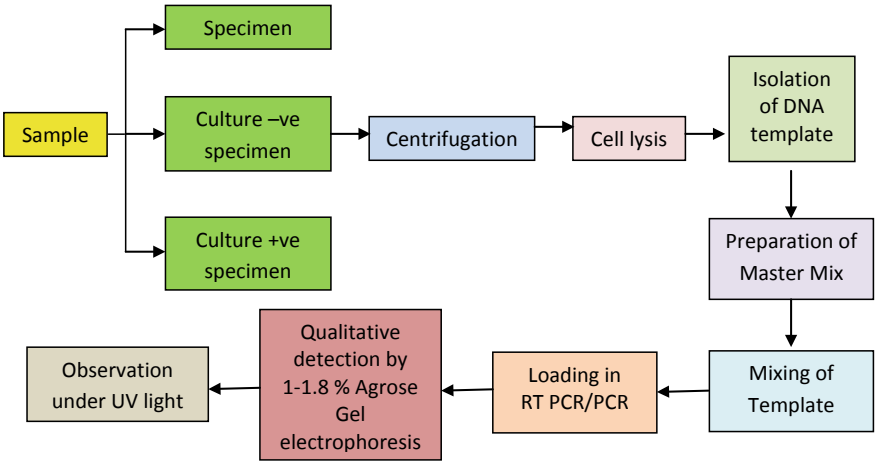

Fig. 6.3 Laboratory workflow of PCR

6.5.1 Advantage of PCR

- Real-time PCR can capitulate usable information on bacterial load and identify a restricted number of microbial species within the limited time of period.
- PCR improves clinical outcomes in chronic infections produced by biofilm phenotype microorganism (Wolcott and Cox 2014).
- Nondividing bacteria can identify by this method.
- PCR has proven reliable complement to solve undiagnosed cases of bacterial infection by using 16S ribosomal ribonucleic acid (rRNA).
- It is also used to evaluate culture-negative samples for the clinical significance (Lleo et al. 2014).

6.5.2 Workflow of PCR

See Fig. 6.3.

6.5.3 Procedure and General Protocol

DNA Extraction

In PCR, DNA has to be first extracted and purified because it cannot be applied

directly to the clinical samples. Amplification products were amplified by using different commercially available kits from Qiagen or Invitrogen (Fig. 6.4).

PCR Steps

The complete **denaturation** of PCR template and the PCR product is very important to yield the good PCR result. Typically, denaturation done at intense heat (95 °C or 97 °C) with specific time (15 s or 30 s) for optimum 25–45 cycles depends on the protocol used. It takes only few seconds to denature or separate DNA strands. The

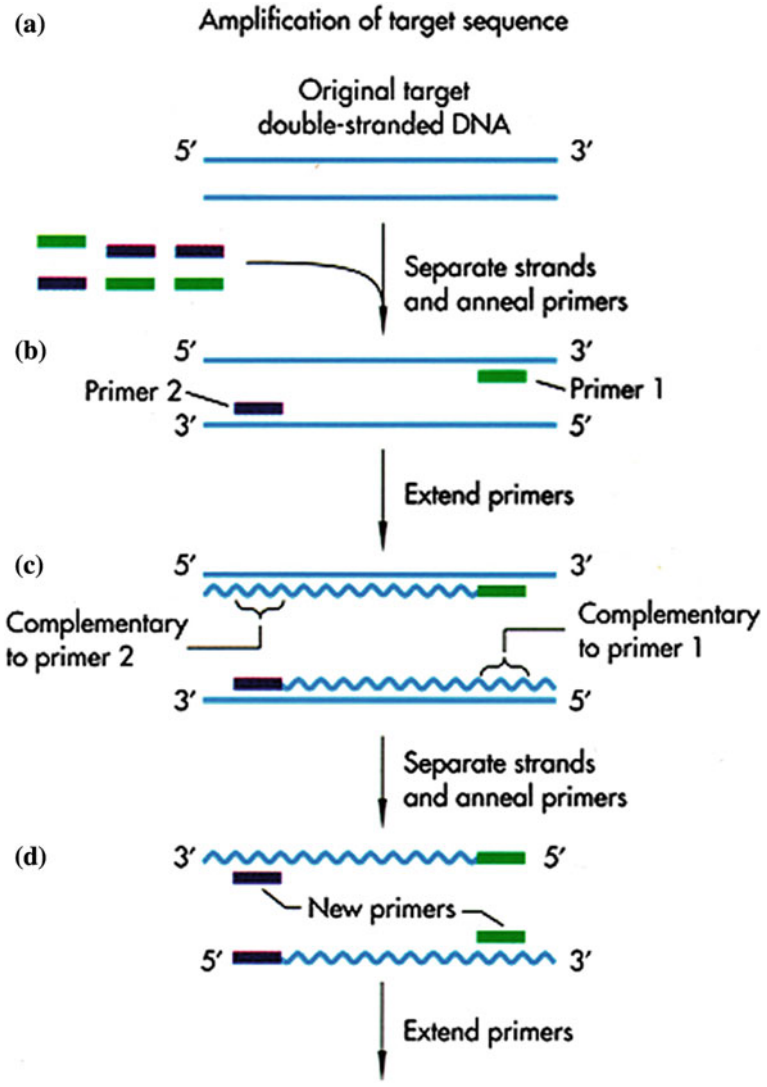

Fig. 6.4 Application of PCR (Michael and Gelfand 1999)

product yield is reduced in incomplete denaturation, which enables the DNA strands to "snap back" (Michael and Gelfand 1999). The most critical factor in designing the high specificity PCR is probably the choices of the primer **annealing** temperature. No annealing occurs if the temperature is too high, or if it is too low, there is increase in nonspecific annealing. The specific temperature and time required for primer annealing lie on the composition of base, length, and concentration of the amplification primers. Temperature for annealing varies in the range of 55–72 °C generally yields the best result. Annealing requires only for few seconds by using specific concentration of primer (0.2 μm). **Extension** time depends upon the temperature, length, and concentration of the target sequence used in particular reaction. Primer extension is traditionally done at 72 °C because this is the optimum temperature for extending primers (Cho and Tiedje 2001).

6.5.4 1–1.8% Agarose Gel Electrophoresis

Gel electrophoresis of PCR products is performed on 1–1.8% agarose with 1X TBE buffer using a molecular weight marker. It is visualized by ethidium bromide staining for 1 h 30 min at 80 V (Miguel).

Amplified product separated on the basis of molecular weight which can be visualized by taking image under UV light box (Fig. 6.5).

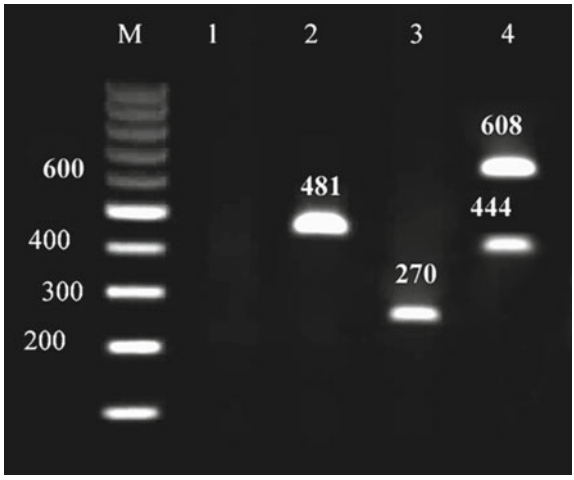

Fig. 6.5 Protein band separation under U.V. light box

6.5.5 *Application of PCR*

Application of PCR techniques to biofilm samples provided promising results in the assessment of potential pathogens. This method enables simultaneous analysis of a high variety of samples and also the identification of the origin of contamination and may be quantitative in an absolute sense (Miguel).

In pleural effusion, samples tend to be culture-negative even once the patient has definite signs of infection, the application of universal 16S PCR, "bacterial load" diagnose bacterium in 82% of the clinically infected samples, whereas different clinical cultures grew bacteria only 55% of the time. Employ a single molecular test enhanced bacterial identification by 27%. Here, it should also be noted that the only PCR test had only 0.9% false positives, whereas clinical cultures had a 2.6% false positive rate. (Wolcott and Cox 2014)

According to Mouraviev and Mcdonald (2018), the quantitative PCR is very useful for the diagnosis of bacteria and fungi like *Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogens, Escherichia coli*, and *Streptococcus pneumoniae* with 100% sensitivity and 99.4% specificity.

By developing TaqMan, real-time PCR quantitative technique is very feasible to calculate number of early colonizer microorganisms like *Actinomyces naeslundii, Actinomyces viscosus, Streptococcus mitis*, and *Streptococcus gordonii* in dental biofilm (Suzuki et al. 2004).

In a study among 135 patients suffering from prosthetic joint infection, PCR had an overall concordance of 90.2% in comparison with tissue culture (Gomez et al. 2012) but it has several limitations; Real-time PCR needs a primer sequence to be developed specifically for each and every species of microorganism present in the sample. Thousands of various microorganisms that may be in human chronic infections, constructing thousands of primers for each analysis is inefficient, costly, and currently not feasible (Wolcott and Cox 2014).

6.6 DNA–DNA Hybridization

DNA–DNA reassociation methods are used in various purposes, but in the area of microbial systematic, they are in most cases linked to the circumscription of prokaryotic species. Use of whole-genome hybridizations in the definition of prokaryotic species has had a vast influence since the origin of the polythetic classification system (Mora 2006).

DNA–DNA hybridization (DDH) is especially used in phylogenetic reconstruction. DDH has been used as the gold standard techniques for the genomic similarity study of pair-wise set of strains for classification purposes (Rosselló et al. 2011). During hybridization, the DNA of each species melted due to the specific temperature to produce single strands. Then a strand of each DNA from each species is combined and allowed to anneal together (recombine) (Robb).

All accepted variations of DDH determination are technically demanding, labor-intensive, and long procedures, thus DDH determination is now performed by few specialized laboratories and microbial taxonomists apply DDH only in case of where the strains to be differentiated have antecedently been shown to be closely related in terms of their 16S rRNA gene sequences.

6.6.1 Principle of DNA–DNA Hybridization

(i) Shorten the gDNA of the assayed microorganism and the gDNA of the reference organisms into minor fragments of 600–800 bp.
(ii) Fragmentation of the double-strands DNA of both strains disassociates by providing heat.
(iii) Decrease the temperature subsequently until in reannealing of fragments.

Therefore, the motive behind that the melting temperature of a double-strand depends on the degree of matching base pairings between strands, genomic similarity and dissimilarity can be terminated from the melting temperature. It is usually specified the DDH value is comparable to the DDH value obtained from hybridizing a reference genome with itself. The DDH values $\leq$70% are considered as an indication that the tested microorganism belongs to multiple species of biofilms than the type strain applied as reference (Auch et al. 2010).

Whole genomic DNA–DNA hybridization has been a keystone for the detection of bacterial species but is not widely accepted because it is not easily implemented.

6.6.2 Major Disadvantages

- Strenuous nature of pair-wise cross-hybridizations.
- Necessity for isotope use.
- Unsustainable of establishing a central database.

6.6.3 DDH Protocol and Procedure

- DNA is absorbed non-covalently to microplates (black MaxSorp, FluroNunc; Nunc) by incubating in denatured DNA solution [10 ng DNA/mL phosphate-buffered saline/$MgCl_2$ (8 mM NaH_2PO_4, 1.5 mM KH_2PO_4, pH-7.2, 137 mM NaCl, 2.7 mM KCl, 0.1 M $MgCL_2$)]
- Plates are sealed by using self-adhesive vinyl tape before incubation,
- Wash plates once with 300 ml PBS per well with the use of a multichannel pipette, dried at 45 °C for 15 min, and stored in desiccators at 4 °C.

- Probe DNA is labeled by missing 10 ml DNA solution [0.5 mg ml 21 in 0.16 SSC (16 SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0 ± 0.2)] and add 10 mL photobiotin solution (Sigma) (0.5 mg mL 21 in water) in a 1.5 mL Eppendorf tube and illuminating this mixture for 30 min under a 400 W mercury vapor lamp and kept the open tube upright in a cooling block on ice.
- Dilute the labeled probe DNA by adding 185 mL 0.1 M Tris/HCL (pH-9.0) and remove photobiotin by extracting twice with 200 mL saturated I-butanol water.
- Probe DNA is then fragmented with 30 ultrasonic pulses (W-385 sonicator) at 70% output; denatured at 100 °C for 10 min and immediately cooled by using ice.
- Perform prehybridization step by adding 200 ml prehybridization solution (26 SSC, 56 Denhardt's solution, 50% formamide, 100 mg denatured salmon sperm DNA ml21) per well, seal the microplate with vinyl tape, and incubate it for 30 min at the appropriate hybridization temperature in the hybridization oven.
- Prehybridization solution is removed and 100 mL hybridization solution (prehybridization solution plus 2.5% dextran sulfate and 1 mg probe DNA mL) is added per well.
- Microplate is sealed again by using vinyl tape and incubated for 3 h at the 5 °C higher hybridization temperature (stringent conditions) than the optimal renaturation temperature calculated as [0.516 (G + C mol%) + 47] 36 °C.
- Microplate washed three times with 300 mL 16 SSC per well for the enzymatic development.
- 100 mL streptavidin-b-D-galactosidase (Gibco BRL) solution is added per well (0.5 U mL 21 in PBS plus 0.5% BSA) and the microplate is incubated for 10 min at 37 °C after covering with a preheated empty microplate.
- Subsequently, the plate is washed three times with 300 mL 16 SSC per well, using the microplate washer.
- Finally, the substrate for b-D-galactosidase, 4-methylumbelliferyl b-D-galactopyranoside (Sigma), is added (100 mL per well, 0.1 mg ml21 in PBS plus 1 msM MgCl2) and the plate is incubated at 37 °C. The reaction product, 4-methylumbelliferone (excitation max, 360 nm; emission max, 465 nm) is quantified using a Spectra Max M2 microplate reader (molecular devices) at 0, 15, 30, and 45 min and data are immediately transferred to a personal computer.
- Calculate the DDH values using the fluorescence measurements at 30 min a homologous reaction is regarded as representing 100% reassociation (Goris et al. 2007).

6.6.4 Application of DNA–DNA Hybridization

Genomic subtractive hybridization developed to identify genomic differences between the two closely related strains of *Streptococcus* or *Actinomyces* spp. and designed species-specific primers and probes for TaqMan real-time PCR. Subtractive hybridization was initially developed to identify differences in cDNA pools, but it has also been successfully used to identify genomic differences between different

strains of *Helicobacter pylori*, *Mycobacterium tuberculosis* and *Neisseria meningitidis* (Suzuki et al. 2005).

Initially, oligonucleotide adaptors used in the subtractive hybridization were 5′-GATCCTCGGTGA-3′ and 5′-AGCACTCTCCAGCCTCTCACCGAG-3′, the second adaptors were 5′-GATCCGTTCATG-3′ and 5′-ACCGACGTCGACTATCCATGAACG-3′ (Suzuki et al. 2005).

Fluorescent in situ hybridization (FISH) permits the visualization and identification of cause of disease in human being by bacteria. Traditionally labeled DNA probes hybridize to their complementary nucleic acid targets according to Watson–Crick base-pairing rules but peptide nucleic acid (PNA) probes have superior hybridization characteristics, including high specificity and improved hybridization kinetics. The specificity and sensitivity of the PNA probes for planktonic bacteria in both single species and mixed bacterial populations are very high (Malic et al. 2009).

6.7 Microarray Technology

It is a robust technology to investigate the interference between a pathogen and the host as it assesses complete genomic expression profiles in response to disease. Usually, traditional methods used on a few genes as suspected virulent factors. However, exploration of genome expression is validated by microarrays technology. They enabled the discovery of groups of genes involved in the similar biological process and virulent pathways that involve many genes that may not have been previously known (Herrera-Rodriguez 2013).

Pathogenic microorganism (viruses, bacteria, and fungi) often employ complex mechanism of virulence developed over millions of years of evolution, which have resulted in a variety of diverse ways for a pathogen.

Several methods for DNA microarray analysis have derived from the latest microarray technologies. Most importantly, the compatibility of microarrays with mini devices resulted in speed enhancement, portability, and sensitivity which are more important factor in the field of diagnostics (Herrera-Rodriguez 2013).

6.7.1 Application of Microarray

Microarray (cDNA) analysis is used for the identification and change in the gene expression profile of different causative organism of biofilm. It also has the ability to analyze thousands of genes at the same time not only in respect of identification but also that of several genes associated with drug resistance. (Cao et al. 2005).

Autogenomics biofilm microarray consists of multiple layers of porous hydrogel matrics 8–10 μm thick on a polyester solid base which provides an aqueous microenvironment that is highly compatible with biological materials. The biofilm microarray

is configured with 240 spots per chip, suitable for current diagnostic applications and permits analysis of both nucleic acid and proteins (Hardiman 2008).

Initiating events in biofilm formation is characterized by Murillo et al. (2005). A detailed transcriptional analysis of the early biofilm stages and its development in *Candida albicans* using an affymetrix oligonucleotide gene chip representative of the entire genome of *C. albicans* was performed by him. They design gene annotations for the 7116 open reading frames (ORFs) in the microarray. RNA labeling, target hybridization, washing, staining, and scanning were performed by using a GeneChip Hybridization Oven 640, a GeneChip Fluidics Station 400, and a GeneArray scanner. DNA microarray data set showed 95% reproducibility which is derived by using two independent experiments.

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Chapter 7
Biofilm-Mediated Dental Diseases

Seema Dubey, Shirish Dubey, Ajay Gupta and Vikash Sharma

Abstract A human body is estimated to be made up of around one hundred trillion cells of which 90% is microflora. Bacteria are the predominant colonizers in the mouth, with 500–700 species commonly seen. The various surfaces of the oral cavity provide differing environments forming "microniches." This leads to the development of a highly complex microbiome. Dental plaque is the biofilm which forms on the various tooth surfaces. Oral microflora has a dual role. It plays a part, not just in pathology, but also in defending the host body and in most cases is a true commensal. The most common oral diseases are dental caries and periodontitis, both of which are biofilm-mediated. Dental caries is characterized by the loss of mineralized tooth tissue due to bacterial action. Periodontitis is essentially an inflammatory process which leads to the destruction of the supporting tissues of the teeth. Several systemic diseases have been shown to be influenced by dental plaque-associated oral diseases. These include cardiovascular diseases, arthrosclerosis, infective endocarditis, aspiration pneumonia, diabetes mellitus, preterm birth, and low-birth-weight babies. The primary step in management of biofilm-related dental diseases is physical treatment, which aims to reduce the bacterial load in biofilms. However, advanced disease treatment becomes essential. Antimicrobials and antibiotics may be administered to control the disease process and reduce bacterial load and growth.

Keywords Biofilm · Dental plaque · Oral microflora · Dental caries · Periodontitis · Plaque control

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7.1 Introduction

A human body is estimated to be made up of around one hundred trillion cells, with just 10% of them being mammalian. The rest 90% consists of the microflora which has made it their home (Samaranayake and Matsubara 2017; Marsh 2010). Bacteria are the predominant colonizers in the mouth, with 500–700 species commonly seen. However, only around 50–60% can be cultivated. The use of molecular biology led to the identification of many new phyla by their genomic fingerprints. The 16S rDNA studies have been of particular use. Coupled with metagenomics and supported by pyrosequencing and high-throughput sequencing techniques like next-generation sequencing (NGS), large success has been noted in the identification of the bacteria (Samaranayake and Matsubara 2017; Ferrer and Mira 2016). Oral flora consists of a wide variety of microorganisms including bacteria, archaea, fungi, mycoplasmas, protozoa, and viruses. The oral cavity has a distinctive ecology, separated from all other surfaces of the body, which allows only certain microorganisms to colonize. The oral microflora or microbiota is nowadays referred to as "oral microbiome" (Marsh 2010; Ferrer and Mira 2016).

The various surfaces of the oral cavity provide differing environments; thus, the microorganisms which initiate and propagate may also be different. These "microniches" of the mouth show differences in pH, oxygen, temperature, reduction potential, and anatomy, which in turn leads to the development of a highly complex microbiome (Simon-Soro et al. 2013; Kleinberg and Jenkins 1964). A more detailed study utilizing the molecular and culture techniques demonstrated the presence of a microbial flora unique to the different parts of the oral cavity. Further, even more, exact sampling studies demonstrated that the same niche contains different microenvironments which help some bacteria further specializing on different surfaces of the gingival crevice or same tooth (Marsh 2006).

The oral microbiota is different from the other neighboring ecological niches or systems, like the skin or gastrointestinal tract (Moore and Moore 1994). Various studies of the structure, function, and diversity of the microbiota, conducted using molecular techniques, have shown that every individual has a unique microflora (Ding and Schloss 2014). Every adult differs remarkably in the composition of the commensal resident microorganisms. Host genetics, diet, environment, and early microbial exposure are some of the factors responsible for the complex community of microbes.

7.2 Oral Flora

The common oral microbiota includes various types of bacteria, fungi, viruses, and protozoa (Samaranayake 2002). The bacteria present consist of various gram-positive and gram-negative cocci and rods. The gram-positive cocci include various species

of *Streptococcus*. Others like *Enterococcus*, *Micrococcus*, *Staphylococcus*, or *Stomatococcus* are also present. The gram-negative cocci include the *Neisseria* species, *Moraxella* species, and *Veillonella* species. The gram-stain-positive rods or filaments mostly include *Actinomyces*, *Eubacterium*, *Lactobacillus*, and *Propionibacterium* species. Other species belonging to *Cornybacterium matruchotti*, *Rothia dentocariosa*, or *Bifidobacterium dentium* may also present. Both facultatively and obligately, anaerobic gram-negative rods are also seen in the oral microbiota. The *Capnophilic* species are the most common facultative anaerobes. Most common fungi are the *Candida* species, especially *Candida albicans*, *C. Glabrata*, *C.tropicalis*, *C. Krusei*, *C. Parapsilosis*, and *C. Guilliermondi*. *Rhodotorula* species may also be seen. Various *Mycoplasms* seen in the mouth include *M. salivarium*, *M. pneumonia*, *M. hominis*, *M. buccale*, and *M. orale*. Herpes simplex 1 and 2, a number of Coxsackie viruses, and *Cytomegalovirus* are the commonly found viruses. Oral protozoa species commonly seen include *Entamoeba gingivalis*, *Trichomonas tenax*, and *Giardia lamblia*.

7.3 Development

The microflora of the mouth is a highly dynamic structure, modifying and changing, even in composition, over time as the oral biology evolves.

The fetus is normally sterile in the womb. In the process of childbirth, it comes in contact with the microorganisms present in the uterus or vagina of the mother. These organisms, however, do not usually get established and are present only transiently. Although the possibility of contamination is high, usually the mouth of the newborn remains sterile. However, as the feeding starts the mouth frequently comes in contact with microorganisms and the process of resident microflora acquisition begins in the oral cavity. Other sources of contamination include food items, water, and saliva from the mother or other people who remain close to the baby (Nisengard and Newman 1988).

The microorganisms who first establish themselves in the oral cavity are known as pioneer species. These especially include various streptococcal species including *S. salivaris*, *S. oralis*, *S. mitis*, *S. gordonii*, and *S. anginosus*. Gradually, the pioneer flora increases in diversity by incorporating various gram-stain-negative anaerobic bacteria during the first few months of life. The most common of these are the *Prevotella melaninogenica*, *Fusobacterium nucleatum*, *Capnocytophaga*, and *Eikenella corrodens* species. The pioneer community with its metabolic activity changes the oral environment and that helps in colonization by other bacteria genera; an example of this is *S. salivaris* whose extracellular polymers attach *Actinomyces* spp.

Microbiota starts changing in a major way with tooth eruption. Bacterial colonization starts in the specialized niches like the enamel and cementum hard surfaces, and in the gingival crevice and crevicular tissue. Hard surfaces promote the growth of gram-positive species. *Streptococcus mutans*, *Streptococcus sanguinis*, *Actinomyces* spp., *Lactobacillus*, and *Rothia* are commonly seen. Gingival crevice and crevicular

tissue harbor bacteria that prefer anaerobic environments, like gram-negative bacteria. Common species include non-pigmenting *Prevotella* spp., *Porphyromonas* spp., *Neisseria*, and *Capnocytophaga* (Marsh 2004; Kononen et al. 1994).

Change in hormones during puberty leads to an increase in the prevalence of spirochetes and black pigmented anaerobes as the former serves as a novel nutrient source. Pregnancy leads to an increase in Prevotella intermedia, while oral contraceptive use in women leads to an increase in black pigmented, due to similar reasons (Nisengard and Newman 1988).

Resident microbiota coexists in reasonable harmony, and the microflora remains in a relatively stable relationship with the host adult because of various interactions both interbacteria and host bacteria. Alteration in critical environmental factors like dietary changes, hormone levels, and oral hygiene can lead to dysbiosis or dysbacteriosis, i.e., a disruption or an imbalance in the microflora composition (Yang et al. 2012).

Aging, both directly and indirectly, may lead to changes in the microflora. In the case of the latter, the severely perturbed habitat can cause variations. An example may be an increase in cancer risk with age. The disease itself and its associated therapy, with cytotoxic drugs leading to myelosuppression, brings about changes in the oral environment which leads to colonization with uncommon opportunistic pathogens not usually associated with oral flora. These can include various species of *Klebsiella*, *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The growth of *Candida albicans* is also promoted. The use of prosthetic teeth, especially dentures, also increases with age, and this too helps in the increase of *C. albicans*. Xerostomia, caused by medication taken in old age, also unbalances the microbiota of the mouth (Marsh and Martin 1999).

The direct effects of aging have also been detected in oral microflora. *Lactobacilli* and *Staphylococci* have been isolated significantly in higher proportion in old individuals. The cause for such changes has been attributed to decreased innate and specific host defenses in mouth (Marsh and Martin 1999).

7.4 Oral Microbiota: Beneficial Functions

Excluding exogenous, pathological, organisms from colonization is the primary function of the resident microbiota. The term given for such exclusion is colonization resistance. Rapid suppression of the resident microflora breaks this resistance. This may occur due to long-term antibiotic treatment. Decreased colonization resistance leads to an increase in the growth of minor drug-resistant microorganisms of the resident microbiota or even exogenous pathogens which can cause oral or systemic infections (Claffey et al. 2014).

Thus, the innate host defenses always include the resident microflora as its component. The following microbial properties play an important role in this (Samaranayake and Matsubara 2017):

- Production of bacteriocins and hydrogen peroxide acts as inhibitory factors and affects the viability of exogenous microbes.
- The metabolic end products of certain bacteria, like short-chain carboxylic acids, are acidic, and by lowering the pH of tissues makes it difficult for invading microbes to grow.
- Quorum sensing molecules (e.g., homoserine lactone, autoinducers) production dissuades colonization of invading bacteria by helping the resident microbiota of the biofilms maintain their quality as well as quantity.
- Competition for nutrients and cofactors required for the growth of microbes.
- Preventing the attachment of "latecomers" by competing for receptors present on the mucous membrane and prosthetic devices for microbial adhesion.
- Coaggregation of bacteria which could be of same species (homotypic) or different species (heterotypic) leading to the formation of multispecies biofilm and thus reducing the prevalence of single non-coaggregated cells.

7.5 Oral Niches

Oral cavity can be divided into various microniches which include the buccal mucosa, the dorsal surface of the tongue, surfaces of teeth (both supragingival and subgingival), sulcular (or crevice) epithelium, and dental appliances and prosthetics (Marsh and Martin 2009a).

7.5.1 Tongue and Buccal Mucosa

Tongue because of a papillary surface is more densely colonized in comparison to cheek mucosa which is relatively smooth. Desquamation of epithelium is the primary factor responsible for the type and number of the microorganism colonizing it. The epithelium is in constant flux as it gets dislodged and swollen frequently making it difficult for many bacterial colonies to multiply. However, the bacteria having adhesions reattach themselves to the epithelial squames and start a new colony on virgin surfaces.

7.5.2 Tooth Surface

Dental plaque is the term given for the biofilm which forms on the surfaces of the tooth. The amount of environmental exposure determines the nature of the bacterial community: Pits and fissures harbor more bacteria than a smooth surface, whereas supragingival surfaces are more aerobic than the subgingival surface. Niches are

formed, based on the exposed tooth surface and their anatomies, and they dictate the microbiome composition.

7.5.3 Gingival Crevice and Its Epithelium

Initiation and the propagation of gingival and periodontal disease may be caused by the microbiome colonizing the crevicular epithelium and the gingival crevice.

The crevicular fluid flow provides essential nutrients for many of obligate anaerobes of the sulcus. In addition, various chemicals such as lysozyme and lactoferrin, and a spectrum of antibodies, in the gingival exudate, are essential for homeostasis of the crevicular microbiome.

7.5.4 Dental Appliances and Prosthetics

The rough surfaces of appliances, if not properly cleaned, function as reservoirs of bacteria and yeasts. *Candida*-associated denture stomatitis may cause from such unhygienic appliances (Samaranayake and Ellepola 2000).

7.6 Factors Modulating Microbial Growth

Microflora may differ quantitatively and qualitatively depending on the microenvironment in the mouth. Variety factors influence these variations. Anatomic factors, saliva, GCF, microbial factors, environment, etc., are some of them.

7.6.1 Anatomic Factors

There are certain regions of the oral cavity where bacterial stagnation occurs. These include the shape and topography of the tooth (e.g., occlusal fissures), misaligned teeth, improper dental treatment (e.g., restorations and crowns), and non-keratinized sulcular epithelium. These factors have a negative impact on the maintenance of oral hygiene and lead to plaque accumulation.

7.6.2 Saliva

Salivary fluid consists of a mixture of inorganic ions, proteins, enzymes, glycoproteins like mucin, and antimicrobial agents. It is secreted by major and minor salivary glands. The major glands include bilaterally parotid, submandibular, and sublingual glands, while the minor glands are present in the labial, lingual, buccal, and palatal mucosa. The inorganic content is mostly composed of sodium, potassium, calcium, chloride, bicarbonate, and phosphate ions. The concentration of these ions varies both diurnally, as well as in the resting and stimulated saliva.

Saliva constituents get adsorbed onto the tooth surface forming a film, or acquired pellicle, which facilitates bacterial adhesion, and thus playing a vital role in modulating bacterial growth. The organic components of saliva like carbohydrates and the proteins act as a readily available primary source of "food" for bacteria. Growth of exogenous organisms is inhibited by the presence of non-specific defense factors like lysozyme, lactoferrin, and histatins (bactericidal and fungicidal agents) and specific defense factors like immunoglobulin A (IgA) available in the saliva. Saliva besides controlling the local temperature (35–39 °C) also maintains its pH (6.75–7.25) by its excellent buffering capacity which serves as optimal conditions for many organisms.

7.6.3 Gingival Crevicular Fluid

Gingival crevicular fluid (GCF) is secreted continuously in a slow flow rate in health. Inflammation (gingivitis or periodontitis) causes an increase in the flow. The composition of GCF is similar to that of serum. Its major function is mechanical flushing out of the microbes from the gingival crevice. However, GCF, like saliva, acts as a source of nutrients, and thus influences the ecology of the crevice. Peptides, amino acids, and carbohydrates serve as nutrients for the growth of proteolytic and saccharolytic bacteria in the gingival sulcus. The GCF, similar to a serum, also helps in maintaining the pH at optimum levels for bacterial growth. And again similar to saliva, it provides specific and non-specific defense factors. IgG is the main immunoglobulin present with IgM and IgA also present but in lesser quantities. Cells of innate immunity, like neutrophils, macrophages or monocytes, also migrate into the sulcus. They play an important role in the protection of periodontal tissues against the microflora by either carrying out phagocytosis or release lysosomal enzymes into the sulcular fluid (Socransky and Haffajee 1991).

7.6.4 Microbial Factors

Microbial interactions can promote or inhibit the different species inhabiting the biofilm.

7.6.5 Environmental Factors

Temperature, pH, oxidation–reduction (redox) potential, ionic strength, and osmotic pressure are various environmental factors which influence the growth and metabolism of oral microflora (Fey and Conrad 2000; McDermid et al. 1988; Lloret et al. 1995; Oktyabrskii and Smirnovaa 2012; Otto et al. 1999). However, these factors, are not uniform on all the surfaces. Many bacteria require a specific pH for growth, and therefore, local pH is a critical factor in the ecology and homeostasis of the plaque biofilm. Gingival crevice pH generally remains below neutral but may increase to greater than pH 8 in diseased sites (McDermid et al. 1988). The buffering capability of saliva (pH around 6.7) regulates the acidity of most oral surfaces. Yet the plaque biofilm with its unique capacity can allow the pH to decrease as low as 5.0, with the help of bacterial metabolism of host diet sugars. This would again bring about changes in the microbiome. The oxidation–reduction potential is one of the physicochemical parameters which is used in accessing the state of growth of oral bacteria. Oral cavity, with its many variations and fluctuations in pH, favors the growth of a variety of bacterial species. Microbial growth is governed by the presence of active gases like oxygen, hydrogen or hydrogen sulfide, involved in oxidation or reduction reactions and changes in pH (Oktyabrskii and Smirnovaa 2012).

7.6.6 Miscellaneous

Antibiotic use, systemic or topical, as well as the use of antiseptics, may bring about changes in the microflora; especially, broad-spectrum antibiotics may favor the emergence of yeast species by removing most of the endogenous flora. Thus, candidiasis is commonly seen in post-long-term antibiotic therapy. The microbial growth is also modulated by the host diet. A diet rich in easily fermentable carbohydrates like sucrose increases the growth of acidogenic flora by acting as a major energy source. The dentist by performing dental procedures, such as dental scaling, or restoration of carious teeth can bring about a drastic change in the microflora composition of the periodontal pocket or on diseased tooth surfaces. The microbiome ecology can thus be modified, leading to a shift in balance toward health. Age-related factors (in elderly people), including a compromised immune system, reduction in salivary flow or long-term medications, can increase the probability of contamination from non-oral bacteria (e.g., staphylococci and enterobacteria).

Hence, the oral microbiome is affected by a variety of factors that influences microbial growth and brings about the development of a complex system.

7.7 Nutrition

The oral microflora depends upon substrates derived from the host's diet or bacterial products of different species. The pioneer species that colonize oral sites generate the end products of metabolism that are utilized by other bacteria. This is especially true in dense bacterial communities as exist in plaque biofilm (Samaranayake and Matsubara 2017).

7.7.1 Host Resources

The host resources are derived from either the diet or from host secretions. They include the following.

- Host diet constituents especially easily degradable carbohydrates like sucrose or starch.
- Some components of saliva including glycoproteins, vitamins, and minerals.
- GCF (gingival crevicular fluid) constituents like serum proteins and related chemicals, break down products of hemoglobin, etc.
- Inflammatory exudate (mainly for anaerobes)
- Components of desquamated epithelial cells
- Oxygen for aerobic bacteria.

7.7.2 Microbial Resources

- Neighboring bacteria's extracellular microbial products
- Glycogen granules for intracellular food storage.

7.8 Dental Plaque

Dental plaque is a specialized biofilm of the oral cavity which forms on the exposed surfaces of teeth. It is a complex community of multiple bacterial species, embedded in a matrix derived from their extracellular products and salivary constituents (Lang et al. 1997). It should be noted that not all biofilms are dental plaque.

Dental plaque supports an extremely diverse collection of microflora, and on average contains between 12 and 27 species (Marsh and Martin 2009b; Aas et al. 2005; Papaioannou et al. 2009). The development of plaque usually occurs in a specific manner. Clean tooth surfaces (as the tooth erupts into the oral cavity or after the professional cleaning of the tooth) become coated with a layer or film of

molecules, consisting of glycoproteins and biologically active proteins, within seconds. Saliva, and to some extent, gingival crevicular fluid and bacterial products are the main sources of these molecules (Hannig et al. 2005). This film, or acquired pellicle, offers attachment to a limited number of bacterial species only. Early colonizers, mainly Streptococcus mitis and *S. oralis*, are initially held near to tooth surface by reversible weak, long-range physicochemical forces. However, they soon express molecules, known as adhesions, which bind to the complementary receptors on the pellicle, making the process irreversible (Whittaker et al. 1996). These pioneer species, then, start to proliferate. The pioneer species, with their metabolism and their by-products, bring about changes in the local environment, for example, their consumption of oxygen makes it more anaerobic.

Coadhesion or coaggregation is the term used to describe the attachment of more fastidious secondary colonizers, utilizing receptors on already attached bacteria. This microbial succession helps make the biofilm more diverse (Kolenbrander et al. 2000).

The plaque matrix is an extremely important component of the system, not just a scaffold. It helps bind the extracellular polymers that the attached bacteria create, including enzymes, and as well helps in preventing the entry of charged molecules inside the biofilm (Allison 2003; Vu et al. 2009). Mature plaque is highly organized, both spatially and functionally, and this helps induce novel patterns of bacterial gene expression. Bacterial interactions are aided by the close proximity of different species (Kuramitsu et al. 2007; Hojo et al. 2009).

These interactions involve

(1) Food chain developments (metabolism of one organism produces the end product used by other bacteria). Metabolic cooperation among species helps catabolize complex macromolecules and thus increases the efficiency of the whole community.
(2) Cell–cell signaling. Helps in the transport of information between cells of similar species, for example, coordinating gene expression with the secretion of small peptides.
(3) Confer antibiotic resistance genes, and
(4) Antagonism. Production of inhibitory molecules can lead to competitive advantage as well as exclude undesirable microbes (Marsh and Martin 2009b).

7.9 Dental Plaque and Caries

Dental caries is an ideal example of a biofilm-induced disease. It is characterized by the loss of mineralized tooth tissue due to bacterial action (Takahashi and Nyvad 2011; Pitts et al. 2017; Koo et al. 2013). The microorganisms, together with a host diet which is rich in sugar, help in the creation of the cariogenic biofilm which is necessary for the development and growth of caries (Takahashi and Nyvad 2011; Pitts et al. 2017). The sugar-rich diet helps in the assembly of extracellular polymeric

substances (EPS) matrix, which leads to a selective increase in acidogenic and acid-tolerant microorganisms.

Various studies have been conducted with the aim of identifying the specific species which could be directly implicated in causing dental caries. However, the cariogenic traits such as the production of acids, tolerance to acidic pH, and polysaccharide production, are not species specific. Several streptococcal species express similar traits (de Soet et al. 2000).

The disease-causing potential of the cariogenic bacteria depends upon the interspecies interactions, which are allowed by the presence of plaque biofilm. However, acid production can also be ameliorated food chain development with Veillonella species, or by the production of bases by other organisms.

Caries risk increases with an increase in the proportion of acidogenic bacteria, especially mutans streptococci (Loesche 1986; Bowden 1990; Marsh 1999; Becker et al. 2002).

However, the association between these species and caries is not unique as even their absence can lead to disease development. Streptococcus mutans presence has also been shown to exist without any detectable tooth damage (Bowden et al. 1976; Marsh 1989). Non-mutans streptococci have also been implicated in these cases (Marsh 1999; Sansone et al. 1993; Brailsford et al. 2001).

7.9.1 Caries Origin Hypothesis

The etiology of biofilm-induced dental diseases, that is caries and periodontal diseases, is usually discussed under two main schools of thought. The "Specific Plaque Hypothesis" proposes that only a few species actively cause disease (Loesche 1976). Thus, preventive measures and treatments directed against these species would be sufficient in controlling disease. "Non-Specific Plaque Hypothesis," however, considers that the whole microflora of the plaque contributes to disease development (Theilade 1986). A varied mix of microorganisms, thus, play an important role in disease. Both arguments appear true to some extent. Biofilm-mediated diseases are essentially multispecies infections, but only a few specific species are able to predominate.

A recent alternate hypothesis, known as "Ecological Plaque Hypothesis," attempts to reconcile the main elements of the earlier hypotheses (Marsh 2003). Various mixed culture studies show that plaque-mediated diseases may occur due to imbalances in the microflora population, with increased number or "enrichment" of "oral pathogens." Cariogenic bacteria form a natural part of dental plaque, but at neutral pH are only weakly competitive, and thus their levels are clinically insignificant. A natural process of de- and re-mineralization occurs and is in equilibrium. However, if fermentable carbohydrate intake and frequency increase, then the pH remains below the critical pH (approximately pH 5.5) for the enamel demineralization for longer durations. Low pH favors the growth of cariogenic bacteria (acidogenic and acid tolerating) and also disrupts the balance toward demineralization. Increased

numbers of cariogenic bacteria, especially mutants streptococci and lactobacillus, result in faster acid production with the next carbohydrate intake, which would further increase demineralization. Other bacterial species which also make acid may be responsible for initial demineralization, or, in absence of more overt cariogenic bacteria, cause disease (de Soet et al. 2000).

Salient points of the ecological hypothesis include:

(a) Changes in the environment are directly linked to the selection of "pathogenic" bacteria.
(b) Disease-causing traits, not specific species is relevant. Diseases need not have specific etiology; any species may contribute to the process (de Soet et al. 2000; Sansone et al. 1993; Brailsford et al. 2001).

A direct corollary of this hypothesis is that disease can be prevented by interfering with the selection pressures which are responsible for proliferation of the pathological bacteria, and not just by targeting the pathogens directly (Loesche 1986). Two main mechanisms that disrupt microbial homeostasis include regular sugar/carbohydrate and associated acidic pH and reduction in salivary flow. An important consideration is the presence of the excellent buffering of saliva and the mildly alkaline pH of the normal oral cavity. In spite of this the occurrence of localized acidification within biofilms and the consequent caries raises questions on the role of the extracellular matrix of the dental plaque. The extracellular polymeric substances matrix, as per one explanation, with its diffusion modifying properties, helps create a microenvironment next to the tooth surface, where it allows the sugars (fuel) to diffuse in, but prevents saliva from reaching (Koo et al. 2013; Hwang et al. 2016; Guo et al. 2015. Thus, dental caries, as a pathological process, should not be considered to be just a microbial disease, but sufficient emphasis should also be given to milieu within which these microorganisms flourish and interact and which is responsible for the accumulation of acid (Bowen et al. 2018). Preventive strategies consistent with the ecological hypothesis may include the following:

(a) Prevention or inhibition of acid production in plaque, e.g., by utilizing fluoride products or other metabolic inhibitors. Fluoride, a key anti-caries product, helps improve enamel resistance and inhibits several enzymes, especially those involved in glycolysis and in maintaining intracellular pH (Marquis et al. 2003). Hence, fluoride reduces acid production after sugar metabolism (Bradshaw et al. 2002).
(b) Dietary regulations, that is avoidance of foods and drinks containing fermentable sugars in between the primary meals, will reduce repeated low pH in plaque milieu. Similarly, use of sugar substitutes such as aspartame or polyols will produce same results.
(c) Saliva flow stimulation utilizing sugar free gum after main meals will introduce host response components, improve buffering capacity, wash away the fermentable substrates, stimulate re-mineralization, and help in rapidly returning plaque pH to resting levels.

7.10 Dental Plaque, Dental Calculus, and Periodontitis

Periodontitis is essentially an inflammatory process, caused due to a mixed bacterial infection, leading to the destruction of the supporting tissues of the teeth (Lamont et al. 2014). While multifactorial, the primary cause is dental plaque microflora (Hajishengallis and Kawai 2014). Loss of support results in avulsion of the tooth. Periodontitis, thus, is the primary cause for loss of teeth during adulthood (Natto et al. 2014).

7.10.1 Calculus

Dental plaque, sometimes, undergoes mineralization due to the precipitation of mineral salts. Entire biofilm, however, does not become calcified (Samaranayake and Matsubara 2017).

7.10.1.1 Formation

Degenerating bacteria, in a mature undisturbed plaque, may become seeding agents for mineralization. Salivary and GCF calcium and phosphate ions start getting deposited within the deeper layers of dental plaque. Bacterial phosphatases and proteases inhibit salivary statherin- and proline-rich proteins, which prevent calcification. This further accelerates calcification. Insoluble mineral crystals are formed which coalesce to a highly calcified mass. This is similar to bone, cementum, or dentin (Roberts-Harry and Clerehugh 2000). Intercellular matrix and bacterial surfaces provide the initial points for the development of these crystals. Later on, they even form intracellular within bacteria (Zander et al. 1960). The overlying surface of calculus is always covered by a layer of non-calcified plaque. The mineral crystals are firmly adherent to the tooth surface, as they fill the pits and irregularities in enamel, cementum, or dentin. This helps the calculus to be firmly attached to the tooth (Jepsen et al. 2011).

7.10.1.2 Classification

Calculus is classified as

(a) Supragingival, that is calculus coronal to the gingival margin, and
(b) subgingival, which is calculus located apical to the gingival margin.

Subgingival calculus is mainly influenced by the GCF components, especially hemorrhagic components, and by the anaerobic microorganisms mineralization. This leads to the black pigmentation of the same.

Supragingival calculus, similarly, is more affected by contact with substances like food pigments and tobacco. It usually has a clay-like consistency and can be easily removed from teeth (Jepsen et al. 2011).

7.10.1.3 Composition

Calculus consists of mineralized and organic material. The inorganic part (around 80% by dry weight) of both types of calculus consists of mostly calcium phosphate $Ca_3(PO_4)_2$, which forms crystals of hydroxyapatite, octacalcium phosphate, magnesium whitlockite, and brushite. The organic part (around 20%) consists of desquamated epithelial cells, leukocytes, protein polysaccharide complexes, and various microorganisms. Cocci, bacilli, and filaments are commonly seen, and occasionally, spiral organisms. Supragingival calculus predominantly has gram-positive organisms, whereas in subgingival calculus, gram-negative species are more common. An organic matrix consisting of proteins, lipids, and carbohydrates is also present. Subgingival calculus has a less extensive matrix compared to supragingival.

Dental calculus, with its rough surface and porous nature, serves as an ideal reservoir for the bacterial toxins like lipopolysaccharides (LPS) which harm the periodontium.

Individual variations in the composition of calculus make it unique. Persons may range from heavy to moderate and slight calculus formers, and occasionally non-calculus formers are also found throughout the populace. Calculus deposition is influenced by numerous variables and also varies from site to site in the same mouth, as well as over time (Corbett and Dawes 1998; White 1997).

7.10.2 Classification of Periodontal Disease

Periodontal disease is subdivided into two main types: gingivitis and periodontitis. While there are many subtypes of each, chronic gingivitis and chronic periodontitis are the most common (Papapanou 2014; Hinrichs and Novak 2012). Gingiva is a collar of keratinized mucosa surrounding the neck of teeth. Inflammation of gingiva is known as gingivitis. It is characterized by redness, swelling, and bleeding from the gingival sulcus or crevice on mechanical stimulation, with either dental instruments or a toothbrush or floss. Gingivitis is considered to be a reversible condition (Papapanou 2014; Scannapieco 2014).

Periodontitis, on the other hand, is a comparatively serious condition, irreversible, and follows generally from gingivitis in susceptible individuals (Papapanou 2014). Deeper supportive components of the tooth get involved as the inflammatory lesion progress. These components include the periodontal ligament, the alveolar bone, and cementum. Damage and destruction of these cause the tooth to become mobile in its socket, and ultimately results in avulsion if not arrested in time (Papapanou 2014).

7.10.3 Etiology of Periodontal Disease

Periodontal disease is a multifactorial disease. The primary cause, however, is bacterial plaque or biofilm (Hajishengallis and Kawai 2014). Secondary etiologic factors which influence disease development are case specific, but include mechanical plaque retentive features (e.g., calculus, developmental grooves on teeth, overhanging or rough restorations), systemic factors (e.g., hormonal especially pregnancy, diabetes mellitus, medications), genetic factors (e.g., congenital immune disorders), and nutritional deficiency (e.g., scurvy) (Preshaw and Taylor 2012; Hinrichs 2012; Diehl et al. 2012; Novak et al. 2012; Carranza and Hogan 2012).

7.11 The Systemic Connection of Oral Biofilms

Several systemic diseases have been shown to be influenced by dental plaque-associated oral diseases, especially periodontitis. Periodontal inflammation may alter both the course and pathogenesis of these diseases. "Focal infection theory" explains the role of localized infection, often asymptomatic, in disseminating microorganisms or their products to distant sites causing disease.

Microbial pathogens of the plaque biofilm have been linked to atherosclerosis and coronary heart disease. They may cause deregulation of the immune system, with progressive inflammation, and hence, disruption of endothelial cell function, an early indicator of cardiovascular disease (Slocum et al. 2016). Poor oral hygiene and the presence of dental calculus have also been linked with arthrosclerosis, which may lead to myocardial infarction, stroke, or death (Soder et al. 2014; Dai et al. 2015).

The risk of infective endocarditis from oral bacteria is well documented (Parahitiyawa et al. 2009). Poor oral hygiene and plaque increase the risk of bacteremia when dental procedures like tooth extraction, or even tooth brushing, are carried out. Thus, antibiotic prophylaxis in susceptible individuals has become mandated and an improved oral hygiene can decrease the risk of infective endocarditis (Lockhart et al. 2009). Aspiration of biofilm organisms could also lead to development of pneumonia, especially in non-ambulatory patients (Ewan et al. 2015).

Critically ill patients, in many cases, require mechanical ventilation as an essential intervention. An endotracheal tube is the most common mode for providing the same. The most common hospital acquired infection in critical care is ventilator-associated pneumonia. An incidence of 9 to 24% is seen in patients who are mechanically ventilated for longer than 2 days. The microbiomes of dental plaque, non-directed bronchial lavages (NBLs), and endotracheal tubes show high similarity suggesting the role oral cavity may play as a source of microorganisms involved in aspiration to the endotracheal tube and the lower airway (Marino et al. 2017).

Diabetes mellitus has chronic periodontitis as one of its long-term complications; however, a "two-way relationship" between blood glucose control and periodontal disease is now being considered. Pro-inflammatory mediators, such as interleukin-6

and tumor necrosis factor-α, which are produced as a result of microbial insult in periodontitis sites, may reach the systemic circulation and can interfere with the functioning of insulin receptors. This would lead to developing insulin resistance and thus impaired glucose homeostasis (Gurav 2012).

Maternal periodontitis is considered to be a risk factor for the baby's health. Preterm birth and low birth weight have been linked to periodontal disease in mothers (Ide and Papapanou 2013). The role of inflammatory cytokines or direct dissemination of bacteria and its products to the fetoplacental unit are thought to be the mechanism by which plaque biofilm may influence outcome of pregnancy (Pitiphat et al. 2008).

7.12 Approaches for Control of Dental Biofilm

As per "National Centre for Health Statistics" in USA, approximately 37% children in the age-group of 2–4 years and 2.4 billion people in the world have dental caries (Dye et al. 2015; Kassebaum et al. 2015), while 15–20% of populace in the age-group of 35–44 years has severe periodontitis (WHO 2012a). Severity of the situation is self-evident. The primary step in management of biofilm-related dental diseases is physical treatment, which aims to reduce the bacterial load in biofilms. It also helps in preventing maturation of the biofilm. Regular, proper brushing with flossing and frequent routine dental check-ups can diminish the risk and help keep plaque-associated diseases at bay.

However, advanced disease treatment becomes essential. Antimicrobials and antibiotics may be administered to control the disease process and reduce bacterial load and growth. Systemic conditions, or co-existence of multiple disorders like diabetes mellitus, acquired immune deficiency syndrome (AIDS), and other immune suppressing conditions and genetic mutations like human Beta-defencin B1 make treatment much more complex and difficult (WHO 2012b). Biofilm pathogens, if allowed to proliferate, may directly or via their products enter the systemic environment (Cullinan et al. 2009; Cullinan and Seymour 2000) and cause further complications including diabetes mellitus (Holmstrup and Flyvbjerg 2016), cardiac diseases (Lockhart et al. 2012), osteoporosis (Wang and McCauley 2016), pneumonia (Laurence et al. 2015), stroke (Palm et al. 2016), etc.

7.12.1 Conventional Treatment

The main modality for control of supragingival plaque is mechanical debridement. Additionally, chemical agents in the form of mouth washes and antimicrobial agents may be used for supplemental therapy. Novel treatment methodologies are also being explored. The treatment approach for disruption of dental plaque is designed based on the status of periodontitis. If reversible, that is gingivitis, then conservative techniques

for plaque removal are preferred. These may also be carried out by the patient. If the disease has progressed to periodontitis, then the severity of the disease is used to define the scope of the treatment. The most common technique for judging the severity of periodontitis is the "probe test" wherein a periodontal probe, an instrument with grading, is used to measure the depth of the "periodontal pocket," a pocket formed between the tooth surface and the gingiva due to apical migration of the junctional epithelium from the cementoenamel junction (Slots et al. 1985).

A 5 mm or less depth of the pocket indicates that non-invasive techniques may be sufficient. Professional plaque removal from the subgingival region by scaling is carried out together with root planing and smoothening of cementum surface of the tooth.

Surgical intervention is indicated when the pocket depth increases beyond 5 mm. These procedures commonly include flap surgery, which can be supported by soft tissue grafting and/or bone grafting (Fernandesa et al. 2018).

Flap surgery refers to elevation of a "flap" of gingival tissue, which allows cleaning of tooth surface, as well as the tissue part. This is then sutured back, either in the same place or apically or coronally depending on the treatment plan. Soft tissue grafting involves the placement of a "graft" tissue harvested from another site (commonly the hard palate) in order to restore the lost or damaged soft tissue of the gingiva. Bone grafting, similarly, involves the replacement of destructed bone by an autologous harvested bone or by alloplastic materials (Fernandesa et al. 2018).

7.12.2 Mechanical Plaque Control

Daily disruption of dental plaque, at and above the gingival margin, prevents maturing of the plaque. This is essential for reducing the gingival inflammation caused by dental plaque (Cancro and Fischman 2000). Effective control depends on the individual's skill as well as acquired behavior patterns (Cancro and Fischman 2000).

Tools to achieve the same are well known and readily available to a large extent. They include oral hygiene products, toothpastes or dentifrices, mouth rinses, oral cleaning aids, and toothbrush. Effective use of the cleaning devices on a daily basis can disrupt plaque growth.

Mechanical aids for oral biofilm control may be classified as following (Mandal et al. 2017):

1. Chewing sticks
   These include neem or miswak sticks and mango leaves.
2. Toothbrushes
   a. On basis of power supply as
      i. Manual
      ii. Powered toothbrushes. Powered brushes can be further divided as single, double, or triple headed.
   b. Bristle diameter

i. Ultrasoft
ii. Soft
iii. Medium
iv. Hard

c. Tuft number
i. Space tufted
ii. Multitufted

3. Interdental cleaning devices

a. Dental floss
i. Twisted or non-twisted
ii. Bonded or non-bonded
iii. Waxed or unwaxed
iv. Thick or thin
v. Floss/knitting yarn combinations
vi. Monofilament
vii. Manual or power

b. Interproximal brushes
i. Cone or cylinder shaped
ii. Reversible handle with small insert
iii. Wire handle brushes
iv. Marginal brushes with single-tufted or multitufted interproximal

c. Toothpick
d. Wooden tips
e. Rubber tips.

Traditional methods utilizing fingers or plant twigs and sticks have slowly fallen to the wayside as the effectiveness of modern toothbrushes (manual) increased. Further advances are continuing and include electric rotation–oscillation, sonic, and even solar-powered toothbrushes. These motorized toothbrushes improve and promote a better oral hygiene with minimal effort (Mandal et al. 2017).

A common adage, however, still stands true: "Best toothbrush is the one being properly used" (Cancro and Fischman 2000).

Electric motorized toothbrushes have become nowadays comparatively common. They have a brush head which is capable of a range of motions and having a power source. Initially developed to enhance oral care, especially the handicapped people or for those who lack in manual dexterity. In addition, powered toothbrushes may be indicated for poorly motivated people, as may influence them to better clean their teeth (Cancro and Fischman 2000; Wright et al. 1976).

Plaque build-up in the interproximal regions is an important cause for gum bleeding and gingivitis. Dental floss is the most commonly used adjunct for interdental plaque control, with numerous studies reporting its effectiveness. A study showed that professional flossing of children participating in a dental caries trial, done periodically, reduced proximal decay in children by 50% (Wright et al. 1976). Other interproximal cleaning aids which require less skill to use include interdental brushes and

wooden interdental cleaning aids. Barton and Abelson (Barton and Abelson 1987) in a study showed that use of interdental wooden picks helps improve interdental cleaning by over 50%. Tooth brushing, in interdental areas, improves cleaning by 8% only (Cancro and Fischman 2000).

7.12.3 Oral Irrigators (Mandal et al. 2017)

Newer devices which utilize a pulsating fluid stream, under pressure, for interproximal and around dental crowns and bridges plaque control have become available on the market. These "oral irrigation devices," "dental water jet," "water flosser," "waterpik," and "oral irrigator" can be used to deliver medicaments like chlorhexidine, stannous fluoride, iodine solution, antibiotics like 5% tetracycline hydrochloride, which can be added to the water for reduction of the microbial load. They are used as an adjunct to brushing, especially for patients with a lot of dental work in mouth. These devices, both power and manual, may be used in a professional setting or at home by the patient.

7.12.4 Chemical Plaque Control

Mechanical plaque control methods may be supplemented by chemical agents, especially in established diseases for maintenance and inhibition of plaque growth. Dual treatment strategy has shown improved results compared to debridement alone (Tariq et al. 2012). It commonly includes local drug delivery of antimicrobials and antibiotics in the form of powders or gels or systemic administration in form of oral tablets and capsules (Preshaw et al. 2004). Cationic agents like Chlorhexidine and Cetylpyridinium chloride are also widely used. They inhibit bacterial growth, and thus anti-plaque, by binding to dental plaque, dental pellicle, and mucous membrane (Eley 1999).

Chlorhexidine has been used as an anti-plaque agent for more than twenty years. It is a highly effective anti-plaque agent, which has survived the test of time (Loe and Schiott 1970; Schiott et al. 1970). Chlorhexidine is considered to a very potent anti-bacterial agent due to its ability to break up existing plaque. It is bactericidal at higher concentrations, and as it dilutes over time due to saliva and, it becomes bacteriostatic (Collaert et al. 1992).

Side effects of chlorhexidine use appear to be reversible and localized, and it generally causes staining of teeth and tongue in a dose dependent manner. Higher concentrations may lead to taste changes and mucosal desquamation (Collaert et al. 1992; Addy et al. 1985; Flotra et al. 1971; Siegrist et al. 1986; Addy et al. 1994; Jenkins et al. 1994).

Plaque control strategies can be thus divided in the following manner (Brecx 1997):

1. Caries prevention.
    a. Based on "Specific Plaque Hypothesis"
        i. Anti-Streptococcus mutans: Chemical agents like chlorhexidine, xylitol, amine fluoride, or stannous fluoride.
        ii. Anti-plaque: Mechanical cleaning with anti-plaque agents like amine fluoride/stannous fluoride, triclogard, chlorhexidine.
        iii. Anti-demineralization: Fluoride agents, especially amine and stannous fluoride, or xylitol.
2. Gingivitis prevention.
    a. Based on "Non-specific Plaque Hypothesis"
        i. Anti-group: Mechanical cleaning with anti-plaque agents like amine fluoride, stannous fluoride, triclogard, or chlorhexidine. Motivation of patient.
3. Periodontitis.
    a. Based on "Group-Specific Hypothesis"
        i. Antimicrobials especially metronidazole.
        ii. Anti-plaque treatment targeting subgingival plaque, utilizing subgingival scaling, root planning, irrigation with betadine, and/or chlorhexidine.

7.12.5 Local Delivery of Drugs

Local drug delivery usually encompasses delivery inside periodontal pockets. Hollow drug reservoirs, known as fibers, are fabricated using polymers, which allow for a sustained release of the drug molecule with either through erosion of the carrier or by diffusion. Most common example is tetracycline loaded fibers in collagen which are used for periodontal pocket therapy (Demirel et al. 1999; Sinha et al. 2014). Also, various polymers like PGA, ethyl cellulose, etc., have been used in the form of strips or packs to deliver antibiotics such as gatifloxacin, metronidazole, or tetracycline (Schwach-Abdellaoui et al. 2000).

An additional procedure called guided tissue regeneration is sometimes carried out as well. It utilizes a biocompatible membrane to prevent migration of epithelial cells into the periodontal defect, in order to allow connective tissue cells to form a normal attachment to the tooth surface, thus allowing the formation of healthy bone (Barrington 1981).

7.12.5.1 Newer Developments

Newer drug delivery systems are also being developed, the most exciting of which include "Nano Drug Delivery Systems." They are supposed to increase the retentiveness of the carrier, and thus drug, for longer periods while also showing the capability to penetrate regions which were considered inaccessible by older systems. Hence, they more easily penetrate the pocket and reach effective concentration in GCF. Additional advantages conferred by this system include a larger surface area with the flexibility to regulate drug release as per the requirement (Fernandesa et al. 2018). A size of 1–10 nm for the carrier appears to provide maximum anti-bacterial activity (Perez-Diaz et al. 2015).

Liposomes have the ability in the, presence of dental pellicle, to get adsorbed on the enamel hydroxyapatite. The lipid bilayer allows them to carry antimicrobial agents. Besides, they get adsorbed on hydroxyapatite present on the tooth enamel especially due to the presence of dental pellicle or extracellular mucopolysaccharide. Hence, a targeting mechanism utilizing liposomes which carry triclosan and chlorhexidine (water insoluble and soluble, respectively) in differing lipid systems can be used to target Streptococcus sanguinis biofilms specifically (Jones et al. 1997).

Gas-filled nanosized cavities of small organic molecules, around 200 nm in size, in an aqueous solution produce free radicals by the process of cavitation. These "nanobubbles" thus have an antimicrobial action via these free radicals (Agarwal et al. 2011). A study has shown the efficacy of ozone nanobubbles, which were used for debridement of whole mouth, for a period of 4–8 weeks. Probing depth of the periodontal pocket was reduced when a comparison with placebo (water) was done (Hayakumo et al. 2013). Fluorescent microscopy showed eradication of Streptococcus mutans and a decrease in plaque accumulation (Nagayoshi et al. 2004).

Phages are viruses that infect bacteria. They are composed of DNA or RNA genome and encapsulated by proteins. Specialized phages may help in production of hydrolytic enzymes which can lead to biofilm breakdown in caries. Their bactericidal effect can decrease the bacterial load of biofilms as well (Kasimanickam et al. 2013). Numerous clinical trials are ongoing to study the use of bacteriophages.

Other treatment modalities are also being explored, including glucansucrase inhibitors, photodynamic therapy, and probiotics, considering the activity they may have against the biofilm microflora (Fernandesa et al. 2018).

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Chapter 8
Biofilm-Mediated Diseases of the Eye

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Abstract Ophthalmology is a rapidly growing discipline of medicine with newer ocular implants and prostheses and improvements over the older ones being constantly introduced to reduce visual morbidity. These implants and devices are an easy target for biofilm formation and predispose to various ocular infections, which at times may lead to vision-threatening complications. One of the most feared complications in ophthalmology is postoperative endophthalmitis with majority of them occurring after cataract surgeries caused by the formation of biofilm over the intraocular lenses. Similar biofilms have been found over the contact lenses, lacrimal devices, ophthalmic implants, scleral plugs, and glaucoma drainage devices, leading to infections and their subsequent failures. Biofilms also disrupt the normal physiology of the eye and cause dry eye disease. Graft rejections after penetrating keratoplasty have commonly been attributed to the formation of biofilms leading to crystalline keratopathy. Current interventions aim at prevention of biofilm formation on the devices and implants by introducing antimicrobial-coated devices and by using biomaterials which have a lesser tendency of formation of biofilm. Prevention of biofilm formation in ophthalmology is an ongoing research with newer modalities being introduced consistently for the same.

Keywords Biofilm · Intraocular lens · Endophthalmitis · Ocular implant · Keratoplasty · Scleral buckle · Ocular device

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8.1 Introduction

Ophthalmology is a rapidly growing discipline of medicine and has been at the forefront of medical innovation. Newer ocular devices and implants are constantly being introduced to reduce visual morbidity. The earliest known account of ocular prosthesis dates back to as long as 2900–2800 BCE, and the evidences also suggest that Sushruta was one of the first surgeons to have performed cataract surgery. The revolution in ophthalmic implants started around world war which also gave us the first PMMA intraocular lens, courtesy of Sir Harold Ridley. Ophthalmology, since then has come a long way with constant introduction of newer implants and prosthesis and improvements over the older ones like contact lenses, scleral buckles, glaucoma drainage devices, etc. However, these devices and implants are not exempted from the clutches of the biofilm formation. As a result, device-related ocular infections pose a risk to the success of such procedures. Also, biofilms disrupt the natural ocular physiology predisposing to various ocular infections. These infections may progress to devastating levels and cause permanent complications which may cause poor visual outcomes and occasionally blindness. Role of biofilms in the diseases of the eye had been underestimated in the past, but with better diagnostic and isolation modalities, more and more disease conditions of the eye are being attributed to the formation of biofilms.

The common biofilm-related infections of the eye include endophthalmitis, keratitis, scleral buckle infections, lacrimal system infections, and periorbital infections.

8.2 Endophthalmitis

Endophthalmitis is undoubtedly one of the most dreaded conditions in ophthalmology accounting for the most number of clinical eviscerations (45.5%) (Chaudhry et al. 2007). Organisms may enter intraocular tissues exogenously (after intraocular surgeries, intravitreal injections, penetrating open globe injury, and perforating corneal ulcers) or endogenously from a distant focus (Sadaka et al. 2012).

Postoperative endophthalmitis forms the majority of the cases, among which the most commonly encountered is the entry of bacteria post-cataract surgery. Cataract extraction along with intraocular lens implantation is the most commonly performed procedure by ophthalmologists worldwide, and postoperative endophthalmitis is the most common complication of cataract surgery causing blindness. The incidence of endophthalmitis after cataract surgery ranges from 0.028 to 0.2%, depending upon the technique used as well as according to the geographical distribution (Taban et al. 2005; West et al. 2005; Wykoff et al. 2010).

Organisms forming the ocular surface flora are the predominant causative agents of the endophthalmitis after cataract surgery. 76–90% of the culture-positive pseudophakic endophthalmitis is caused by gram-positive bacteria. In 38–59% cases of

acute onset postoperative endophthalmitis, the cultures have been found to be positive for *Staphylococcus epidermidis (*a coagulase-negative staphylococci—CoNS (Driebe et al. 1986). While delayed-onset endophthalmitis is commonly caused by Propionibacterium acnes (Shirodkar et al. 2012), rarely, enterococcal endophthalmitis may also be seen after cataract surgery (Scott et al. 2003). Despite rigorous measures and intensive interventions, enterococcal endophthalmitis is associated with poor visual outcomes. Electrostatic forces cause bacterial adherence to the intraocular lenses which may get attached to the lens surface (26%) due to wiping of the lens around the wound at the time of intraocular lens implantation (Vafidis et al. 1984). Periocular skin and the eyelashes form the major pool of endophthalmitis causing bacterial source. Evidence suggests that bacteria are also capable of forming biofilms on the posterior capsular bag (Sawusch et al. 1989). After uneventful cataract surgery, the contamination of the anterior chamber has been reported to be found in 2–46% of the cases, the rate of which is higher than that of postoperative infection. This suggests that rapid turnover of the aqueous humor causes clearing of the bacterial inoculum from the anterior chamber which prevents its progression to endophthalmitis. Vitreous, on the other hand, is more stable and hence clearing of microbes attached to IOL surface is slow and inefficient. The intraocular lens provides an abiotic surface for the bacteria to form a biofilm; therefore, normal clearance mechanisms from anterior chamber are rendered futile.

The capability of bacteria of forming biofilm over IOL depends largely upon the material of the IOL as well as the species of organism. While *S. epidermidis* adheres firmly to polymethylmethacrylate (PMMA), Staphylococci have been found to have better adherence to polypropylene than PMMA (Sawusch et al. 1989). Epidemiological evidences suggest that polypropylene haptics increase the risk of endophthalmitis up to 4.5 times (Menikoff et al. 1991).

The ica locus plays an important role in staphylococcal biofilm formation. Studies from Japan, Mexico, and India have shown CoNS to be positive for icaA and icaD as well as icaAD gene locus (Suzuki et al. 2005; Makki et al. 2011; Juarez-Verdayes et al. 2013). In in vitro models, silicone is shown to have higher vulnerability toward biofilm formation, which is closely followed by hydrophobic acrylic and PMMA. Hydrophilic acrylic shows least propensity toward bacterial adherence (Baillif et al. 2018).

Various modifications in the surface of IOLs have been made to increase their water content by the use of different agents like methacryloyloxyethyl phosphorylcholine (MPC), heparin, and fluorine. In order to decrease the incidence of endophthalmitis, further developments are required in the form of medications which may cause disruption of the biofilms apart from making of IOLs of such material which can prevent the formation of biofilm altogether.

8.3 Contact Lens Associated Keratitis

Biofilms have been observed on contact lenses which are believed to cause microbial keratitis.

Of the predisposing risk factor for infectious keratitis, most common is the use of contact lenses. Both gram-negative (*Pseudomonas aeruginosa* and *Serratia* spp.) and gram-positive organisms (*Staphylococcus aureus*) have been found to cause bacterial keratitis (Cheng et al. 1999). Among protozoa, Acanthamoeba is the most common pathogen causing infectious keratitis, the incidence of which is rare but vision-threatening and often takes an aggressive course (Hammersmith 2006). The causative organisms gain access through contaminated lens care materials, lens cases and manual contaminations due to improper cleaning of the contact lenses, poor hygiene, and long-wearing time. Extended wear soft contact lenses, as well as daily disposable and silicone hydrogel contact lenses, pose a greater risk of keratitis in contrast to daily-wear rigid gas permeable lenses (Dart et al. 2008).

The incidence of infection being more among the contact lens users may be attributed to a combined effect of corneal epithelial damage and inoculation of contact lenses by colony-forming bacteria. Wearing contact lens leads to reduced corneal epithelial barrier function, either mechanically, due to accumulation of debris under the lens during night-time wear or due to friction and pressure from normal blinking during daytime wear. This causes the progress of infection originating from ocular surface, adnexa or biofilms over the contact lens, into the deeper corneal layers. These biofilms render the bacteria resistant to host mucosal defenses and antimicrobial treatment. The periodic release of organisms or their products such as endotoxins further damages the corneal epithelium, making it more vulnerable to infections. (Willcox et al. 2001; Zegans et al. 2005)

Contact lenses also induce corneal hypoxia and hypercapnia, thus affecting the epithelial response to the damage. Compromisation of the tear fluid exchange also limits the antimicrobial properties of the lens by alterations in the tear film composition.

Hence, contact lenses contribute to corneal infections by providing an adequate surface for bacteria to form biofilms, inducing corneal hypoxia and damage to the corneal epithelial cells.

8.4 Crystalline Keratopathy

Infectious crystalline keratopathy (ICK), a rare form of microbial keratitis, may occur in both normal as well as compromised corneas, following penetrating keratoplasty in corneal grafts or around sutures (Gorovoy et al. 1983; Reiss et al 1986). The main feature of ICK is branching crystalline opacities in the corneal epithelium and stroma and minimum inflammatory response. The most common pathogen associated with

ICK is viridans streptococci. Other bacterial and fungal species like staphylococci, Candida, and Enterobacter and Acanthamoeba are also known to cause ICK.

The periodic acid–Schiff (PAS) stain of the corneal samples obtained from the patients of ICK indicates high concentrations of polysaccharides. The levels of polysaccharides are associated with the ability of bacteria to form biofilms which are well-organized multicellular structures. Topical corticosteroid therapy for extended period of time and prior penetrating keratoplasty has been found to be important risk factors of ICK (Fulcher et al. 2001). The features of ICK can be explained by the formation of a biofilm on the corneal lamellae, though the underlying mechanism is yet to be explained. It is proposed that anatomical changes resulting from keratoplasty cause inflammation and immunological activity, which encourage biofilm growth by the causative organism.

ICK is highly resistant and hence is unresponsive to rigorous antimicrobial treatment. More recently, disruption of biofilms using Nd:YAG laser along with further antimicrobial therapy has been proposed (Masselos et al. 2009).

8.5 Dry Eye

Dry eye disease is a complex of multiple etiologies; hence, the disease presentation is usually overlapping. A new theory of dry eye has recently emerged which combines blepharitis and dry eye into one simple condition, dry eye blepharitis syndrome (DEBS) (Rynerson and Perry 2016).

It is proposed that biofilms have a significant role in causation of DEBS. Biofilms are formed on the surfaces that provide moisture and nutrients. The eyelid margin provides an ideal habitat for the bacterial biofilm to thrive due to the presence of moisture, nutrients, and warmth. It is proposed that biofilm formation commences right after birth with colonization of the lids by bacteria. Many factors such as medications, hormonal state, and reduced blinking, exacerbate dry eye, but the underline etiology originates from a biofilm, which is present from infancy. This biofilm eventually achieves quorum-sensing gene activation and releases virulence factors.

DEBS is caused by *Staphylococcus aureus* in all age groups but how early in life is the presentation of symptoms of DEBS is dependent on the strain of *S. aureus*.

Four stages of DEBS are suggested being affected by biofilm in a sequential manner:

Stage I—folliculitis: inflammation and edema of lash follicles.
Stage II—meibomian gland dysfunction: impaction and inflammation of the meibomian gland.
Stage III—lacrimitis: impaction and inflammation of the glands of Krause and Wolfring.
Stage IV—breakdown of structural integrity of eyelids leading to chronic inflammatory lid disease presenting as lid laxity, entropion, ectropion, and floppy eyelid syndrome.

Hence, it is required that patients should be educated about eyelid hygiene and prevention of blepharitis so that the chronic problem of DEBS can be drastically reduced. With growing knowledge regarding the role of biofilms in dry eye syndrome, microblepharoexfoliation using a device called BlephEx has shown promising results in treatment of DEBS. In this procedure, a rotary device with a sponge tip is used along with an eyelid cleanser to remove biofilms from the eyelid margins and eye lashes. This has proven more effective than any other measure.

8.6 Ocular Implants and Biofilms

8.6.1 Conjunctival Plug

Conjunctival plugs employed to treat dry eye are made of silicon, hydrophobic acrylic, collagen, and hydrogels. Secondary infections can occur following implantation of these plugs. These conditions usually have delayed onset and are unresponsive to treatment, hence, they are presumed to be caused due to biofilm formation on the implant (Yokoi et al. 2000). These infections can range from canaliculitis, dacryocystitis to conjunctivitis. When punctal plugs from patients without any symptomtic infection were removed and examined, 53% of the samples showed the presence of bacterial biofilms (Sugita et al 2001). Yokoi et al. demonstrated *S. haemolyticus* and *Candida tropicalis* in the conjunctival plug removed from a case reported to have developed conjunctivitis in the eye following the implant.

8.6.2 Scleral Buckles

Scleral buckles made of silicon are largely employed in the treatment of rhegmatogenous retinal detachment. Gram-positive cocci, in particular coagulase-negative staphylococci, nontuberculous mycobacterium (*M. chelonae*) and *Proteus mirabilis* are commonly found to cause infections of the scleral buckles. Presence of biofilms has been found on 65% of scleral buckles removed for infection and extrusion, as demonstrated by electron microscopy (Holland et al. 1991; Pathengay et al. 2004).

It is possible that bacteria attach to the buckle at the time of surgery and form biofilms which lead to indolent infections. Biofilm formation on these prosthetic devices may cause chronic inflammation and tissue damage due to cytotoxic damage by the bacteria as well as the host response to the planktonic cells shed by the biofilm (Costerton et al. 1999).

8.6.3 Lacrimal Intubation Devices

Lacrimal stents and Jones tube often employed in the treatment of chronic dacryocystitis provide a surface for biofilm formation by microorganisms. Failure of polyurethane stents, as well as infections following placement of silicone or Jones tube, has been attributed to formation of biofilms on these devices as demonstrated by scanning electron microscopy. *Staphylococcus aureus*, *Streptococcus epidermidis*, and *Pseudomonas aeruginosa* have been found to cause the majority of these implant-related biofilms (Kim et al. 2018).

Ali et al. (2017), in a study, found that the Monoka stents removed showed evidence of biofilm formation and physical deposits. The external surfaces, cut ends as well as intraluminal surfaces were all involved with the ampullary portion of the stent head being the most common site of deposition. Longer duration of the stent retention was associated with more extensive biofilm formation with more widespread deposits in stents retained for three months than those retained for six weeks.

8.6.4 Orbital Implants

Samimi et al., in a study, demonstrated biofilm formation in periorbital biomaterials. The orbital implants such as orbital plates and anophthalmic socket implants, all demonstrated biofilm deposits. The organisms demonstrated, varied from *S. aureus*, gram-negative bacilli such as *Achromobacter* and *Pseudomonas*, *M. chelonae*, *Pantoea agglomeran*s, to yeasts such as *Candida* and *Trichosporon*. A greater undertaking of the role of biology of biofilms may help prevent complications related to these prosthetic devices.

8.6.5 Other Biomaterials Used in Ophthalmology

Recently, biofilm formation has been implicated in the pathogenesis of infections associated with keratoprosthesis and glaucoma drainage devices as they may provide a suitable surface for bacterial inoculation and environment for biofilms to thrive. A few cases have recently been reported in which role of biofilm formation in glaucoma drainage device has been suspected (Masood et al. 2016; Esporcatte et al. 2016). Jassim et al. (2015) found that 85% of the eyes with Boston type 1 K-Pro Keratoprosthesis had positive cultures of which 57.7% had biofilm-forming capacity. The coagulase-negative staphylococcus isolated from these K-Pro eyes had reduced susceptibility to vancomycin. Further investigation is required to look for the contribution of biofilms in the causation of these infections.

8.7 Prevention and Treatment of Biofilms

Given the dreaded outcomes of ocular biofilms, it becomes important to incorporate practices to remove the biofilm or reduce its formation. Current interventions aim at prevention of biofilm formation on the devices and implants. Biocidal molecules can be covalently attached to slowly release antibiotics or modify the surface to prevent colonization of surfaces (Bispo et al. 2015).

Biocide-coated and antimicrobial-releasing ophthalmic devices are in research. IOL coated with antimicrobials like rifampicin, doxycycline, and norfloxacin are being tested on animal models. Other substances that have shown promising results in preventing biofilm formation are gallium nitrate and silver. However, the long-term exposure of ocular tissue to these antimicrobial treatments and development of resistant strains may pose a threat to the advancement of these implants.

Biofilm-related infections can also be reduced by using materials with a lower predisposition for biofilm formation such as one-piece PMMA intraocular lenses (Elder et al. 1995). Polymers, such polyacrylamide, dextran, or polyethylene glycol, and also nanopores, nanotubes, and nanopillars made of anodized aluminum, titanium dioxide, or polymethylmethacrylate prevent adherence of biofilm-forming organisms (Samimi et al. 2013). However, changes in the material surface may cause opacities, hence limiting its use.

New therapeutic strategies are being suggested and experimented everyday to prevent the formation of biofilm and elimination of organisms in a formed biofilm. Strategies are required to reduce enzymatic degradation of antibiotics within the biofilm, to change nutrition to the biofilm-forming bacteria, and possibly, to alter gene expression which can offer resistance to biofilm formation.

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Chapter 9
Biofilm-Mediated Diseases of the Ear, Nose, and Throat (ENT)

M. Ravi Sankar, M. Arulalan and Amit K. Keshri

Abstract Biofilms play an important role in many chronic infectious diseases of the ear, nose, and throat (ENT), including rhino-sinusitis, otitis media with effusion, cholesteatoma, and chronic adenotonsillitis, as well as infections associated with implants and prostheses. As a consequence of an increased use of implants and prostheses in ENT patients, there has been an increased incidence of infections and associated biofilms. Common strategies behind the treatment of biofilms include antimicrobial neutralization, the dispersion of existing biofilms, and the disruption of quorum sensing. Prevention is the most efficient way of combating biofilms, achieved by aseptic precaution, ultraclean operating theaters, sterilization of surgical instruments and implants, and the use of prophylactic antibiotics and antibiotic-coated implants. There is still a significant lack of knowledge about the stages of biofilm formation, making its management more challenging. Ear, nose, and throat surgeons need to understand the role of biofilms and be aware of methods of treatment and prevention.

Keywords Biofilm · ENT · Chronic rhinosinusitis · Otology

9.1 Introduction

Biofilms play an important role in many chronic human infectious diseases as well as having an important role in many chronic infectious diseases of the ear, nose, and throat.

The role of biofilms is well established in diseases of the ear, nose, and throat, such as chronic rhino-sinusitis, otitis media with effusion (OME), cholesteatoma, chronic adenotonsillitis, and implant and prosthesis infection (Hall-Stoodley et al. 2006; Zuliani et al. 2006; Brady et al. 2010; Fastenberg et al. 2016).

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9.2 Chronic Rhino-sinusitis

The etiology of chronic rhino-sinusitis (CRS) is usually multifactorial, that is, including local factors, environmental factors, and genetic factors. A higher incidence of biofilms in CRS patients suggests they play a role in its pathogenesis, however, there is no correlation between biofilm and CRS severity. However, the presence of biofilm is associated with many of the worst post-operative outcomes after Functional Endoscopic Sinus Surgery (FESS) (Chen et al. 2012). Common organisms associated with biofilm formation in CRS patients are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenza*, viridans streptococci, coagulase negative staphylococci, *Enterococcus fecalis*, and *Propinobacterium* and *Corynebacterium* species. In a few cases, fungi like *Candida albucans* are also associated with biofilm formation and are resistant to amphotericin B, nystatin, and echinocandins treatment (Fastenberg et al. 2016).

The possible factors that initiate biofilm formation on the sinonasal mucosa include defects in the adaptive and innate immunity of an individual and a defective ciliary function in the nasal mucosa. Once a biofilm forms over the sinonasal mucosa it induces the over expression of cytokines and cell surface proteins that result in a local inflammatory response (Fig. 9.1).

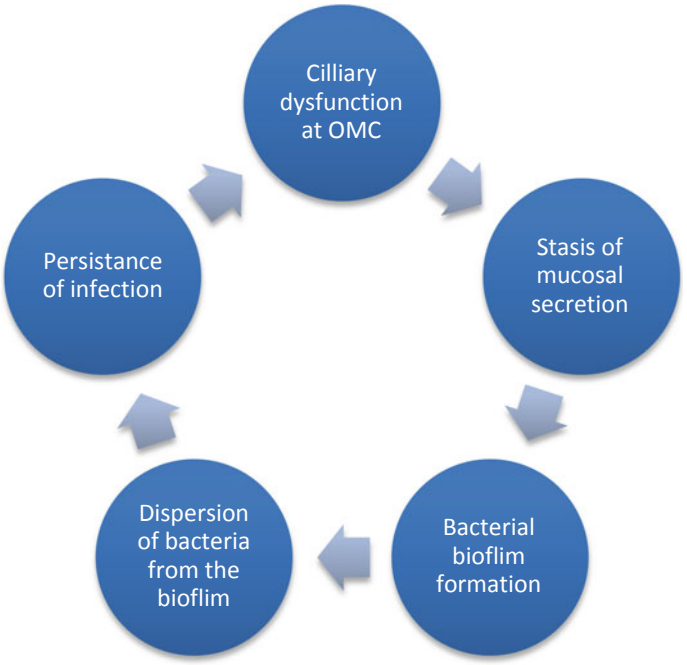

Fig. 9.1 The role of biofilms in chronic rhino-sinusitis

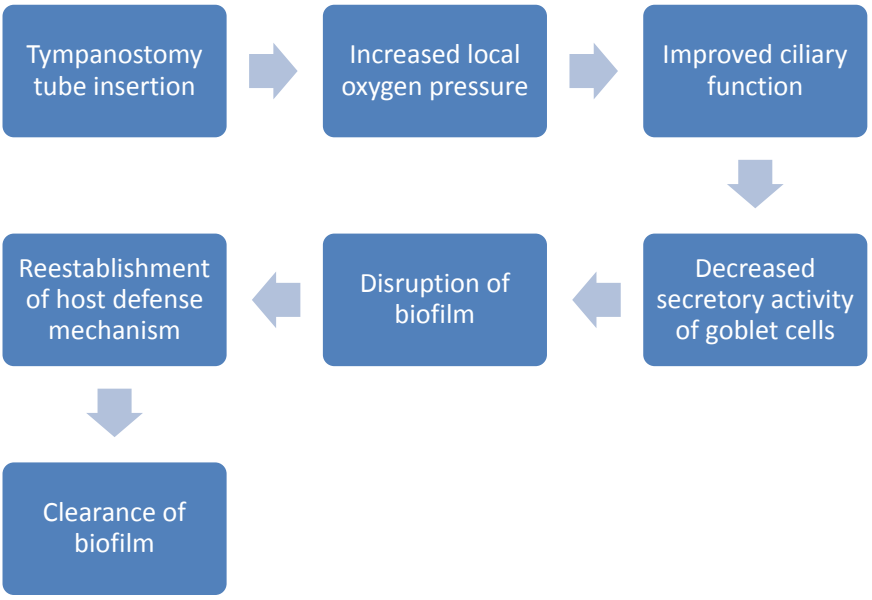

Fig. 9.2 Mechanism of action of a tympanostomy tube for clearing biofilms in cases of otitis media with effusion

9.3 Otitis Media with Effusion

In cases of recurrent otitis media with effusion, more than 90% of specimens show the presence of biofilms (Post et al. 2007). One common source of biofilms in recurrent OME is adenoid tissue. Endotoxins produced by biofilms are responsible for the chronicity of OME. Tympanostomy tube insertion is the main mode of treatment for OME (Mena Viveros 2014) (Fig. 9.2). The most common organisms responsible for biofilm formation are *P. aeruginosa* and *Streptococci pneumoniae*.

However, a tympanostomy tube can itself act as surface for biofilm formation. Methods to prevent the formation of biofilms over tympanostomy tubes include minimizing bleeding during surgery (clots provide an apt environment for *P. aeruginosa* colonization) and bombarding the silicon of the tube with ions and coating it with albumin (Malaty and Antonelli 2008).

9.4 Cholesteatoma

In cholesteatoma pathology, the keratin matrix provides an ideal environment for the formation of biofilms. *Pseudomonas aeruginosa* is the most common organism associated with such growths, having the ability to adhere to keratinocytes (Mena Viveros 2014).

9.5 Adenotonsillitis

There is a well-established association between biofilms and chronic adenotonsillitis (Coticchia et al. 2007; Kania et al. 2007).

The most common organisms associated with adenotonsillitis are *P. aeruginosa*, *S. aureus*, *S. pneumonia*, and *Moraxella catarrhalis*. In chronic cases an adenotonsillectomy is the treatment of choice.

9.6 Biofilms in Ear, Nose, and Throat Implants and Prostheses

The most common implants associated with the formation of biofilms in Oto-rhino-laryngology (ORL) are:

1. Cochlear implants.
2. Speech prostheses.
3. Tracheostomy tubes.
4. Tympanostomy tubes.
5. Bone-anchored hearing aids (BAHAs).

Cochlear implants

A cochlear implant (CI) is a bionic device which provides hearing to severe to profound hearing loss patients. It has an external component which sits behind the ear and an internal component that is implanted under the skin. Very rarely, even after good antibiotic coverage, the internal component can become infected—with the chance of salvage being minimal. The formation of biofilms over implants is one of the major causes of infections resistant to treatment. The most common organism responsible for this is MRSA and *P. aeruginosa*. The formation of biofilms over implants is mediated by polysaccharides (intercellular adhesions) and proteins (cooperation) (Brady et al. 2010).

Possible ways that biofilms can cause the failure of devices include where biofilms protect bacteria from the effects of antibiotics and as a consequence of biofilm-induced allergic reactions resulting in persistent inflammation (Im et al. 2015). Most biofilm-induced infections require implants to be removed and replaced. Very few cases are successfully managed using local therapies, such as tea tree oil or hydrogen peroxide, to salvage implants (Brady et al. 2010).

Speech prostheses

Speech prostheses are used as voice rehabilitation aids post laryngectomy—as a consequence of larynx carcinoma. The formation of biofilms over such prostheses represents the most common cause of failure and salivary leakage. The most common organisms responsible for this are *Rothia dentacoriosa*, *Streptococcus salivarius*, *S.*

aureus, *Streptococcus epidermidis*, *Candida albicans*, and *Candida tropicalis*. Measures that reduce the formation of biofilms over prostheses include the topical use of *N*-acetyl-cystine, application of 7% silver oxide, or use of a prosthesis made of silicon, modified with per-fluro-alkyl siloxane (Macassey and Dawes 2008). Consumption of probiotic drinks, containing *Lactobacillus casei*, 3 times daily for 6 months reduces the likelihood that voice prosthesis replacement is required (Smith et al. 2011).

Tracheostomy tubes

Some studies have shown that 90% of the tracheostomy tubes removed after 7 days have biofilms (Mena Viveros 2014).

Tympanostomy tubes

Tympanostomy tubes or grommets used for secretory otitis media have in a few cases shown biofilm formation.

Bone-anchored hearing aids

Biofilm formation in BAHAs is less common than in cochlear implants because BAHAs are made of titanium and are not in contact with the middle ear mucosa (Macassey and Dawes 2008).

9.7 Treatment

Common strategies behind the treatment of biofilms include (Fastenberg et al. 2016):

1. Antimicrobial neutralization.
2. Dispersion of the existing biofilm.
3. Disruption of quorum sensing.

Antimicrobial neutralization

In order to avoid biofilm infection an acute infection regime of single drugs in minimal doses for short periods is recommended—preventing resistance and tolerance (Høiby et al. 2010). Commonly use systemic antibiotics include macrolides and fluoroquinolones. Certain studies have shown that long-term use of macrolides provides some benefit. It is thought that they act by interfering with autoinducers and that they have an immune-modulatory function. Mupirocin irrigation has shown a beneficial effect in *S. aureus*-induced CRS. Non-antibiotic antimicrobial agents like *N*,*N*-dichloro-2-2-dimethyl-taurine, manuka honey, and gentian violet have also shown proven efficacy. Biofilms under the effect of electric currents, ultrasonic radiation, pulsed ultrasound, pressure waves, and hydrodynamic flushing have been found to be more susceptible to antibiotics (Smith et al. 2011; Mena Viveros 2014).

The intake of probiotics containing *Lactobacillus* species has shown a dubious beneficial effect on the prevention of biofilms. The clinical use of antimicrobial photodynamic therapy is currently under trial. This process involves the act of destroying

cells in the presence of a photoreactive dye and a laser. The use of hyaluronic acid has shown good antiadherence and antibiofilm action during in vitro studies and has provided promising results in vivo, particularly in the form of nebulization using sodium hyaluronate and saline (Marcuzzo et al. 2017).

Dispersion of the existing biofilm

- Surfactants are commonly used as dispersion agents.
- Baby shampoo at a concentration of 1% is the most commonly used agent.
- Originally, citric acid/zwitterionic molecules and sinosurf were used but were withdrawn due to their toxicity (Fastenberg et al. 2016).
- Currently, targeting enzymes, such as dispersin B and bacterial deoxyribonuclease, are under trial (Donelli et al. 2007).

Disruption of quorum sensing

- An in vitro study has demonstrated that macrolides and azithromycin significantly affect quorum sensing but its role in CRS patients has yet to be studied (Fastenberg et al. 2016).
- Recently, the important role paraoxonases play in the fight against biofilm formation has been demonstrated (Camps et al. 2011).

9.8 Prevention

Prevention is the most efficient way of combating the development of biofilms (Babb et al. 1995; Chow and Yang 2004). Methods of prevention include:

1. Extreme aseptic precaution during implant procedures.
2. Ultraclean operating theaters.
3. Sterilization of surgical garments, instruments, and implants.
4. The use of prophylactic antibiotics and antibiotic coated implants.

9.9 Conclusion

There is still a lack of knowledge regarding the stages of biofilm formation. This makes its management more challenging. New strategies are being developed and tested like the implementation of antibiotic-coated catheters. Ear, nose, and throat surgeons need to develop an understanding of biofilms and should develop their knowledge of treatments and prevention strategies (Figs. 9.3, 9.4 and 9.5).

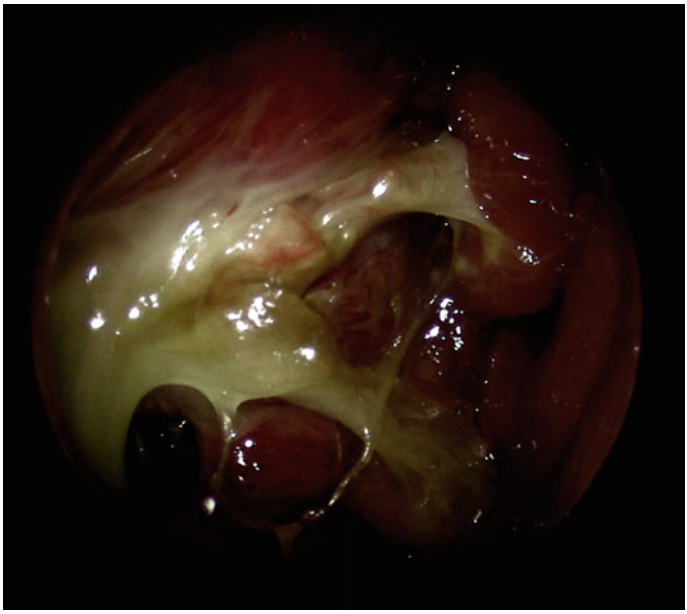

Fig. 9.3 Endoscopic view of a biofilm in the nasal cavity (paranasal sinus)

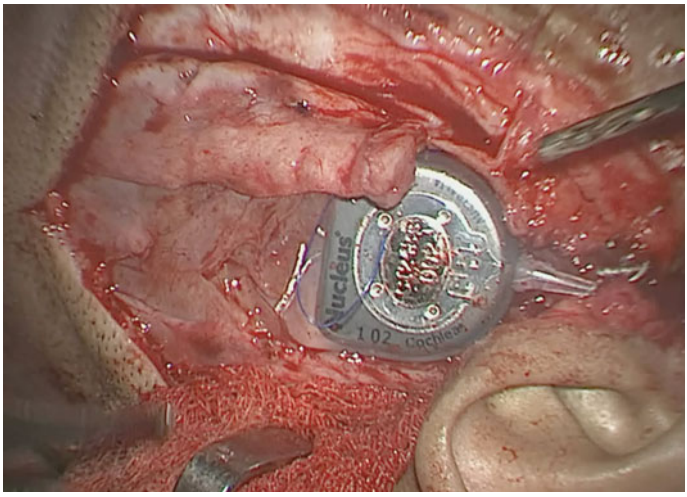

Fig. 9.4 Biofilm over an infected cochlear implant

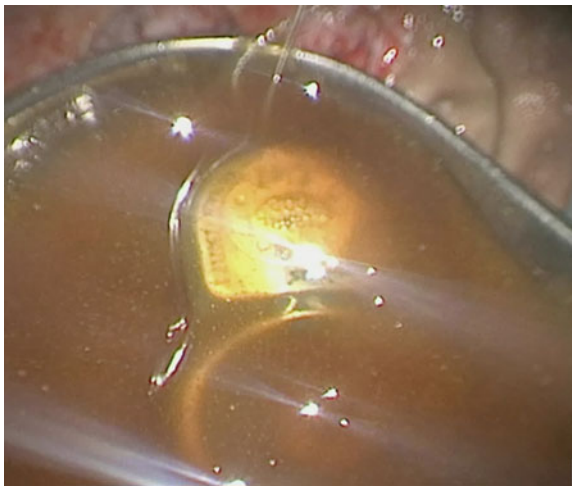

Fig. 9.5 Cochlear implant dipped in manuka honey after the removal of a biofilm

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Chapter 10
Biofilm-Mediated Diseases of the Heart and Lungs

Surojeet Das

Abstract Research and studies have undoubtedly established the fact that microbial biofilms have the capacity to inhabit human tissues and medical devices as well as playing a role in microbial pathogenesis. Biofilms are present across the entire environment and are linked with almost 75% of nosocomial infections. Growths of microorganisms in a multicellular form present an incredible challenge to a host's defenses and antimicrobials. Therefore, biofilms create elaborate chronic and subacute infections which are not easy to overcome. Extensive insight regarding genetic, microbiological, molecular, and biophysical processes within biofilm formations has been acquired. This knowledge has influenced our understanding and management methodologies for several infectious diseases. Our knowledge to date has also enabled the development of unique antimicrobial treatments targeted at biofilms. Fungal and bacterial biofilms play significant roles in a variety of pulmonary and heart diseases, of which cystic fibrosis lung disease, pneumonia caused by ventilators, infective endocarditis, pulmonary infections, and atherosclerosis are noteworthy.

Keywords Biofilms · Ventilator-associated pneumonia · Cystic fibrosis · Infective endocarditis · Atherosclerosis

10.1 Introduction

The prevalent view of infectious diseases in recent times has primarily been dependent on our comprehension of infections. This understanding has been developed by growing planktonic microorganisms in liquid culture media in the laboratory. It was not until the early 1980s that biofilm function in microbial pathogenesis became so perceptible (Costerton et al. 1999; Parsek and Singh 2003). Largely, biofilms are associated with biotic surfaces, such as dental and epithelial, or abiotic surfaces. Untethered microbial aggregates can represent other biofilms that inhabit compartments of tissues that are compromised, like the sputum found within the lumen of the

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airways of cystic fibrosis (CF) sufferers. These types of restricted microorganisms characteristically cause chronic diseases which are slow, progressive, and localized rather than being acute and invasive. Growth on medical devices and on hot tissues has emerged as a significant means of virulence for pathogens such as *Pseudomonas aeruginosa*, staphylococcal species, and fungi *Candida albicans* and *Aspergillus fumigatus* (Boisvert et al. 2016).

There are extensive differences in gene and protein expression archetype, along with physiological differences, between biofilm cells and planktonic cells (Sauer et al. 2002; Whiteley et al. 2001). Most particularly, this form of growth presents phenotypic traits that encourage microbial survival against antimicrobial abuse and in antagonistic environments, including drug and host resistance. Therefore, a primary characteristic of biofilms is strong defiance to treatments for antimicrobial occurrences. This leads to the requirement for complex treatments, incurable or irreversible infections, or even the physical removal of contaminated tissues or medical devices. Biofilms are found in copious subacute and chronic infections that are persistent in nature, for example, infections associated with catheters, endocarditis, and CF lung disease with chronic bacterial infections (Costerton et al. 1999; Parsek and Singh 2003). There has been an increased use of ingrained medical contrivances and catheters and therefore it has been approximated that 60–70% of nosocomical infections are linked to biofilms (Wenzel 2007).

10.2 Biofilms Related to Endotracheal Tubes and Ventilator-Associated Pneumonia

One of the main nosocomial infections is ventilator-associated pneumonia (VAP) which has noteworthy morbidity and mortality. Within hours of endotracheal intubation (Adair et al. 1999) bacterial colonization takes place and biofilms grow rapidly on the surface of endotracheal tubes. It is during the process of mechanical ventilation that the aerosolization of biofilms takes place, this is sometimes a consequence of interference during suctioning of the trachea, releasing bacteria that might cause pneumonia (Luna et al. 2009; Inglis et al. 1998). Although biofilms produced on endotracheal tubes are insufficient to cause pneumonia associated with ventilators, they do represent a significant reservoir of microbes (Cardeñosa Cendrero et al. 1999; Inglis et al. 1989; Perkins et al. 2010). During one study on patients experiencing mechanical ventilation, 72 out of 75 had biofilms in their endotracheal tubes, discovered during electron microscopy. In almost 50% of VAP cases, one of the causes identified for the failure of treatment was that similar pathogens were recognized in the bronchoalveolar lavage fluid and biofilms found in endotracheal tubes (Gil-Perotin et al. 2012).

It has been observed during studies using culture-based methods, as well as those independent of culture, that endotracheal tubes are polymicrobial and are constituted of various organisms established within enteric and oropharyngeal flora (Adair et al.

1999; Gil-Perotin et al. 2012; Cairns et al. 2011; Vandecandelaere et al. 2012). Aspiration of secretions into the subglottic area or retrograde colonization are observed to be a noteworthy path for colonization of bacteria of the distal endotracheal tube (Adair et al. 1999; Luna et al. 2009; Feldman et al. 1999). The most common members of such colonies are from oral flora (e.g., *Prevotella* and *Streptococcus* species), however, frequent recovery of ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, *Enterobacter* spp.) organisms have been identified, created from biofilms of endotracheal tubes (Adair et al. 1999; Gil-Perotin et al. 2012; Feldman et al. 1999).

It has been observed that there is also the possibility of elevated formations of biofilms and parallel augmented antimicrobial resistance with dental biofilm coaggregation and mutual interactions between different varieties of microbes (Bousbia et al. 2013). Several experimental studies on polymicrobial infections challenge the idea of oral commensal organisms traditionally being non-pathogenic. For instance, there have been increased instances of virulence in lung infections caused by interactions between oral commensal organisms and *P. aeruginosa* (Sibley et al. 2008). Furthermore, polymicrobial infections of VAP, set off by oral commensal organisms, are likely underrated by current standard microbiological approaches.

There has been clinical testing of the inhibition of bacterial adhesion and/or biofilm formation and strategies that integrate materials into endotracheal tube biomaterials and vascular catheters (Fernandez et al. 2012). Silver-coated endotracheal tubes have shown great potential, being linked with radically reduced biofilm formation, risk of VAP, and bacterial lung colonization (Kollef et al. 2008; Tokmaji et al. 2015). Other methods incorporate antiseptic-coated endotracheal tube surfaces (Raad et al. 2011) or metal nanoparticles (Machado et al. 2010). Despite the fact these approaches are potentially promising, their routine use to date is precluded on the grounds of cost effectiveness and, for some devices, safety.

10.3 Biofilms in Cystic Fibrosis

Most patients suffering from CF experience mucociliary clearance along with other damaged host defenses, initiated by mutations in the conductance transmembrane regulator CF gene. This gives way to chronic lung ailments, characterized by constant bacterial infections of the airways and disparaging lung inflammations (Gibson et al. 2003). In adult patients with CF the major pathogen detected is *P. aeruginosa* which causes a lasting chronic airway infection that defies treatment by antibiotic therapies and the host's immune system. It has been established that the growth of biofilms is primarily due to the chronic, non-invasive, and drug intractable nature of infections with *P. aeruginosa* (Costerton et al. 1999; Parsek and Singh 2003; Singh et al. 2000).

Growth of *P. aeruginosa* biofilm is linked with pervasive modification in gene manifestation and up-regulation of exo-polysaccharide fabrication, while demonstrating down-regulation of motility and acute virulence genes for, for example,

secretions of type III (Mikkelsen et al. 2011; Ventre et al. 2006). This leads to bacteria that cause fewer incursions of host cells and cytotoxicity. Clinical observations corroborate with experimental results that patients who have CF give refuge to pulmonary infections with *P. aeruginosa*, that are chronic in nature, for long periods of time without showing signs of invasive disease (Singh et al. 2000). This is in contrast to patients with *P. aeruginosa* acute pneumonia, who often succumb within a few days.

P. aeruginosa, unlike biofilms associated with catheters or experimental models of biofilms that are surface attached, develops untethered biofilm that accumulates in CF airways within the sputum. During the phase when bacterial motility is constrained, suitable conditions exist—linked with chronic inflammation and CF sputum, together with the existence of neutrophil elastase, amino acids, and DNA—to encourage the formation of biofilm aggregate bacteria developing similar biofilm aggregates to those within high-density gels (Caceres et al. 2014; Sriramulu et al. 2005). It is also important to note that this growth of non-attached biofilm also offers resistance to antibiotics and neutrophils in vitro. However, it is important to note that we have a limited understanding of *P. aeruginosa* biofilms in CF due to deficient models of in vivo lung infections.

Multidrug intolerance, mediated by biofilms, significantly hampers the treatment plan for *P. aeruginosa* chronic lung infections. There have been significant improved outcomes in treatment of CF lung disease by routine treatment with inhaled antibiotics like tobramycin (Quon et al. 2014). However, this does not help in eradicating chronic *P. aeruginosa* infections despite high pulmonary concentrations. There has been significant in vitro activity shown by unique compounds, such as antimicrobial peptides (de la Fuente-Núñez et al. 2015) and metal nanoparticles (Martinez-Gutierrez et al. 2013), however, these are yet to be used clinically.

10.4 Biofilms in Pulmonary Infections

It is only recently that the significance of biofilm development in the pathogenesis of *A. fumigatus*, a ubiquitous filamentous fungus, has emerged. In immune compromised patients *A. fumigatus* triggers invasive respiratory infections and inhabits the airways of patients with chronic pulmonary diseases like CF. It has been derived from histopathologic studies of tissues from human and animal models that *A. fumigatus* grows as a biofilm composed of a multicellular aggregation of hyphae embedded within an extracellular matrix (Morisse et al. 2013). It has been demonstrated in experimental studies that biofilm growth is a factor affecting fungal virulence by encouraging observance of hyphae to host cells (Sheppard 2011) and augmenting resistance to antifungals (Seidler et al. 2008) and the host's immune system. Hence, the formation of pulmonary biofilms by *A. fumigatus* may add to the phenomenal failure rate of antifungal therapies in the treatment of invasive aspergillosis. Therefore, an immediate requirement for the preclinical evaluation of antibiofilm strategies

needs to comprehend their full potential to further improve the current increases noted in invasive and chronic *A. fumigatus* infections.

10.5 Biofilms in Indwelling Vascular Catheters

It has been demonstrated by scanning and transmission electron microscopy that in effect all indwelling central venous catheters are inhabited by microorganisms entrenched in a biofilm matrix (Raad 1998). The most common organisms isolated from catheter biofilms are *K. pneumoniae*, *S. aureus*, *P. aeruginosa*, *Staphylococcus epidermidis*, *C. albicans*, and *Enterococcus faecalis* (Elliott et al. 1997; Raad et al. 1992).

The origination of these organisms is from the microflora found on the skin of patients, contaminated infusates, or exogenous microflora from healthcare personnel. Such organisms gain access to catheters along their exteriors by movement externally from the skin along the exterior catheter hub or port. There is the possibility of quick colonization of these devices (within less than 24 h) perhaps as a consequence of conditioning films produced by the host (plasma, platelets, and tissue proteins) (Maki 1994). Raad et al. (1993) discovered that biofilm formation on central venous catheters is universal, but the duration of catheterization was the basis for the extent and location of biofilm formations: in the short term, that is, less than 10 days, catheters had more biofilm formations on their outer surfaces; in the long term, that is, around 30 days, catheters had more biofilm formations on their inner lumen. Microbial growth can be affected by the character of the fluid dispensed through central venous catheters: Gram-positive organisms (*S. aureus* and *S. epidermidis*) did not show growth in intravenous fluids. On the other hand, Gram-negative aquatic organisms (e.g., *P. aeruginosa*, *Serratia* spp., *Enterobacter* spp., and *Pantoea* spp.) demonstrated sustainable growth (Maki and Mermel 1998; Maki and Martin 1975; Anderson et al. 1986; Failla et al. 1975; Donlan et al. 1999). Because these fluid solutions have limited nutrient content, they demonstrate rare turbidity, suggesting numbers are $<10^7$ organisms per milliliter. Occurrence of bloodstream infections in patients is directly proportional to the number of organisms on the tip of a catheter (Aufwerber et al. 1991; Corona et al. 1990; Anaissie et al. 1995), supporting the concept of biofilm development requiring a critical level, with greater numbers leading to substantial cell detachment and embolism.

There have been several studies to determine the impact of different types of antimicrobial treatments on influencing the formation of biofilms on devices. It was discovered by Freeman and Gould (1985) that to eliminate microbial colonization of catheters requires the addition of sodium metabisulfite to the dextrose-heparin flush. Similarly, Darouiche et al. (1999) discovered that reduced colonization occurs if catheters are infused with minocycline and rifampin compared with catheters permeated with chlorhexidine and silver sulfadiazine. Kamal et al. (1991), found that if catheters were layered with a cationic surfactant (tridodecylmethylammonium chloride) they had a reduced probability of contamination and biofilm development

in comparison with untreated catheters. Another discovery made by Flowers et al. (1989) suggested that a local application of polyantibiotic ointment has a protective impact on catheters, leading to lower rates of contamination. Maki (1994) made several suggestions for the control of biofilms on central venous catheters, including the use of topical antibiotics, aseptic techniques during implantation, reduced catheterization times, the introduction of mechanical barriers to thwart incursions of organisms by connecting catheters to a surgically implanted cuffs, use of in-line filters for intravenous fluids, removal of contaminated devices, and use of antimicrobial agents for coating the inner lumen of catheters.

10.6 Mechanical Heart Valve Biofilms

There is the possibility that components of mechanical heart valves and tissues surrounding the heart may have microorganisms attached to them, leading to a condition called prosthetic valve endocarditis. The major organisms responsible for this state are *S. aureus*, diphtheroids, *S. epidermidis*, *Streptococcus* spp., Gram-negative bacilli, enterococci, and *Candida* spp. The origination of these organisms can be from various sources such as the skin, dental work, or other indwelling devices like central venous catheters (Braunwald 1997). Source is an important parameter for identifying causative microorganisms as is time of exposure to contaminating organisms during surgery (early endocarditis is mostly caused by *S. epidermidis*)—usually invasive procedures like dental work (*Streptococcus* spp.) or from indwelling devices. The insertion of mechanical heart valves causes a lot of tissue damage, resulting in the accumulation of circulating platelets and fibrin where the valve is attached—there is a greater chance of microorganism colonization in such areas (Braunwald 1997). As a consequence biofilms usually develop on the tissues surrounding prostheses or on the sewing cuff fabric used to attach devices to tissues (Illingworth et al. 1998; Carrel et al. 1998) rather than on the valve itself (Karchmer and Gibbons 1994). Administration of antimicrobial agents usually happens during valve replacement or during dental work to prevent initial attachment by killing all microorganisms that may have entered the bloodstream. As far as other indwelling devices are concerned, comparatively fewer patients are cured of biofilm infections by means of antibiotic therapies alone (Hancock 1994). Illingworth et al. (1998) discovered, via experimentation, that a silver coating on a St. Jude mechanical heart valve (St. Jude Medical Inc., St. Paul, MN), when implanted into a guinea pig artificially infected with *S. epidermidis*, resulted in reduced inflammation compared with when an uncoated fabric was used. Despite no confirmed determination of the number of attached organisms, the authors inferred there was a direct relationship between inflammation and number of visible organisms. This was later confirmed by Carrel et al. (1998), finding the approach to be effective during in vitro studies with various organisms (Donlan 2001).

10.7 Biofilms in Infective Endocarditis

Despite extensive research, improvements in diagnostic techniques, improvisation in surgical management, and variety of antibiotic choice, infective endocarditis (IE), an uncommon condition, still carries high morbidity and mortality. The incidence of this condition has not changed for over 3 decades. However, changes have been noted in patterns of IE occurrence, due to progress in socio-economic conditions in developed countries, longer lifespans, and as a consequence of invasive methods used to treat many diseases (Fong 2009).

Of significance for IE in developed countries for the past several decades is rheumatic heart disease. It has been superseded by degenerative mitral and aortic valvular disease, that influences IE, amplifying with age and with greater predominance in males (Prendergast 2006). However, rheumatic carditis has common occurrence in developing countries. Observational studies in France in 1999 recorded that almost 47% of patients with IE had no known previous heart disease (Hoen et al. 2002). The study also reported that only 16% of IE was prosthetic-valve endocarditis (PVE), however, data collected in 2001 suggests that PVE was linked with 26% of occurrences of IE, with almost 74% affecting native valves (Tornos et al. 2005).

The highest incidences of IE, that is, 1–5% per year (Mir et al. 2002), have been reported in cases of intravenous drug abuse (IVDA), followed by patients with prosthetic valves, 1–3%, with rates dropping after 1 year to 0.3–0.6% patients per year (Morellion and Que 2004). The median incidence in the general population is 3.6/100,000 people per year (Prendergast 2006). Almost 22% of infections are accounted for by nosocomial infections with associated mortalities greater than 50%, majorly associated with long-standing catheters and surgical procedures—less than 50% have essential valvular disease (Bourza et al. 2001). With mortality percentages greater than 50%, primary pathogens are *Staphylococcus* and *Entercoccus*.

Very insignificant changes have been witnessed over the last decade with regards to the microbiology of IE. At 34%, *Staphylococcus* is the most common organism associated with native valve IE and 23–30% PVE, followed by 14–17% oral *Streptococcus*, group D *Streptococcus*, and *Enterococcus* for native valve IE, and about 17% coagulase-negative *Staphylococcus* (CoNS) for PVE (Braun et al. 2007). The structure of pathologic lesions formed by IE suggests biofilm activity. Marrie and colleagues, even before the hypothesis of biofilms in 1987, studied bacterial vegetations formed on aortic valves of 6 IE cases under an electron microscope. Populated bacterial micro-colonies entrenched in amorphous material were located within all observed sections, suggesting biofilm formation. The authors suggested that along with a mix of extracellular amorphous material, fibrin and platelets along with bacterial micro-colonies, made up the flora and also guarded organisms against contact with antibiotics. In 2012, Bosio and his team established a *Mycobacterium fortuitum* biofilm on a freestyle bioprosthetic valve infected using immune fluorescence staining and electron microscopy. Recently a report from Europe, identified 10 patients with disseminated *Mycobacterium chimaera* 7 infections following open-heart surgery in 3 different hospitals. Of these, 8 of the 10 patients required

surgical intervention despite receiving targeted antimicrobial therapy. Failure of antimycobacterial-directed therapy was quoted as the reason for biofilm formation on prosthetic surfaces.

It is well established that almost 80% of IE cases contain *Streptococcus*, *Enterococcus*, and *Staphylococcus*—all known to form biofilms (Donlan and Costerton 2002). Their pathogenicity is determined according to biofilm—with differences between species having already been identified, such as surface adhesion proteins, system for quorum sensing, virulence factors, and composition of extracellular polymeric substance (EPS) matrix (Speziale and Geoghegan 2015; Mohamed and Huang 2007). Many studies have recognized that when separated from patients with IE these species have the competency to form biofilms in vitro (Presterl et al. 2005; Fey et al. 1999; Chuang-Smith et al. 2010). A higher tolerance to antibiotics in comparison to planktonic organisms derived from the same species has been observed in bacteria inhabiting such biofilms formed in vitro (Chifiriuc et al. 2011).

Four criteria, indicative of infections arising from clinical biofilms have been identified by Parsek and Singh: (1) The infecting bacteria is adherent to some substratum or surface. (2) From observations of infected tissue it is clear that bacteria reside in cell constellations or small colonies surrounded by an extracellular matrix. (3) The infection is limited to a fastidious location; however, there is a significant likelihood of dissemination. (4) There is the presence of antibiotic resistance even though the vulnerability of the planktonic cells of the organism is in question (Parsek and Singh 2003). All the above criteria are satisfied in the case of IE (Elgharably et al. 2016).

10.8 Biofilms in Atherosclerosis

Atherosclerosis is a disease resulting from the deposition of fatty plaque within arterial walls. It is the main cause of ischemia which is basically a restriction of blood. Atherosclerosis leads to the obstruction of peripheral arteries, heart failure due to blood congestion, heart attack, and even strokes. The link between bacteria and atherosclerosis has been studied at a very basic level, with negligible attention given to the fact that bacteria have the potential to form biofilms within arterial plaques. Lanter and his team established that bacteria can generate from the deposits within carotid arterial plaques. It was established that *Pseudomonas aeruginosa* biofilms were stimulated in vitro and experieced a biofilm dispersion response when tested with norepinephrine in the presence of transferrin. Dispersion of biofilm was considered to be linked to the liberation of bacterial enzymes into the environment surrounding biofilm micro-colonies. This process allows bacteria to escape the biofilm matrix. Therefore, this study establishes a potential mechanism linking hormonal state and the possibility of suffering strokes or heart attacks (Lanter et al. 2014).

10.9 Cardiovascular Implantable Electronic Devices

It is very common for cardiovascular implantable electronic devices, that also comprise cardiac resynchronization therapy devices and cardioverter defibrillators, to acquire biofilm infections (Viola and Darouiche 2011; Deva et al. 2013). Skin commensals such as *P. acnes* and *S. aureus* have arisen out of enrichment cultures and sonication. There has been an increased trend in clinical infections of such devices.

10.10 Conclusion

Despite biofilm infections being extremely common and the cause of many fatal infections, we are still at a nascent stage in terms of understanding their precise role in pulmonary and heart infections, particularly those caused by non-typable *Haemophilus influenzae* and mycobacteria. Treatment of such infections is restricted by the antimicrobial activity of the available antifungal and antibacterial drugs. There have been several novel discoveries in terms of materials and drugs connected to effective therapies against biofilms, demonstrating potential both in vitro and in vivo. However, there is considerable work to complete before such therapies find their way into clinics.

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Chapter 11
The Role of Biofilms in Medical Devices and Implants

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Abstract Biofilms assume a vital job in medicinal-related contamination, particularly being identified with the embedding of restorative gadgets like intravascular catheters, urinary catheters, dental inserts, breast implants, and orthopedic inserts. Biofilms are an intricate gathering of microbial cells that have the ability to cling to the exopolysaccharide lattices available on the outside of various medicinal gadgets. Currently, biofilm-related contaminations of therapeutic gadgets represent a challenge to the general wellbeing of patients and antagonistically influence the capacity of gadgets. Medicinal inserts that are utilized in oral and orthopedic medical procedures are created utilizing amalgams including hardened steel and titanium. During the last decades, strong efforts have been made to improve osteointegration and prevent bacterial adhesion to these surfaces. The embedding of therapeutics on surface medical structures by different physical and synthetic procedures is designed to improve their surface properties, that is, to encourage bio-combination and furthermore counteract bacterial bonding. Biofilms have great importance for public health because of their role in certain infectious diseases and importance in a variety of device-related infections.

Keywords Biofilms · Bacterial infections · Medical devices · Implants · Healthcare-associated infections

11.1 Introduction

A biofilm is a network of microscopic organisms appended to a substratum or surface. Microscopic organisms in biofilms are inserted in an extracellular polymeric lattice of their own creation. Microbes create biofilms on submerged surfaces, for

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example, common amphibian frameworks, living tissue, and the surfaces of teeth, as well as inhabiting therapeutic gadgets and inserts (Donlan 2002). Biofilms that develop on restorative instruments have become both a human wellbeing and financial issue (Francolini and Donelli 2010; Sousa et al. 2011). Therapeutic gadgets, including sutures, catheters, heart valves, vascular unions, orthopedic inserts, and intrauterine devices are inclined to encourage the development of biofilms (Costerton et al. 1999), prompting noteworthy danger in terms of contamination and patient outcomes. Healthcare-associated infections (HCAIs) can occur in homes or clinics (van Kleef et al. 2013). Therapeutic gadget-related contaminations represent a significant financial burden and are related to an expansion of infections and mortality (Donlan 2008). The most common HCAIs include ventilator-associated pneumonia (VAP) and lower respiratory tract diseases and catheter-related urinary tract contaminations. For the most part, the microorganisms associated with HCAIs include Gram-positive microbes, for example, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Enterococcus faecalis*; Gram-negative microscopic organisms including *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*; and yeasts (Donlan 2001). It is the development of these microorganisms inside biofilms that forms the basis of one particular test for treating HCAIs linked to biofilms offering protection against antimicrobial treatments. Biofilms are networks of microorganisms that can connect to both abiotic and biotic surfaces and have consequently been linked to the improved treatment of diseases associated with wounds, non-healing wounds, and restorative gadget-related contamination (Percival et al. 2012; Seth et al. 2012; Vinh and Embil 2005). One noteworthy component of a biofilm is its self-generated extracellular polymeric substances (EPSs) (Lindsay and von Holy 2006). These, for the most part, comprise of polysaccharides, nucleic acids, and proteins which help to shield microorganisms from external dangers, including invulnerable framework segments and antimicrobials. The association between biofilms and medicinal gadget-related diseases was initially perceived in 1972 (Johanson et al. 1972)—biofilms being generally connected with a wide range of polymeric therapeutic gadgets, for example, catheters and cardiovascular pacemakers (Hall-Stoodley et al. 2004; Marrie et al. 1982; Peters et al. 1982). The increase in biofilm-related contamination—caused by the use of restorative gadgets in medicinal services—saw the ascent of the term "polymer related disease." HCAIs happen because of contamination by various, normally microscopic, organisms. The growth of parasites and infections are the most common type of HCAIs. However the emergency clinical 'superbug' meticillin-resistant S. *aureus* (MRSA) is a typical reason for septicaemia in clinical settings.

11.2 Mechanism of Biofilm Formation

The development of biofilms by bacteria on surfaces begins when free-drifting, planktonic microscopic organisms bond with surfaces and collect into small gatherings of microbes known as small-scale provinces. The connection procedure of cells to a

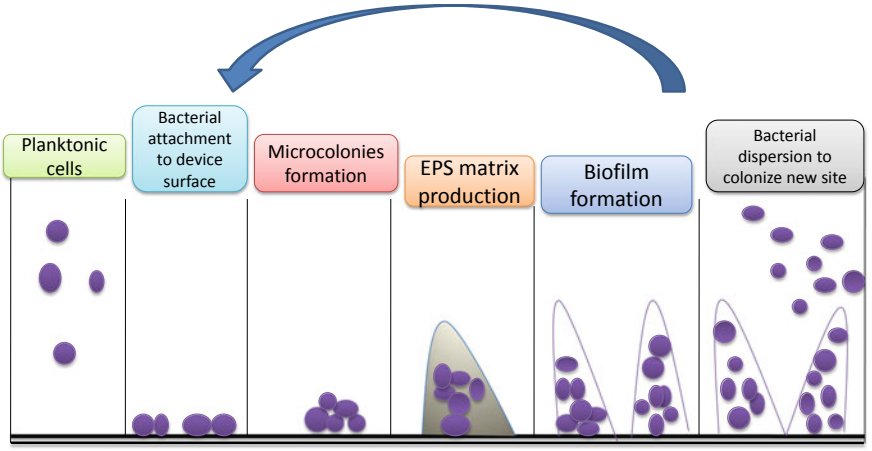

Fig. 11.1 Diagrammatic representation of microbial biofilm formation

surface enacts qualities that are required for the amalgamation of extracellular polymeric substances (EPS) involving polysaccharides and different biopolymers such as proteins, glycoproteins, glycolipids, and extracellular DNA (Flemming et al. 2007). Cells then have the inclination to deliver and implant themselves in such an EPS grid—that acts to shield them from the cells and humoral-resistant responses of the host (Costerton et al. 1999). As the biofilm develops, the undifferentiated connected microbes separate into phenotypes that are significantly extraordinary to planktonic cells (Stoodley et al. 2002). The procedure of separation is activated by amassing *N*-acylhomoserine lactones that the individual bacterial cells produce in the lattice of atoms found in EPSs. They secrete things that allow them to stick to a surface & are encase in a hard/crunchy matrix composed of many different things including DNA, proteins, lipids, polysaccharides, dead cells. The development of biofilms commonly takes 1–2 weeks after colonization and is first noticeable as a foul superficial covering. A schematic representation of the formation of a biofilm is shown in Fig. 11.1.

11.3 Prevention and Control of Biofilms

A few methodologies involve preventing microbial cells attaching to surfaces, hence preventing the development of biofilms (Francolini and Donelli 2010; Sousa et al. 2011). Similarly, there are a few strategies that control the development of biofilms on the surfaces of medicinal gadgets.

11.3.1 Cell Repellent and Non-adhesive Coatings

A few materials like silicon are utilized in the development of urinary catheters and contact lenses. However, cells can promptly cling to surfaces of hydrophobic materials, for example, polydimethylsiloxane (PDMS) elastomers, because of the impact of van der Waals interactions and hydrophobicity. The functionalization of gadget surfaces with self-assembled monolayers (SAMs), polymer brushes, and polymer coatings is a profitable and successful methodology for forestalling cell bonding on these surfaces (Hou et al. 2007; Raad et al. 2008).

11.3.2 The Active Release of Antimicrobial Compounds and Biofilm Inhibitors

Coatings that effectively discharge antimicrobial mixes or biofilm-inhibitory mixes can be utilized to avert biofilm development and gadget-related diseases in patients (Wenderska et al. 2011; Worthington et al. 2012). Such coatings comprise PDMS elastomers and ceragenin—a cholic corrosive inferred antimicrobial operator that has a quick, expansive range, and a nonspecific strategy for assault on bacterial cell films (Epand et al. 2010).

11.3.3 Antimicrobial Coatings with Tethered Biocides

The coatings comprising certain cationic mixes, in a similar manner to polymers, anticipate biofilm arrangement by killing or hindering microorganisms after their adherence to a surface. Their mode of operation is for the most part connected to changes in film porosity or layer disturbances in cells (Gottenbos et al. 2002).

11.3.4 Competitive Adherence by Benign Organisms

Coatings that consolidate antimicrobial peptides (AMPs) can prevent biofilm development on the surfaces of various restorative gadgets (Bahar and Ren 2013).

11.4 Biofilms and Healthcare-Associated Infections

Tainting of restorative gadgets for the most part happens as a consequence of a few microorganisms that move to a gadget from the skin of patients or medical staff, polluted water, or numerous other external ecological sources (von Eiff et al. 2005). A

wide range of microorganisms have been ensnared within therapeutic gadget-related contaminations—of which *S. epidermidis* and *S. aureus* are most regularly connected with biofilms and are generally referenced as causes of HCAIs (Gotz 2002; von Eiff et al. 2005; Vuong et al. 2004). According to past investigations, roughly 80% of the microorganisms engaged in material-related contaminations are *S. epidermidis*. Most of them are multidrug resistant isolates, which is one of the greatest challenges in clinical practice. Multidrug resistance is amongst the top three threats to global public health and is usually caused by excessive drug usage or prescription, inappropriate use of antimicrobials, and substandard pharmaceuticals. These species are regularly identified as the cause of biofilm-based HCAIs, including catheter-associated urinary tract infection (CAUTI). In addition, several biofilm-forming bacteria can be found in different medical devices (Table 11.1).

11.4.1 Central Venous Catheters

Focal venous catheters are used to convey liquids, medicines, blood components, or drugs, and are further used in dialysis treatments (Donlan 2008; Percival and Kite 2007). Both the external parts of the catheter and catheter lumen can become sullied and thereby offer opportunities for biofilm arrangements—the length of catheter in situ affecting areal extent and level of colonization (Donlan 2008). It has been documented that within the initial 7-day period after catheterization, extraluminal biofilm is considered a significant reason for catheter-related circulation system contaminations. In actual fact, vascular catheters that had been in situ for more than 30 days showed proof of heavy luminal colonization and biofilm development (Raad et al. 1993). Consequently, patients who require the utilization of such gadgets for intravenous access over long periods of time, for example, bone marrow transplant patients, may indeed face the very real danger of circulatory system contamination (Donlan 2001). It has also been noticed that catheter colonization and biofilm development in focal venous catheters happens rapidly.

11.4.2 Urinary Catheters

Urinary catheters are cylindrical latex or silicone gadgets that are utilized to quantify urine yield and furthermore to gather urine during medical procedures, counteracting urine maintenance and controlling urinary incontinence. For patients, associated dangers increase by roughly 10% each day after catheterization. Biofilms can promptly occur on both the internal and external surfaces of urinary catheters (Donlan 2001), and rising colonization cannot be prevented by cleanliness measures alone. In anticipation of such issues, it is important that clinicians only use catheters when absolutely essential and for limited periods of time (Talsma 2007).

Table 11.1 Biofilm-producing microorganism found in medical implants/devices

S. no.	Medical implant/device	Biofilm-producing microorganism	References
1.	Artificial voice prostheses	*Candida albicans*, *Streptococcus mitis*, *Streptococcus salivarius*, *Rothia dentocariosa*, *Candida tropicalis*, *Streptococcus sobrinus*, *Staphylococcus epidermidis*	Bryers (2008), Rodrigues et al. (2007)
2.	Cardiac pacemakers	*S. aureus*	Darouiche (2001)
3.	Central venous catheters	*S. epidermidis*, *S. aureus*, *E. faecalis*, *K. pneumoniae*, *P. aeruginosa*, *C. albicans*	Bryers (2008), Darouiche (2001), Rodrigues et al. (2007)
4.	Central-line-associated septicaemia	*C. albicans*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. epidermidis*	Douglas (2003), Pannanusorn et al. (2013)
5.	Cerebrospinal fluid shunts	*S. aureus*, *S. epidermidis*, *Enterococcus*	Darouiche (2001)
6.	Contact lenses	*P. aeruginosa* and Gram-positive cocci	Bryers (2008), Darouiche (2001), Rodrigues et al. (2007)
7.	Dental implants	Acidogenic Gram-positive cocci (e.g., *Streptococcus*), Gram-negative anaerobic oral bacteria	Bryers (2008), Darouiche (2001), Rodrigues et al. (2007)
8.	Endotracheal tubes	*S. aureus*, *S. epidermidis*, *C. albicans*, *P. aeruginosa*	Darouiche (2001)
9.	Orthopaedic implants	*Hemolytic streptococci*, *Enterococci*, *P. mirabilis*, *Bacteroides* sp., *P. aeruginosa*, *E. coli*	Rodrigues et al. (2007)
10.	Peritoneal dialysis catheters	*Streptococci*, *Staphylococci*	Bryers (2008), Darouiche (2001), Rodrigues et al. (2007)
11.	Prosthetic heart valves	*Streptococcus viridans*, coagulase-negative *Staphylococci*, *enterococci*, *S. aureus*	Rodrigues et al. (2007)
12.	Replacement joints	*S. aureus* and *S. epidermidis*	Bryers (2008)
13.	Surgical wounds, prostheses-related infections	*Candida*, *E. coli*, *Staphylococcus* spp., MRSA, *P. aeruginosa*, *S. aureus*, *S. epidermidis*	Douglas (2003), Edmiston et al. (2013), Kathju et al. (2009)
14.	Urinary catheters	*S. epidermidis*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *P. mirabilis*	Bryers (2008), Darouiche (2001), Rodrigues et al. (2007)

11.4.3 Ventilator-Associated Pneumonia and Endotracheal Tubes

Ventilator-associated pneumonia has been recorded as pervasive after 48–72 h in patients who have been intubated and are on mechanical ventilation. Diagnosing ventilator-associated pneumonia (VAP) requires a high clinical suspicion combined with bedside examination, radiographic examination, and microbiologic analysis of respiratory secretions. Aggressive surveillance is vital in understanding local factors leading to VAP and the microbiologic milieu of a given unit. The increased danger of triggering VAP following intubation with mechanical ventilation is 6–20 fold. Mortality rates are 24–76%, that is, fundamentally higher than death rates for urinary tract and skin diseases (Chastre and Fagon 2002; Craven and Hjalmarson 2010). Endotracheal tubes (ETTs) are often associated with the development of biofilms and the multidrug-safe bacterium MRSA and Gram-negative bacilli, such as, *K. pneumoniae*, *E. coli*, *P. aeruginosa*, and *Acinetobacter* spp. (Bauer et al. 2002; Inglis et al. 1989).

11.4.4 Surgical Site Infection

Surgical site infections (SSIs) are contaminations that happen following surgery (Graves 2004). These SSIs can occur due to the sullying of an injury by microscopic organisms from a patient's skin. The most common organism associated with SSIs is *S. aureus*—ordinarily found in typical skin. Kathju et al., during examinations, showed that by confocal microscopy the similarity of bacilli and cocci inside biofilms on explanted sutures taken from a perpetual SSI. Further examination utilizing fluorescence in situ hybridization identified parts of biofilms containing *Staphylococcus*—confirmed using a *Staphylococcus*-explicit test (Kathju et al. 2009). Ongoing investigations have identified similarities between biofilms found on two unique types of suture—absorbable and non-absorbable—from tainted and non-contaminated injuries.

11.4.5 Mechanical Heart Valves

A few microorganisms may join and create biofilms on various segments of mechanical heart valves and furthermore in the encompassing tissues of the heart, prompting a condition known as prosthetic valve endocarditis. The microorganisms associated with prosthetic valve endocarditis are *S. epidermidis*, *S. aureus*, *Streptococcus* spp., Gram-negative bacilli, diphtheroids, enterococci, and *Candida* spp. These may originate from skin or from associated medical gadgets, for example, focal venous catheters or from dental work.

11.4.6 Contact Lenses

There are various kinds of polymeric contact lens materials created to avoid biofilm development. Biofilms of specific species, for example, *Candida*, *P. aeruginosa*, and *Fusarium*, are impervious to biocides found in standard contact lens arrangements, however they are likewise defenseless to hydrogen peroxide (Szczotka-Flynn et al. 2009). Contact lenses produced using hydrogels that discharge ceragenin are apparently equipped to oppose colonization by *P. aeruginosa* and *S. aureus* for a period of several months (Gu et al. 2013).

11.4.7 Orthopedic Implants

A few contamination related to hip embed cuases biofilm infection required substitution of medical procedure that are because of bacterial biofilm arrangement which may cause aggravation and tissue demolition around inserts significantly more quickly than the harm brought about by gum disease (Belibasakis 2014).

11.4.8 Dental Implants

Biofilms located on the surface of teeth are called dental plaque. Microscopic organisms multiplying in the dental plaque are associated with various diseases, for example caries, gum disease, periodontitis, and peri-implantitis. Such microbial assaults represent a significant reason for dental implant failure (Paquette et al. 2006). Periodontal diseases and peri-embedded infections are explicit contaminations initiated by microbial species when the balance between host and microbial pathogenicity becomes unbalance.

11.4.9 Breast Implants

Biofilm diseases of breast implants fundamentally potentiates capsular contracture and anaplastic large cell lymphoma. Past investigations have considered the development of capsular compressions around breast implants as a consequence of subclinical diseases. There are a small number of microscopic organisms that are associated with biofilm advancement in breast implants. One recent study considered how *S. epidermis* created biofilms on the internal surfaces of breast implants while paying little attention to external surfaces (Ramasamy and Lee 2016). In addition, due to their expanded surface area, inserts with rougher surfaces may harbor more prominent biofilm loads than those with smooth surfaces (James et al. 2018).

11.5 Detection and Diagnosis of Bacterial Biofilms on Medical Devices

There are some biofilm-forming bacteria that are connected with several human diseases—some are listed in Table 11.2. The use of traditional culture methods to determine colonization is not indicative of biofilm growth (Hall-Stoodley et al. 2012). There are some barriers that can make successful diagnosis difficult—one being the emergence of small-colony variants (SCVs). These SCVs are a subpopulation of biofilm bacteria that produce small colonies as well as developing resistance to antimicrobial action and evading detection—a consequence of their slow growth rate (Neut et al. 2007). In order to improve the diagnosis of device-related infections different methods, like the sonication of infected implants, may improve culture positivity (Achermann et al. 2010). Several more sophisticated molecular methods of identification, including PCR and fluorescence in situ hybridization, are also being used to identify bacteria in complex biological samples and are proving to be a more accurate means of detection. Several publications and studies have indicated the differences between culture and molecular diagnostic methods (Hall-Stoodley et al. 2006).

Table 11.2 Biofilm-forming bacteria associated with human disease

S. no.	Disease	Biofilm-forming bacteria	References
1.	Bacterial prostatitis	*E. coli* and other Gram-negative bacteria	Mazzoli (2010)
2.	Biliary tract infection	*E. coli* and other enteric bacteria	Urdaneta and Casadesus (2017)
3.	Cystic fibrosis pneumonia	*P. aeruginosa* and *Burkholderia cepacia*	Eberl and Tummler (2004)
4.	Dental caries	*Streptococcus* spp. and other acidogenic Gram-positive cocci	Zhu et al. (2018)
5.	Meloidosis	*Pseudomonas pseudomallei*	Sawasdidoln et al. (2010)
6.	Musculoskeletal infections	*Staphylococci* and other Gram-positive cocci	Otto (2008)
7.	Necrotizing fasciitis	Group A streptococci	Siemens et al. (2016)
8.	Otitis media	*Haemophilus influenzae*	Van Hoecke et al. (2016)
9.	Periodontitis	Gram-negative anaerobic oral bacteria	Larsen and Fiehn (2017)
10.	Urinary catheter cystitis	*E. coli* and other Gram-negative rods	Jacobsen et al. (2008)

11.6 Preventive Measures for Biofilm Control and Future Perspectives

Research has centered upon various complex techniques for sanitization and the alteration of therapeutic gadgets to avoid microbial development and biofilm arrangement. The development of antimicrobials attached to the outside of medicinal gadgets like catheters incorporates connection of a flimsy film on the outside of catheters, that is, bound to their surface, or attached to their surfaces within a polymer lattice. Various elements impact the viability of catheters. Their method of treatment with antimicrobial agents, including solvency, hydrophilicity, and fondness to penetrate tissue are for the most part factors that influence their ability to fight against infection. The utilization of bioactive atoms and catalysts is a novel methodology used as an anticipatory action against biofilm development on embedded materials. In one investigation, Ren and colleagues utilized a counterfeit biofilm model to evaluate different cleansers for their capacity to evacuate *E. coli* from adaptable endoscopes. This examination underscored that increasingly bacterial biofilms are discovered utilizing enzymic cleanser treatments rather than non-enzymic cleanser treatments (Ren et al. 2013). In an ongoing examination by Gawande and colleagues the adequacy of a normally occurring protein, combined with a gel, is being assessed with respect to constant injury-related microorganisms (Gawande et al. 2014). The diverse methodologies for preventing biofilm development on therapeutic gadgets are provided in Fig. 11.2.

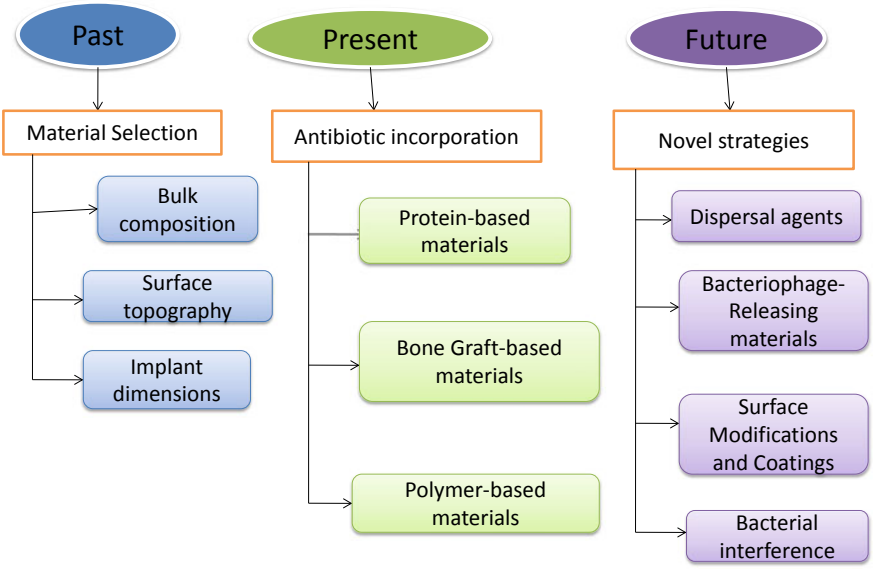

Fig. 11.2 Strategies for prevention of biofilm on implantable materials

Future research should expand our understanding of microbial biofilms and their cooperation with biotic and abiotic surfaces and furthermore build up conceivable control systems including the utilization of antimicrobial-treated therapeutic gadgets and locks for biofilm avoidance and control. A perfect inhabiting therapeutic gadget should have surfaces that are similar to those found in the human body, providing no more hospitable surfaces and thereby anticipating and preventing contamination. To accomplish biocompatibility, the outside of restorative gadgets ought to be smooth and uniform to permit the development of solid tissue and the avoidance of pathogens. The utilization of infection causing agents, taking the surface physico-substance properties of the therapeutic gadgets is the key factors which lead to medicinal gadgets pre-treated with antimicrobials.

In the future, to better comprehend and control biofilms inhabiting medicinal gadgets, science must pursue advancements in several areas. A few solid procedures for gathering and estimating biofilms need to be created. For instance, for focal venous catheters, the reference strategy for measurement of biofilms on catheter tips is the move plate method, where the tip of the catheter is expelled and moved over the outside of a non-selective medium. The procedure used to measure biofilms relies upon the quantity of microorganisms recouped by contact with an agar surface.

11.7 Conclusion

Biofilms are critical to the time patients spend in hospital as well as to several different diseases. This is not just because of their capacity to represent a place of refuge for microorganisms but additionally because of their innate resilience and "opposition" to antimicrobials. Analysis of biofilm-related diseases raises grave concerns that infectious diseases develop more easily on abiotic surfaces, following evacuation of a medicinal gadget, than on biotic surfaces. The pathogenesis of gadget-related diseases identifies with microbes that connect to and develop on surfaces in complex networks. Biofilm microscopic organisms are becoming progressively impervious to antimicrobial operators. A few novel demonstrative methodologies coordinated towards recognizing biofilm microbes have been created. Similarly, imaginative ways of dealing with aversion and treatment incorporate the utilization of antisense particles, majority-detecting inhibitors, bacteriophages that hydrolyze biofilm grids, and bacterial impedance. Ultrasound waves in combination with gentamycin entrapped in bone cements has been shown to prevent 70% of biofilm formation in a rabbit model.

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Conflict of Interest None

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Chapter 12
Biofilm-mediated Gastrointestinal Diseases

Satish K. Nayak

Abstract The gastrointestinal tract is a unique organ system in the human body that communicates with both external and internal environments. The microbes in the gastrointestinal tract exist both in a planktonic form free in the lumen and as part of biofilm attached to the epithelium. Biofilms have been implicated in pathogenesis of many GI diseases like Barret's esophagus, malignancies of the esophagus stomach and colon, inflammatory bowel disease, infectious diarrhea, irritable bowel syndrome, etc. Not only do they contribute to the pathogenesis of these diseases but also hamper with treatment by inducing resistance or by acting as a barrier to the host immune system and antimicrobials. This chapter gives a brief overview of role of biofilm in common GI diseases, its implication in pathogenesis, diagnosis, and treatment.

Keywords Gastrointestinal tract · Gastroesophageal reflux disease · Irritable bowel syndrome · Carcinoma colon

12.1 Introduction

The gastrointestinal tract is a unique organ system in the human body that communicates with both external and internal environments. Presence of microorganisms in certain parts of this organ system is considered normal. Human Gastrointestinal tract is colonized by a large number of microbes—about 40 trillion microbes of more than 1000 species (Sender et al. 2016). The density of microbial colonization depends on the specific part but, in general, it increases from cephalad (esophagus) to caudad (colon). The interaction between the microbiome and the host would be most in the gastrointestinal tract not just because of the high number but also because of balanced interaction with the host immune system. The phenomenon of immune tolerance, autoimmunity, and many of the systemic disorders are directly or indirectly contributed by these host–microbiome interactions.

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The microbes in the gastrointestinal tract exist both in a planktonic form free in the lumen and as part of biofilm attached to the epithelium. Due to difficulty in sampling, earlier studies were more concentrated on the luminal microbes. Recently, there has been more interest in the biofilm as it is found to be more closely associated with the epithelium and immune system and found to be causing or contributing to the pathogenesis of GI diseases. Mixed species biofilms are mostly pathogenic in nature and have been observed in esophageal, gastric and intestinal diseases, and colonic malignancies. Apart from appendix, most site of GI tract may not harbor polymicrobial biofilm in health. The biofilm gives microbes protection against antimicrobials and antibiotics, hence promoting their survival. It may also promote synergy between host and the consortia helping in nutrient digestion, nutrient processing and acting as an immune barrier. Specific markers and targets are being developed to understand and treat these biofilm-mediated diseases.

Abnormalities within the GI tract are easily amenable to treatment using endoscopic techniques. The instruments used here can potentially harbor biofilms and act as a nidus for microbes.

Normal GI defense mechanisms prevent microbial adherence to the GI surface and hence the formation of biofilm. However, thin biofilms are seen normally is some parts of the gut, especially the colon and presence of polymicrobial biofilm may be normal in the appendix. It possibly acts as a reserve for gut microbiota and appendix would aid in reinstating healthy gut flora following infection or antibiotic exposure. More often, microcolonies of bacteria are seen within the gut and these have potential to form biofilms. Factors that prevent biofilm formation are

1. The rapid transit time of the gut.
2. Presence of mucus layer which acts as a physical and chemical barrier between the lumen and epithelium.
3. The rapid turnover time of intestinal epithelium. Intestinal epithelial cells are shed at a rate of 1–3 billion per hour in the small intestine.
4. Constant mixing movement and propulsion of food and water by peristaltic waves.

When there is a loss of these intrinsic defense mechanisms by mucosal disease, or otherwise, it leads to microbial attachment and potential to form a biofilm leading to further disease subsequently. Studies have shown some diseases to have definite associations with biofilms.

12.2 Esophagus

Esophageal microbiota was earlier thought to be predominantly similar to oral microbiota. However, recent studies have shown the esophageal microbiome to be unique. An endoscopic sampling of the esophageal mucosal tissue has shown the presence of biofilm in the lower esophagus the type of which varies between patients with

different esophageal diseases. There are no studies on normal people as most of the sampling was done in patients with GI symptoms.

12.2.1 Gastroesophageal Reflux Disease and Barret's Esophagus

Gastroesophageal reflux disease is one of the most common GI disorders caused by reflux of gastric contents into the lower esophagus. Loss of anti-reflux barriers with impaired clearance of gastric refluxate from the esophagus has been considered pathogenic mechanisms (Günther et al. 2014). Persistent GERD is a predisposing factor for Barret's esophagus (replacement of esophageal epithelium with metaplastic specialized intestinal epithelium) and esophageal adenocarcinoma. About 6–10% of GERD progress to Barret's esophagus and about 0.5–1% of Barret's progress to EAC (Wheeler and Reed 2012).

One of the first studies by Osias et al. showed colonization of bacteria in the epithelium of Barret's (Osias et al. 2004). They concluded that these are resident bacteria and may play a role in the pathogenesis. Following this, many other studies have tried to look at the esophageal microbiome in these conditions. Difference in the microbiome colonizing the distal esophagus was shown in patients with and without Barret's (Macfarlane et al. 2007). Another study used 16 s ribosomal sequencing and cluster analysis showed two distinct clusters of the esophageal microbiome—type 1 which predominantly consisted gram-positive bacteria, aerobic *streptococci* (mostly belonging to phylum *Firmicutes*) and type II which consisted more of gram-negative species (phylum *Bacteroides*, *Fusobacteria*) (Yang et al. 2009). The type II microbiome was more consistently seen with Esophagitis and Barret's esophagus. Further studies on analysis of esophageal microbiota using culturing techniques showed the presence of about 111 species belonging to 26 genera. There was a shift in the biofilm composition in the patients with GERD and Barret's without any significant changes in overall bacterial counts. Nitrate reducing *Campylobacter consicus* and *Vielonella. Neisseria* and *Fusobacterium* were shown to be present in significantly high levels in these patients suppressing the growth of other bacteria (Blackett et al. 2013; Norder Grusell et al. 2018). However, aerobic streptococci and other bacteria still persisted in these biofilms.

Production of N-nitroso compounds by nitrate-reducing bacteria may play a role in carcinogenesis (Macfarlane and Dillon 2007). High levels were demonstrated at the site of Barret's and esophageal adenocarcinoma compared to GERD. There was overexpression if IL-18 in these tissues. IL-18 is associated with tumor proliferation, local invasion, and metastasis in tumors of stomach and breast (Pages et al. 2000). Its significance has not been clearly demonstrated in the esophageal lesion.

12.2.2 Carcinoma of the Esophagus

The incidence of esophageal carcinoma is gradually increasing and is the sixth most common cause of cancer-related death worldwide and eighth most commonly diagnosed cancer (Parkin et al. 2002). Squamous cell carcinoma and adenocarcinoma are the two common subtypes. Adenocarcinoma is associated with premalignant lesions like Barret's esophagus. Characterization of the microbiome in the cancer tissue of about 20 patients undergoing resection showed the presence of *Streptococcus mitis*, *Streptococcus anginosus*, and *Treponema denticola* (Narikiyo et al. 2004). Esophageal microbiome with a predominance of gram-negative bacteria mainly campylobacter consicus has been shown in foci of Barret's with dysplasia, thereby supporting the role in carcinogenesis.

Another study has shown increasing levels of *Fusobacrerium nucleatum* in esophageal carcinoma. Resected specimen of 325 esophageal carcinomas was analyzed for the presence of *F. nucleatum*. It was positive in about 23% of cases. Its presence has been associated with poor prognosis, rapid progression, and overall survival. *Fusobacterium* was found to be closely associated with the esophageal epithelium causing oncogenesis via high cytokine levels and immune inhibition via T cell suppression (Yamamura et al. 2016). Superficial layers of the tumor showed high levels of *F. nucleatum* DNA in comparison with the deeper layers. The oncogenesis is probably due to the activation of chemokine CCL 20 within the tissue which may contribute to aggressive tumor behavior (Baba et al. 2017). Further studies are needed to demonstrate the biofilm formation and its role in tumorigenesis.

12.3 Stomach

A highly acidic environment in the stomach inhibits the growth of microbes in the stomach. Very few organisms adapt and survive this microenvironment with the best example being *Helicobacter pylori*.

12.3.1 Helicobacter pylori *Infection*

H. pylori is one of the most common infections worldwide involving more than half of the global population. It is implicated in the pathogenesis of gastric ulcer, duodenal ulcer, and gastric cancer. It colonizes the gastric mucosal layer and the gastric glands and survives for long periods of time unless treated (Carron et al. 2006). The survival in this harsh acidic environment is due to various mechanisms including urease production, motility, and immune evasion properties. Formation of biofilm has been recently studied and it is supposed to be one of the survival factors for *H. pylori.*

Initial studies on *H. pylori* were on the free-living planktonic forms but recent studies demonstrated these organisms a part of biofilms both in vitro and in vivo studies. Biofilm of *H. pylori* has also been demonstrated in vegetable and nongastric in vivo environment suggesting that these may act as reservoirs of infection (Ng et al. 2017).

H. pylori strains from clinical, laboratory, and mouse-adapted strains have shown to produce biofilms in vitro environments. Two dominant morphological forms have been observed in *H. pylori* on analysis using scanning electronic microscopy—a bacillary form and a coccoid form. The coccoid form is considered viable but non-culturable. It is found to be associated with stressors like antimicrobials, starvation, and prolonged culture. As *H. pylori* is a fastidious organism which thrives in the microaerophilic condition, the bacterial morphology depends on the environment.

Biofilm-associated *H. pylori* have also been demonstrated in vivo studies both in mouse and human models. Biopsy specimens analyzed using scanning electron microscopy showed a thick layer of bacteria involving the mucosa and gastric glands, and these were not present in noninfected specimens.

Genomic, transcriptomics, and proteomics-based strategies have been used to study *H. pylori* biofilms. Many genes including flagellar protein, outer membrane protein, and Cag pathogenicity island are associated with biofilm formation. Mutations in CAG showed lesser biofilm formation (De La Cruz et al. 2017). Using proteomic analysis, 35 different proteins were shown to have different expressions between free-living and biofilm-associated *H. pylori* (Shao et al. 2013). These proteins were associated with virulence, motility, and signaling. This variation suggests strongly that the protein expression is significantly different between the two forms and can have bearing on the chronicity of the infection.

The implication of *H. pylori* biofilm:

a. Antibiotic resistance: Bacterial biofilms are known to be resistant to antibiotics due to an additional layer of protection due to the matrix. Bacteria in the biofilm also resist antimicrobials by mechanisms like tolerance which may be transient and nonheritable. Of more importance is the transfer of genetic material between the subpopulations within the biofilm which may lead to newer mutations and make the bacteria less susceptible to the drugs. Studies have shown that biofilm-associated *H. pylori* were more resistant to clarithromycin with MIC more than 16 folds and MBC more than fourfolds when compared to the planktonic state (Yonezawa et al. 2013).
b. Immune evasion: The biofilm matrix component called proteomannan has immune-modulating properties. It was shown to inhibit T and B cell responses and promote mast cell degranulation. These mediators cause breakdown of mucosal layer and induction of Treg cells which further suppress T cell (Harris et al. 2008).
c. Therapeutics: Targeting the biofilm matrix may be a novel approach in *H. pylori* treatment. *N*-acetylcysteine is a mucolytic which acts by breaking thiol bonds in the glycopeptides and thereby destabilizing the biofilm matrix. Studies have

shown that pretreatment or combination of NAC with *H. pylori* eradication therapies significantly decreased the bacterial load and increased clearance rates (Hyunh et al. 2004).

12.4 Intestines

12.4.1 Foodborne Bacterial Disease and Biofilm

Acute infectious gastroenteritis due to foodborne bacterial pathogens is common infectious disease in developing countries. *Salmonella*, *Escherichia coli*, *Listeria,* and *staphylococcus* are important causes, and all these are known to form biofilm both in biologic and inert surfaces. Interspecies and intraspecies interactions among these organisms are important in maintaining the biofilm which provides these organisms a survival advantage and makes them more pathogenic (Giaouris et al. 2015). Biofilms are associated with persistence of foodborne pathogens (Bridier et al. 2015). Studies to decipher the intra- and interspecies interactions in these biofilms are crucial to develop newer strategies to control foodborne infections and epidemics.

Salmonellosis: Non-typhoid salmonella causes acute and persistent diarrhea. *S. typhimurium* and *S. enteritidis* are most prevalent. Biofilm formation in these organisms is characterized by the expression of curli-fimbriae and cellulose. Bacteria deficient in these failed to produce thick biofilms. S*almonella* sp. biofilms are also known to overgrow and replace biofilms produced by other species and thereby increasing the virulence.

Enteroaggragative *E. coli*: Out of all *E. coli* strain enteroaggregative *E. coli* is more common producer of biofilm due to its aggregative property and expression of certain proteins like Ag43, type 1 pili, and curli. The expression of these proteins is seen in biofilm-forming bacteria which is important in intraspecies interaction. EAEC is one of the common causes of foodborne diarrhea in infants and immunosuppressed patients (Huang et al. 2006). Human strains were shown to produce more biofilm compared to animal strains and those in innate objects (Vijay et al. 2015). Studies have shown that *Lactobacillus casei* strains inhibit biofilm formation in EAEC and make them more susceptible to eradication by antimicrobials (Andrzejewska and Sobieszczańska 2013).

12.4.2 Clostridium Difficile

Clostridium difficile has been a cause of nosocomial diarrhea and cause of diarrheal outbreaks in hospital severe form of which is known as pseudomembranous colitis. It is a spore-forming anaerobe with capacity to produce robust biofilm within the colonic mucosa. People with unstable colonic microbiome like elderly and those

exposed to multiple antibiotics are at risk for colonization by *C. difficle* (Smits et al. 2016). Once colonization occurs many factors like intraspecies and interspecies interactions, formation of multispecies biofilm, possible interaction with fungi or bacteriophages lead to pathogenesis. The toxins A and B from the bacteria are the main pathogenic mediators and cause extensive tissue damage.

The role of replenishing the host–microbiome using fecal microbiota transplant in the management of C *difficile* is extensively studied and is treatment of choice in recurrent C. difficile infection.

12.4.3 Inflammatory Bowel Disease

Crohns disease and ulcerative colitis are the two most common forms of inflammatory bowel disease. Ulcerative colitis involves colonic mucosa and occasionally terminal ileum. Crohns disease involves any part of the GI but predominantly the intestines. It can involve layers of the bowel wall and cause complications like strictures and fistulae. Microbial involvement has been studied in the pathogenesis of IBD and studies looking at the gut flora composition have shown reduced diversity of microbiome in these compared to normal controls. Further there has been a significant difference in microbiome between the two conditions. However, all these were estimation of the luminal microbiota and study of mucosal biofilm in these disease has gained importance only recently.

Ulcerative colitis:

- Inflammation of the colonic mucosa of unclear etiology.
- Infectious origin of the disease has been proposed previously and organisms including *shigella*, *E. coli*, and *fusobacterium* have been isolated from the diseased mucosa. None have been conclusively proven.
- Microbial biofilms have close associations with the intestinal mucosa and modulate the immune response.
- Mucosal destruction, crypt inflammation, cytokine release, and chemotaxis play a role in the pathogenesis of the disease.
- Specific bacteria have not been identified in these mucosal layers, however, the usual biodiversity is not seen as compared to patients with IBS or normal subjects.
- Use of pre-probiotics or symbiotics in these patients has demonstrated repopulation of the mucosal layer with *Bifidobacterium longus* and significant reduction in the cryptitis and cytokine level suggesting a definite role of mucosal microbiota in the pathogenesis.
- Ulcerative pouchitis (inflammation of the neorectum after total colectomy) has been managed/prevented by using high-dose probiotic mixture for 3–4 weeks (Holubar et al. 2010).

Crohns disease:

- Crohns disease can involve any part of the GI, however, Ileocolonic followed by small bowel involvement are common presentations.

- Gut microbiome changes in Crohns disease are not well studied. *E. coli*, *Mycobacterium paratuberculosis,* and fungi *Saccharomyces cerevisiae* have been found in various to be contributing to the pathogenesis.
- Markers like anti-sacromyces cervisiae antibody (ASCA), omp-C, cbir-1, AMCA, ACCA which are antibodies against microbes have been used as prognostic markers as well as severity markers in Crohns disease. (Paap M et al. 2008)

12.4.4 Irritable Bowel Syndrome

Alteration in the gut flora and bacterial overgrowth has been the most studied mechanisms for pathogenesis of irritable bowel syndrome. Microbiome exists as mucosa associated biofilm layer within the small bowel and modulates the small bowel barrier function, permeability, immune mechanisms, and local reflexes. This results in a altered sensitivity, change in the mucosal permeability, and intestinal motility producing the symptoms of pain, diarrhea, or constipation which characterize irritable bowel syndrome.

Following an episode of acute infective gastroenteritis, there is a change in the composition of the gut microbiome and possibly within the mucosal biofilm which can lead to alteration in the local homeostasis causing post-infectious IBS. Infection with *Giardia duodenales* has shown to modulate human gut biofilm and possible to play a role in post-infection IBS (Beatty J K 2017).

12.4.5 Colorectal Malignancy

Formation of colonic biofilm has been connected to the initiation and progression of colorectal malignancies. This has been more consistently found in proximal colonic malignancies. (Dejea, C. M et al. 2014) A multispecies biofilm is seen more often than a solitary organism. This results in a more orchestrated and significant cellular response leading to genotoxicity and possibly oncogenesis (Dejea, C. M et al. 2014).

The “keystone-pathogen hypothesis” or the “alpha-bug hypothesis” is based on the studies with enterotoxigenic *Bacteroides fragilis*(Hajishengallis, G et al. 2012). This initiates production of endotoxins that can cause DNA damage, Th 17 dependent inflammatory response, and proliferation of the precancerous lesion.

This is complemented by the driver–passenger hypothesis where alpha bug (ETBF) being the driver which is gradually replaced by opportunistic pathogens (passenger) which outnumber the driver and result in further progression to CRC. *Fusobacterium* dominated biofilms in the colon of patients with CRC support this hypothesis and also suggest that the formation of biofilm may be the key step in the driver–passenger model of colonic cancer (Fearon, E. R., & Vogelstein, B. 1990).

Bacterial biofilms may not be carcinogenic, but the organisms it harbors like Fusobacterium may contribute to the colorectal cancers.

Biofilms from the left colon of healthy people showed the presence of *Bacteroidetes* and *lachnospiraciae* with the conspicuous absence of *Fusobacterium*. Bacterial biofilms can contribute to increasing intestinal permeability and loss of barrier function loss which may be an early step in colon carcinogenesis. This has been supported by the evidence that bacterial invasion is found in all colorectal tumors associated with biofilm and absent in tumors not associated with biofilm. Scanning electron microscopic analysis has shown dense multibacterial biofilms in all right-sided colonic tumors but very few in the left side. Right-sided CRC has a worse outcome than left-sided colon cancers which may be explained with the above phenomenon.

Development of preventive strategies to detect and inhibit such biofilm formation may be useful screening and preventive tool in CRC. Specific drugs targeting the intestinal microbiome which can help to modify the components of the biofilm may be used as a therapeutic target in CRC prevention.

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Chapter 13
Biofilm-Mediated Urinary Tract Infections

Jyotsna Agarwal and Shruti Radera

Abstract Biofilm-forming bacteria may involve approximately in 80% of all infections with urinary tract being one of the main areas where biofilm can become a serious threat. Biofilm plays a major role in causing catheter-associated UTIs and recurrent UTIs. Recurrent UTIs can be categorized as relapse (if all episodes of infections are caused by the same microorganism) and reinfection (if the episodes are caused by different microorganisms). Relapses may be due to biofilm-forming capacity of the microorganisms and involve protected, intracellular bacterial reservoir in bladder mucosa. Urinary catheters allow entry of microorganism, usually the commensal perineal flora into the urinary tract, causing catheter-associated UTI (CAUTIs). CAUTIs account for 40% of all nosocomial infections. Biofilms not only play an important role in CAUTIs but also cause blockage of catheter. Urinary catheter encrustation is another problem of concern as it can cause blockage of catheter, leading to urine retention, which is a painful medical emergency. The organisms most often contaminating these devices and developing biofilms are *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus epidermidis, Enterococcus faecalis*, etc. Biofilms provide high resistance to antibiotics. Several studies have recommended for combination therapy rather than using single antibiotics, with macrolides being the first choice. Another way to use antimicrobials is to impregnate catheters with these antimicrobials agents. This may restrict bacteria to attach themselves to catheter surface and further development of biofilm. Another approach is to find out new therapeutic options in a way that either biofilm does not form and if forms then it could be treated easily. Some of them are coating of catheter with hydrogels, using nanoparticle or iontophoresis to disrupt biofilm, to use non-pathogenic bacteria so that they can competitively impede with pathogens to establish quorum sensing inhibitors, etc.

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13.1 Infections in Urinary Tract

Urinary tract infections (UTIs) are one of the most important causes of diseases affecting individuals of all ages. It is estimated that nearly one in two women would experience one episode of UTI at some time in their lives (Kunin 1999). Females are affected by UTIs more than males, likely due to their anatomic structure, as shorter female urethra facilitates movement of bacteria from urethra to bladder. Even colonization of vaginal introitus by gut flora may also increase the chances of urinary tract infection (Rosen et al. 2007; Weichhart et al. 2008).

On the basis of anatomical site-affected, UTIs can be classified as cystitis (infection of the lower urinary tract or bladder) and pyelonephritis (infection of upper urinary tract or the kidneys) and prostatitis. UTIs can be categorized clinically as acute uncomplicated cystitis, recurrent cystitis, acute uncomplicated pyelonephritis, complicated UTI (UTI related to indwelling catheters, UTI in men), and asymptomatic bacteriuria.

About 80% of uncomplicated UTIs are caused by *Escherichia coli (E. coli)* (Stamm and Hooton 1993). Complicated UTIs may vary from cystitis to urosepsis with septic shock. Nearly 20% of women with acute cystitis suffer from recurrent UTIs (2 episodes of uncomplicated UTIs in 6 months or 3 infections within a year). Recurrent UTIs can be categorized as relapse (if all episodes of infections are caused by the same microorganism) and reinfection (if the episodes are caused by different microorganisms). Relapses are considered as complicated UTIs. Relapses may be due to biofilm-forming capacity of the microorganisms and involve protected, intracellular bacterial reservoir in bladder mucosa (Soto et al. 2006; Barber et al. 2013).

Asymptomatic bacteriuria (ABU) can be defined as the presence of more than 100,000 CFU bacteria per ml of voided urine without any sign and symptoms of UTI (Ipe et al. 2013). Approximately 6% of healthy individuals and 20% of elderly individuals are present with ABU (Ferrières et al. 2007). Strains causing ABU somehow form biofilm in uroepithelial cells and do not cause any symptomatic disease in the host.

Acute pyelonephritis is considered as an organ/life-threatening infection of renal pelvis and kidney. Acute pyelonephritis may be due to ascending infection where microorganism reaches kidney from lower urinary tract, or it may also be due to hematogenous spread, i.e. microorganism reach to renal parenchyma through blood. Microorganisms which adhere to the uroepithelium and form biofilm can also invade the kidney tissue, thereby causing pyelonephritis and even chronic prostatitis (Nickel et al. 1985).

Acute prostatitis is an unusual genitourinary infection in males and is presented as a febrile UTI (Millán-Rodríguez et al. 2006). Most common causative microorganisms associated with acute prostatitis are *E. coli* (Brede and Shoskes 2011), *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella spp.*, *Enterococcus* spp., and *Serratia* pp (Millán-Rodríguez et al. 2006).

Urinary catheters make a way for the microorganisms usually the commensal perineal flora, to enter into the urinary tract; causing catheter-associated UTI (CAUTIs). CAUTIs account for 40% of all nosocomial infections (Tambyah 2004). Biofilms not only play a major role in CAUTIs but may also cause catheter blockage.

Most of the CAUTIs are caused by *E. coli*, *Proteus, P. aeruginosa*, *Enterococci*, *Candida*, *Klebsiella,* or *Enterobacter* spp. (Tambyah et al. 1999).

13.2 Pathogenesis of Biofilm-Mediated UTIs

Biofilm-forming bacteria may involve approximately in 80% of all infections with urinary tract being one of the main areas where biofilm may become a serious threat (Robino et al. 2013). Biofilm can form in the uroepithelium, prostate calculi, and implanted foreign bodies such as catheter (Tenke et al. 2006).

13.2.1 Role of Biofilms in Recurrent UTIs

Despite anatomically and physiologically normal urinary tract, some young healthy women suffer from recurrent UTIs (Finer and Landau 2004). Approximately 20% women with acute cystitis develop recurrent UTI later on, thereby causing substantial economic burden to the healthcare system.

It has been observed in many studies that most of the microorganisms isolated from urine of patients with relapse infections were capable to produce biofilm "in vitro" (Madersbacher et al. 2000). It has been shown in many studies that uropathogenic *E. coli* (UPEC) isolates associated with recurrent UTI are better biofilm producer than UPEC causing only single episode of infection (Agarwal et al. 2014).

In case of relapse, bacteria can also enter a quiescent state by invading bladder epithelial cells and remain over there in dormant state as membrane-bound cells (Barber et al. 2013) (Fig. 13.1).

Bacteria in these quiescent cells are resistant even to 1000 times the antibiotic concentration, which is effective on their planktonic stage, and bacteria are also inaccessible to host defense system in these compartments (Barber et al. 2013). These quiescent cells may get activated by some environmental triggers such as reorganization of actin filaments during terminal differentiation of bladder epithelial cells which in turn activate the regrowth of bacteria, thereby stimulating the development and dispersal of intracellular bacterial communities and the reappearance of clinical symptoms (Barber et al. 2013).

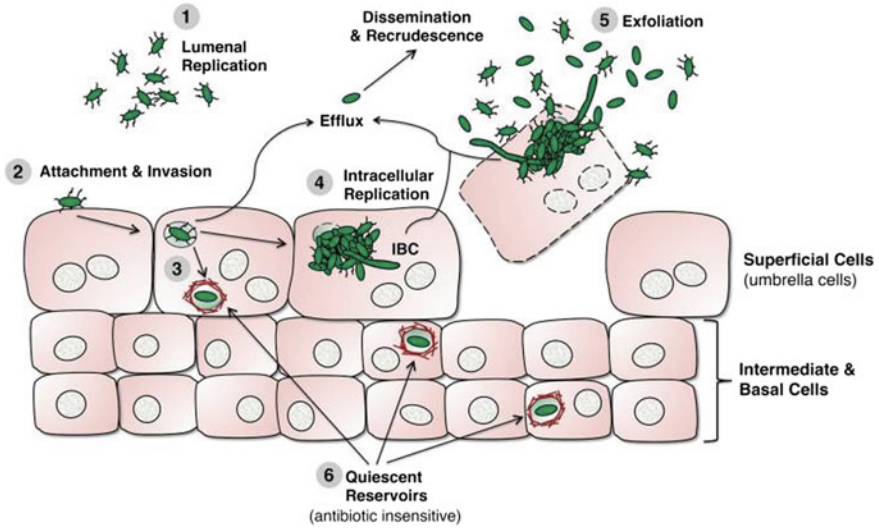

Fig. 13.1 Sequence of events involved in establishment and recurrence of urinary tract infection (UTIs) (reproduced from Barber et al. (2013)). During an episode of UTI, uropathogenic *E. coli* can either multiply within lumen of bladder (1) or may attach to bladder epithelium followed by invasion (2). Following invasion bacteria can either go back to bladder (3) lumen or be engulfed into endosome like compartments (4). Subsequently, these compartments may disrupt leading to release of UPEC in bladder cytosol, thereby leading to rapid intracellular growth of bacteria and formation of intracellular bacterial communities. Within these communities, bacteria can acquire different morphologies such as long filamentous cells which are resistant to host defenses (5). Infection can trigger exfoliation of these bladder cells; disruption of these bladder cells causes elimination of adhered and internalized bacteria, resulting in dissemination of pathogen (6). Some of the bacteria may remain entrapped within endosomes like compartments within bladder epithelial cells, which acts as quiescent reservoir; these compartments are often surrounded by actin filaments (red) and are not easily eradicated by antibiotic treatments. These reservoirs may lead to recrudescence of UTIs later on

13.2.2 Role of Biofilm in ABU

Relatively little is known about the mechanisms, how bacteria responsible for ABU cause bladder colonization. As we know that bacterial adherence is one of the key factors, to make any pathogen capable to cause infection, low abundance of adhesins in bacterial strains associated with ABU could explain why these strains do not cause clinical symptoms in the host; however, it does not explain how these strains are so capable to colonize bladder epithelial cells. However, few studies have demonstrated that the ability to grow fast in urine is one of the possible mechanisms for bladder colonization of ABU *E. coli* (Roos et al. 2006a, b).

Most common microorganisms associated with asymptomatic bacteriuria are *E. coli, P. aeruginosa, Enterococcus,* and *Staphylococcus aureus* (Nicolle 2005).

The most common bacteria causing UTI and ABU, i.e., *E. coli* can be divided into ABU *E. coli* and uropathogenic *E. coli* (UPEC) strains (Ronald 2003).

It is also seen that if individuals with ABU *E. coli* are not treated, they are at reduced risk of developing pyelonephritis in future (Hansson et al. 1989). As patients with ABU do not develop any sign and symptoms of UTI, these individuals do not require any treatment generally. Moreover; it is also seen that colonization with ABU strains helps in preventing infection by other virulent strains (Darouiche et al. 2001).

This observation led to development of an idea that non-virulent but niche-dominant bacteria can be practically exploited to prevent UTI with other virulent strain, called as bacterial interference. Even, ABU strains can be inoculated deliberately to prevent infections from virulent strains. For example, ABU strain 83972 has been used prophylactically as an agent to prevent infections in individuals at risk to infections by harmful bacteria (Darouiche et al. 2001, 2005; Hull et al. 2000; Trautner et al. 2002).

13.2.3 Role of Biofilms in Catheter-Associated Infections

The typical cellular structure of the bladder and the regular emptying of urine do not allow microorganisms to multiply to threatening levels or sticking to the surrounding mucosa. Normally, microorganisms remain freely suspended in urine in urinary bladder and are not able to cause any infection, unless they are present in such a huge number that bladder's innate defense is overwhelmed (Donlan 2001).

Whenever a foreign body such as an urinary catheter or stent is inserted into the bladder, the probability of a patient to have UTIs increases, as these devices allow microorganisms to enter into the urinary tract; thereby helping them to initiate infection (Fig. 13.2).

Urinary catheters are tubular structure made up of either latex or silicone, which when inserted into the urinary tract may develop biofilms either on the inner or the outer surfaces. The majority of these uropathogens are fecal contaminants or patient's own perineal flora or transitory microflora which is present in the periurethral area (Jordan et al. 2015; Daifuku and Stamm 1986; Leranoz et al. 1997; Old et al. 1983; Yamamoto et al. 1985). Transitory microflora are usually the nosocomial microorganisms which may be acquired from healthcare personnel or from contact with other patients. Also, these organisms may originate from tap water or other environmental sources like bedsheets, side table, and bedpan used in hospital.

The organisms most commonly contaminating these devices and developing biofilms are *Staphylococcus epidermidis, Enterococcus faecalis, E. coli, P. mirabilis, P. aeruginosa, Klebsiella pneumoniae*, etc. (Stickler 1996).

The pathogens can enter bladder either at the time of catheter insertion, through the catheter lumen (intraluminal; 34%), or along the catheter urethral interface (extraluminal; 66%) (Warren 1996).

Biofilms may be monomicrobial or polymicrobial, depending on the type of device used and for how long it was present in the patient; it can also be possible that initially biofilm is monomicrobial but prolonged exposure can lead to formation of

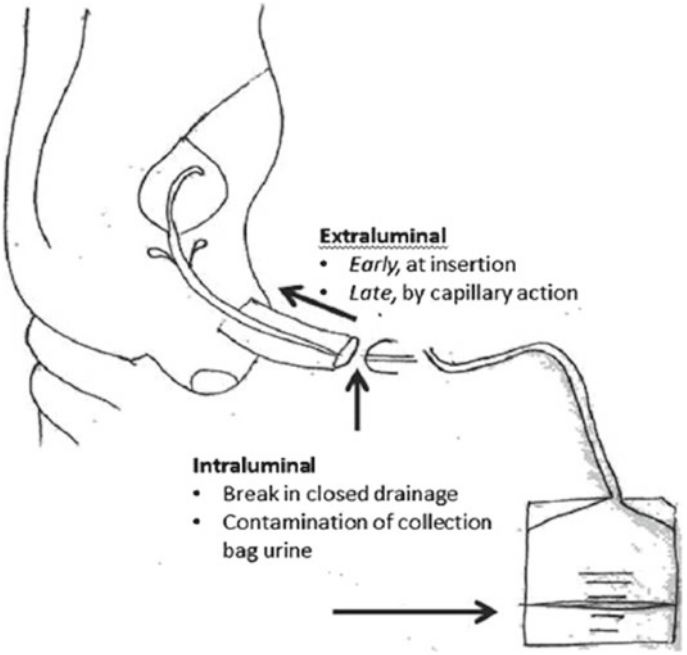

Fig. 13.2 Routes of entry for microbes into urinary tract with indwelling catheter (reproduced from Parida and Mishra (2013))

polymicrobial biofilm and the organisms may vary from Gram-negative or Gram-positive bacteria or yeasts (Stickler 1996; Sayal et al. 2014).

The tendency to form biofilm, thereby leading to UTIs, increases as the duration of catheter use increases. Around 10–50% of patients with short-term urinary catheterization (7 days) become infected, while almost all patients with long-term catheterization (>28 days) suffer from CAUTIs (Stickler 1996).

Indwelling urinary catheters provide a surface for microbial adhesion and thus favor the colonization of uropathogens. Urinary catheters may further damage the protective uroepithelial mucosa during its insertion process, which may lead to the exposure of new binding receptors on bladder epithelium for bacterial adhesins (Garibaldi et al. 1980). Also, catheter in the urinary tract disrupts host defense mechanism, which may further result in over distension of bladder and incomplete voiding of urine, thus leaving residual urine in bladder which creates a favorable environment for microbial growth (Hashmi et al. 2003).

Microorganisms use similar approach to infect urinary tract in uncomplicated UTIs as well as in CAUTIs. However, due to the introduction of a foreign body, organisms causing CAUTIs require lesser virulence factors to colonize and establish infection than those required by pathogens to establish infection in perfectly normal urinary tract.

Bacterial adhesins help bacteria to attach host cell by recognizing host cell receptors located on surfaces of the host cell or catheter (Corpe 1980). These bacterial cell surface structures also help bacteria to recognize extracellular matrix components such as proteins, carbohydrates, glycoproteins, and glycolipids.

After attaching to catheter surface or on uroepithelium, bacteria start to change phenotypically and produce exopolysaccharides. These exopolysaccharides entrap and protect bacteria (Fig. 13.3). These attached bacteria multiply many times to form small bacterial colonies known as microcolonies; these microcolonies further mature into biofilms. Bacteria communicate by quorum sensing, which helps them to form biofilm. Quorum sensing also regulates detachment of microorganism from biofilms after cellular populations reach a threshold value. This rate of exchange of genetic material occurring between microorganisms within the biofilm is much greater than that between planktonic cells; thus, there is rapid spread of antibiotic-resistant gene among microcolonies.

Uropathogens when inside biofilm are potentially resistant to antibiotics and host immune response (Hausner and Wuertz 1999; Roberts et al. 1999; Costerton et al. 1995). The shedding of daughter cells from actively dividing microorganisms and from disruption of biofilm seeds other sections of catheter and urinary tract, thereby leading to spread of infection to other sections.

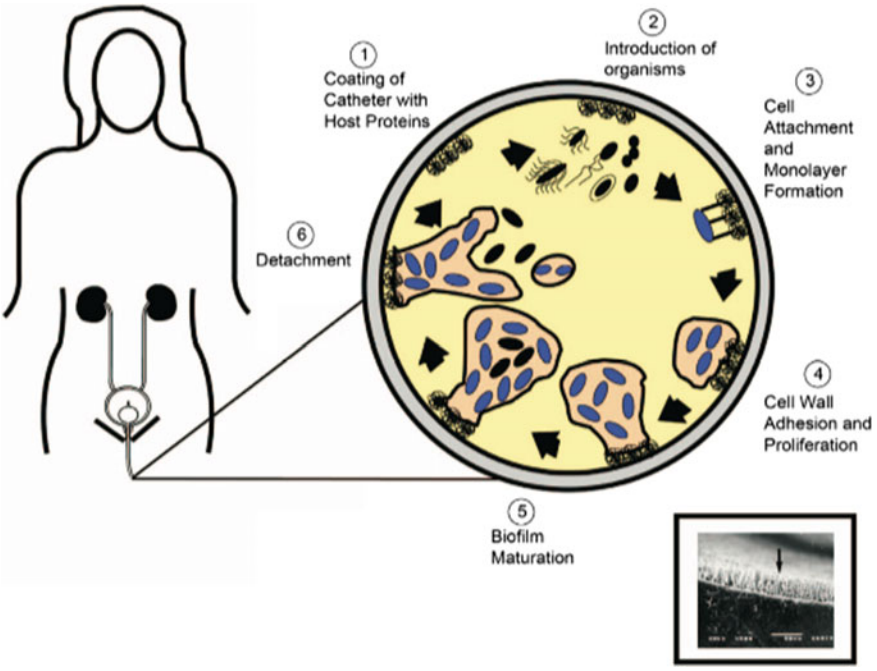

Fig. 13.3 Pathogenesis of biofilm formation on urinary catheters (reproduced from Jacobsen et al. (2008)). **The inset shows a scanning electron microscopic picture of an encrusted urinary catheter due to *P. aeruginosa*** (reproduced from Stickler et al. (1998))

Once uropathogens get colonized in urinary tract or catheter surface, they should adapt themselves to urinary tract environment and acquire nutrients. The production and secretion of degradative enzymes and toxins into the local environment cause damage to tissues. As iron is a limiting nutrient in the human host, uropathogens have developed complex iron acquisition systems, e.g., siderophores, heme transporters, etc. (Weinberg 1984).

Due to the presence of urease enzymes, certain bacteria are capable of using urea, thereby releasing ammonia and carbon dioxide, which makes local environment alkaline, further leading to the precipitation of polyvalent ions that become enmeshed in the biofilms on catheters and urinary epithelial surfaces, thus forming crystalline biofilm (Breitenbach and Hausinger 1988; Jones and Mobley 1987; Griffith et al. 1976).

Evasion from host defense system is a must for maintaining infection in urinary tract. Polysaccharidic capsules help bacteria to evade host immune system by resisting its phagocytosis (Podschun and Ullmann 1998; Williams et al. 1983). Being similar to human cell's polysialic acid residues, capsular structures cause a poor immunogenic response (Troy 1992).

Urinary catheter encrustation is another ongoing problem which causes blockage of catheter, leading to urine retention (Feneley et al. 2015).

Factors causing encrustation of catheter are metabolic dysfunction, colonization of catheter by bacteria; more specifically urease-producing bacteria that form crystalline biofilms, e.g., *P. mirabilis, Proteus vulgaris,* and *Providentia rettgeri* (Jordan et al. 2015; Hola and Ruzicka 2001; Campos et al. 2004; Farnaud et al. 2004; Jones et al. 2004)

There are added advantages of crystalline biofilm; CAUTIs can persist even after catheter removal; this may be due to the fact that when catheter is removed, crystal can break off, thus seeding the site with bacteria (Feneley et al. 2002). These crystal fragments again act as nuclei on which minerals grow ultimately forming bladder stones (infection stones) (Feneley et al. 2002). As the name suggests, these bladder calculi can store pathogens, reinfecting the bladder and allowing the formation of crystalline biofilm of a new catheter, and the vicious cycle goes on.

Not all the urease-producing microorganisms cause catheter encrustation, e.g., *S. aureus, P. aeruginosa, K. pneumoniae, E. coli, Morganella morganii,* and *Providencia stuartii,* do not form crystalline biofilms, as amount of urease produced is too low. These organisms can form biofilm but cannot cause catheter encrustation as amount of ammonia produced after urea hydrolysis is not enough to raise pH > 8, which is needed for apatite and struvite to form (Broomfield et al. 2009).

Though *Klebsiella pneumoniae* and *P. aeruginosa* cannot form crystalline biofilms, they can still block catheters, as these bacteria produce large amount of mucous and cause the same problem of reduced or halted bladder drainage (Stickler 2008).

13.3 Microbial Factors Contributing to Biofilm Formation in Urinary Tract

13.3.1 Escherichia coli *and Urinary Tract Infections*

E. coli, a member of the *Enterobacteriaceae* family, cause majority (80%) of UTI in humans and is one of the most common causes of Gram-negative bacteremia in hospitalized patients.

Being primarily found in human gut and due to close proximity of urethra to anus, *E. coli* contributes to majority of cases of UTI, whether it is uncomplicated or catheter-associated UTI (CAUTI) (Jacobsen et al. 2008).

E. coli strains associated with UTI are special extraintestinal *E. coli* that have acquired some special virulence factors so that they can grow in adverse environment of urinary bladder and are known as Uropathogenic *E. coli* (UPEC) strains (Jacobsen et al. 2008).

UPEC strains are the most common cause of nosocomial UTIs (50%) as well as UTIs in the general public (70–90%) (Jacobsen et al. 2008).

UPEC strains can be classified into four phylogenetic groups, A, B1, B2, and D. Strains of B2 and D are usually considered as pathogenic *E. coli* causing most of extraintestinal infections including UTIs (Nowrouzian et al. 2006). Since these organisms can colonize intestinal and vaginal tracts, these sites serve as potential reservoirs for UTIs and CAUTIs (Donnenberg and Welch 1996; Johnson 1991).

E. coli is a motile bacterium and utilizes its flagella to invade the urinary tract (Jacobsen et al. 2008). Inside urinary tract, with the help of its virulence factors, UPEC contribute to the occurrence and recurrence of UTIs and CAUTIs (Jacobsen et al. 2008). One of the most important virulence factors of UPEC is type 1 fimbriae, which are found in 80–100% of UPEC strains (Jacobsen et al. 2008). Type 1 fimbriae are complex helical structures which consist of repeating major pilin FimA subunits, tip fibrillum (FimF and FimG), and tip adhesion FimH assembled via the chaperone (FimC)-usher (FimD) pathway (Sauer et al. 2000). Type 1 fimbriae (adhesin) helps UPEC strains to adhere to-D- mannosylated proteins, such as uroplakins, which are found in ample amount in the uroepithelial cells lining the urinary tract. Type 1 fimbriae also help UPEC strains to adhere on the surface of a catheter, thus causing establishment of UPEC infection, which can then support complex biofilm formation (Jacobsen et al. 2008; Ulett et al. 2013). FimH of type 1 pili also identifies extracellular matrix proteins including collagen (types I and IV), fibronectin, and laminin. Thus, these bacterial adhesins can recognize bladder epithelial cells, renal epithelial cells, immune cells, and extracellular matrix proteins. So, type 1 fimbriae help bacteria in auto-aggregation and biofilm formation and protect them from host defense system (Pratt and Kolter 1998; Schembri et al. 2001; Schembri and Klemm 2001).

Apart from helping bacteria in adhering to surface, type 1 pili also initiates host and bacterial signaling pathways which help bacteria to deliver its products to host tissues, and also promotes bacterial invasion into host cells (Mulvey 2002).

P fimbriae are the second most common virulence factors associated with UPEC uropathogenesis; these help UPEC strains to attach to the -D-galactopyranosyl-(1-4)--D-galactopyranoside receptor epitope in globoside residues present on renal epithelial cells and thus play a major role in causing pyelonephritis as well as ascending UTI (Dodson et al. 2001; Plos et al. 1995). P fimbriae also play a major role in establishing reservoir in intestinal mucosa (Goetz et al. 1999; Mahmood et al. 2000). Recently, Ulett et al. described F9 fimbriae for UPEC strain CFT073 which play a vital role in biofilm formation (Ulett et al. 2007).

Flagella help bacteria to ascend from catheter surface to the upper urinary tract. Studies have also shown that flagella greatly increase the persistence and fitness of UPEC associated with CAUTI (Lane et al. 2005; Wright et al. 2005).

After the adherence of bacteria on the surface of catheters or uroepithelial cells, next step is to the establish UPEC infection which occurs through the colonization of the bladder, by invading the host cells and the subsequent formation of biofilms. UPEC strains invade bladder epithelium and renal epithelium which is mediated by various virulence factors such as type 1 fimbriae, the Afa/Dradhesin family (Dr, Dr-II, F1845, Afa-1, and Afa-3), S pili, P pili, and CNF1 (Fukushi et al. 1979; Martinez et al. 2000; McTaggart et al. 1990; Donnenberg et al. 1994; Palmer et al. 1997; Springall et al. 2001; Warren et al. 1988) UPEC strains also have the capability to form intracellular bacterial communities, which also helps bacteria to persist in urinary tract (Justice et al. 2004).

Factors contributing in the formation of biofilms by *E. coli* can be enumerated as fimbriae, curli, flagella, antigen 43, and extracellular matrix molecules including cellulose, colanic acid, and poly- -1,6-*N*-acetyl-D-glucos- amine (Danese et al. 2000; Danese et al. 2001; Davey and O'Toole 2000; Donlan and Costerton 2002; Wang et al. 2004; Zogaj et al. 2001).

The adhesin antigen 43 (Ag43), representative member of the auto-transporter (AT) family, is also associated with urovirulence (Ulett et al. 2007). Ag43 (encoded by the *flu* gene) assists bacteria to auto-aggregate and to form a frizzy colony, thus helping in biofilm formation (Ulett et al. 2007). Ag43 is found usually on the surface of those UPEC which are located within intracellular biofilm-like bacterial pods in the uroepithelium; thus, it can be conferred that it may contribute in survival and persistence of UPEC during prolonged infection.

Curli fibers are highly stable, insoluble, extracellular amyloid fibrils that are variably expressed by *E. coli* (Barnhart and Chapman 2006). Biogenesis of these fibers requires both structural (CsgA and CsgB) and non-structural (CsgD, CsgE, CsgF, and CsgG) components encoded by genes on two divergent operons (Hammar et al. 1995; Chapman et al. 2002; Hammer et al. 2007; Nenninger et al. 2011). These are composed primarily of CsgA proteins with CsgB proteins as minor components. During curli assembly, CsgB monomers are exported outside of bacteria through CsgG pores; then, they fold into proper conformation, and associate with bacterial cell surface (Hammer et al. 2007). Chaperoned by CsgE proteins, CsgA monomers are also exported out to the cell surface in the same fashion as unfolded proteins. Out on bacterial surfaces, initially exported CsgA monomers fold into proper conformation upon interaction with CsgB and then associate with CsgB and form nucleation

centers. Subsequently, CsgA monomers are exported out; they assume proper conformation, interact with existing nucleation center, and incorporate into existing CsGA fiber, and in this manner, these fibers elongate.

These fibers can form biofilm on both abiotic and biotic surfaces and also cause immune modulation in mammalian hosts (Cegelski et al. 2009; Vidal et al. 1998; Barak et al. 2005; Torres et al. 2005; Kai-Larsen et al. 2010). It is also shown experimentally in an UTI model of mice that they have the capability of bladder colonization (Cegelski et al. 2009). Based on these findings, curli fibers are being considered as an important virulence factor in human urinary tract infections (UTIs) (Norinder et al. 2012).

These fibers facilitate epithelial cell adherence and increased resistance to the human antimicrobial peptide LL-37, urinary levels of which are increased during UTI (Chromek et al. 2006).

13.3.2 Proteus mirabilis *and Urinary Tract Infections*

P. mirabilis is very well known for causing catheter-associated urinary tract infections (CAUTIs) and urinary calculi. Due to the presence of urease, these bacteria have the tendency to form crystalline biofilms on catheters and calculus in urinary system.

Though *P. mirabilis* is an intestinal organism, it does not cause UTI in patients with normal, unobstructed urinary tracts (Chow et al. 1979). However, the presence of a chronic, indwelling catheter permits bacteria to ascend up the catheter and into the urinary system (Holá et al. 2012; Jones et al. 2004; Sabbuba et al. 2002). *P. mirabilis* ascends with the help of swarming motility (Jones et al. 2004). Urease production and crystalline biofilm formation have also been found correlated with swarming (Jones et al. 2005; Armbruster and Mobley 2012; Schaffer and Pearson 2015). However, contribution of swarming in UTIs is still not clear, as swarming defective mutants also have the capability to form crystalline biofilm (Schaffer and Pearson 2015; Jansen et al. 2003).

Urease activity of *P. mirabilis* leads to formation of crystalline biofilms, which leads to catheter encrustation, thereby causing catheter blockage (Schaffer et al. 2016). Many studies have shown that urease production and mannose-resistant *Proteus*-like (MR/P) fimbriae play an important role in causing CAUTIs. Urease enzyme hydrolyzes urea into ammonia and carbonic acid. Due to ammonia released during hydrolysis, pH increases. In CAUTI, the ammonia and carbon dioxide released bind with Mg^{2+} and Ca^{2+} found in the urine, respectively, resulting in struvite and carbonate apatite which precipitate and form crystalline deposits on catheters and/or aggregate into calculi in an organ within the urinary system (Mobley et al. 1995; Bichler et al. 2002; Castro et al. 1999) (Fig. 13.4).

It is also shown in a mouse model of ascending UTIs that these bacteria also utilizes urease to establish infection in bladder as well as kidney (Johnson et al. 1993). Urease mutant bacteria exhibit a lower bacterial load in both the bladder and kidney and also fail to form urinary stones (Johnson et al. 1993; Jones et al. 1990; Dattelbaum et al.

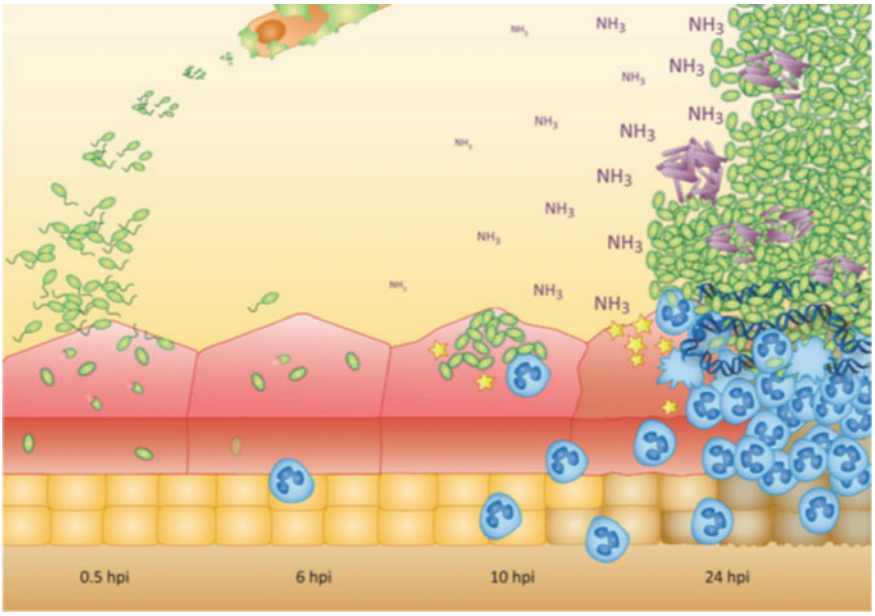

Fig. 13.4 ***P. mirabilis*** **mediated crystalline biofilm formation on catheter surface** (reproduced from Schaffer et al. (2016)). During initial stages of infection bacteria (green) adhere to and invade bladder epithelial cells. However, intracellular bacteria disappear with time and fimbriated bacteria start forming clusters on the surface of bladder epithelium. Size of bacterial cluster increases, leading to increased local concentration of toxin (yellow stars), ammonia (NH_3), and neutrophilic infiltration (blue cells). Altogether, this effect leads to formation of crystalline deposition (gray) and destruction of bladder epithelial surface

2003). Ammonia liberated during urea hydrolysis may cause urothelial damage; thus, many sites on urothelium are available for bacterial adhesion (Johnson et al. 1993).

Formation of crystalline biofilm on catheter surface takes place in the following steps (Morris et al. 1997; Barros et al. 2017):

1. Firstly urinary tract gets infected by a urease-producing bacterial strain.
2. Deposition of urine components and minerals on catheter surface makes it favorable for bacterial adhesion. Thus, urease-producing bacteria get attached to the catheter surface.
3. These bacteria release exopolysaccharide matrix, thereby forming biofilm community.
4. As the bacterial numbers increase, amount of urease increases; thus, amount of ammonia increases, increasing the pH of urine and biofilm.
5. Calcium and magnesium ions are attracted to the biofilm's matrix due to high pH of surroundings.
6. Calcium and magnesium phosphate crystallize, thereby forming struvite and apatite crystals on the catheter surface.

Fimbriae or pili are hair-like appendages which are present on external surface of both Gram-negative and Gram-positive bacteria (Proft and Baker 2009). These pili can help bacteria in biofilm formation, surface attachment, and evasion of the host immune response (Pizarro-Cerda and Cossart 2006; Waksman and Hultgren 2009).

The *P. mirabilis* HI4320-type strain genome contains at least 17 different operons that encode chaperone-usher fimbriae, the most within a single strain of any bacterium known to date (Pearson et al. 2008) Of these 17 known fimbrial operons in *P. mirabilis*, five have been characterized in vivo. At least four fimbrial operons are shown to cause *P. mirabilis* virulence in mouse models of UTI: MR/P (Bahrani et al. 1994; Li et al. 1997, 1999), uroepithelial cell adhesin (UCA, also known as non-agglutinating fimbria, or NAF) (Pellegrino et al. 2013), *P. mirabilis* fimbriae (PMF) (Massad et al. 1994; Zunino et al. 2003), and fimbriae 14 (Himpsl et al. 2008). MR/P fimbriae are the best characterized fimbriae which play an important role in bladder infection.

MR/P fimbriae are critical for bladder and kidney infection in mice. Bacteria in the urinary tract produce MR/P fimbriae at 24–48 h after infection, with the highest percentage found in the bladder (Jansen et al. 2004). Additionally, mrp genes within the mrp operon are highly induced in vivo and are required at early steps of infection, colonization of bladder, and crystal formation (Bahrani et al. 1994; Li et al. 1997, 1999; Pearson et al. 2011; Zunino et al. 2001).

The bladder and kidney receptors for MR/Pfimbriae are unknown. Some evidence suggests that MR/P may be important for bacterial adherence to urothelial cell and/or proper localization of *P. mirabilis* in the bladder (Jansen et al. 2004; Zunino et al. 2001; Li et al. 2002). Studies have also shown that MR/P may be important for initiating or maintaining cluster formation within the bladder. Therefore, MR/P could be responsible for both host binding and cluster formation. Without cluster, mineral does not deposit and chances of stone formation reduce to zero.

13.3.3 Klebsiella pneumoniae *and Urinary Tract Infections*

K. pneumoniae forms biofilms, particularly on indwelling medical devices (Hatt and Rather 2008). Biofilm-forming tendency of *K. pneumoniae* may contribute to bacterial persistence, i.e., alter the innate defense mechanisms of the host (Murphy and Clegg 2012).

Capsular polysaccharides, type 1 and type 3 fimbriae of *K. pneumoniae* contribute to biofilm formation (Li et al. 2014). Type 3 fimbriae initiate biofilm formation and are composed of subunits of the protein MrkA, while MrkD is found at the tip of fimbriae and helps in fimbrial binding. (Murphy and Clegg 2012).

Fimbriae, namely type 1, type 3, Kpc and KPF-28 adhesin, are important role players in the establishment of infection and biofilm formation. Type 1 fimbriae are required for establishment of infection. Type 3 fimbriae play a vital role in biofilm formation in *K. pneumoniae* (Struve et al. 2009). Both types 1 and 3 fimbriae act as colonization factor for *K. pneumoniae* biofilm-associated urinary infections (Murphy

et al. 2013). The Kpc fimbriae are responsible for hypermucoviscous *K. pneumoniae* and may also contribute to biofilm formation (Wu et al. 2010).

The protective effects of biofilms enhance in a synergistic manner when multiple species are present (Burmolle et al. 2014). Whenever biofilm is made of multiple species or strains, these species can organize themselves in three manners, i.e., separate monospecies microcolonies, co-aggregation, and arrangement in layers (Elias and Banin 2012). *K. pneumoniae* are usually found along with *P. aeruginosa* in biofilm in urinary tract infections (Childers et al. 2013). In a joint account, *P. aeruginosa* forms the base structure due to its potential to colonize itself, while *K. pneumoniae* forms a tower-like structure at the top due to its higher growth rate (Chhibber et al. 2015).

13.3.4 Pseudomonas aeruginosa *and Urinary Tract Infections*

P. aeruginosa causes about 12% of nosocomial UTIs (Kunin 1994). Biofilm formed by *P. aeruginosa* consists of an extracellular matrix which consists of polysaccharides, proteinaceous components, and extracellular DNA (eDNA) (Sutherland 2001; Whitchurch et al. 2002; Vallet et al. 2001, 2004; Kulasekara et al. 2005). Non-mucoid strains of *P. aeruginosa* also produce biofilms, but these biofilms do not depend upon alginate biosynthesis. (Wozniak et al. 2003; Starkey et al. 2009). *Pseudomonas* biofilms can colonize any solid surfaces and form ring-like structures in culture tubes and microtiter plates or pellicles at the air–liquid interface (O'Toole and Kolter 1998; Friedman and Kolter 2004a).

PEL and PSL exopolysaccharides are the main polysaccharides, and their production is encoded by *pel* and *psl* genes, respectively (Friedman and Kolter 2004a, b; Jackson et al. 2004). Mutations in any of two genes, i.e., either *pel* or *psl* or both result in mutant bacteria that produce less biofilm; e.g., *P. aeruginosa* strain PA14 lacks *pslABC* genes and does not produce the PSL polysaccharide (Stewart and Costerton 2001; Fux et al. 2005; Stewart 2001). Along with *pel* and *psl* gene, eDNA is also required for the synthesis of biofilm (Whitchurch et al. 2002). Very little is known about how these pseudomonal factors contribute in the biofilm formation in animals and human being.

PEL, PSL, and alginate exopolysaccharides are required for biofilm formation on the catheter and for its spread into the kidneys. In a study, it was shown that PA14 *pelD* mutant could also form biofilm on the catheter surface when PA14 strain was also present as infectious strain; however, it was unable to form biofilm, when it caused monomicrobial infection. Thus, it was shown that PA14*pel*D mutant strain utilizes PEL polysaccharide produced by PA14 strain (Cole et al. 2014).

Since exopolysaccharides are essential for biofilm formation, the ability of exopolysaccharide-deficient *Pseudomonas* to form biofilm could be induced in vitro by mouse and human urine as exposure of *P. aeruginosa* to urea can induce some

of the cells to round and lyse. The released eDNA during lysis process could help in biofilm formation in both PA14 and the *pelD* mutant strains (Cole et al. 2014). If DNAse I is added, then biofilm formation decreases in both strain, as DNAse I causes breakdown of eDNA.

13.3.5 Miscellaneous Microorganisms and Urinary Tract Infections

Factors responsible for enterococcal biofilm development are not very well understood. Various factors are identified for biofilm development in *Enterococci*; however, mediators of dispersion are yet to be identified.

Endocarditis and biofilm-associated pilus (Ebp) mediates attachment of bacteria to the surface both in vitro and in vivo (Nallapareddy et al. 2006, 2011, 2011; Bourgogne et al. 2010; Singh et al. 2007; Nielsen 2012). Deletion of ebpABC causes reduction in attachment and impaired biofilm formation in vitro (Nallapareddy et al. 2011). Similarly, the absence of surface adhesins, including aggregation substance (Agg), enterococcal surface protein (Esp), and adhesin to collagen from *E. faecalis* (Ace), resulted in reduced attachment to cultured human cells and defected biofilm formation in vivo. (Mohamed et al. 2006; Rozdzinski et al. 2001; Sussmuth et al. 2000; Sillanpaa et al. 2010; Toledo-Arana et al. 2001).

After initial attachment, microcolony formation takes place, and rhamnose polysaccharide is released (in vitro, *Enterococcus* biofilm are formed typically as sheets) (Fig. 13.5); however, factors which are responsible for microcolony formation are still unclear. (Ch'ng et al. 2019) Some of these microcolonies get dispersed, while others may further form typical mature biofilm with thick matrix. Mature enterococcal biofilms contain extracellular matrix components such as extracellular DNA (eDNA), polysaccharides, extracellular proteases including gelatinase (GelE), autolysins (Atla), serine proteases (SprE), lipoteichoic (LTA) in matrix. Among all these components, eDNA is best described; it can be seen as part of intracellular filamentous structures, at bacterial septum and part of biofilm matrix; its release from cells is dependent on autolysin AtlA (Leibman 2012; Guiton et al. 2009; Thomas et al. 2009). It has also been seen that treatment with DNase reduced stability of biofilm and increased detachment of bacteria (Dunny et al. 2014; Vorkapic et al. 2016).

Capacity to form biofilms on abiotic or biotic surfaces is an important pathogenic factor of *C. albicans* (Fanning and Mitchell 2012). Biofilms are formed step by step, including adherence of yeast cells to the substrate, multiplication of these yeast cells, formation of hyphal cells in the upper part of the biofilm, accumulation of extracellular matrix material, and dissemination of yeast cells from the biofilm complex (Finkel and Mitchell 2011) (Fig. 13.6).

Dissemination of yeast cells from biofilm complex directly contributes to virulence (Uppuluri et al. 2010). The major heat shock protein Hsp90 acts as a key

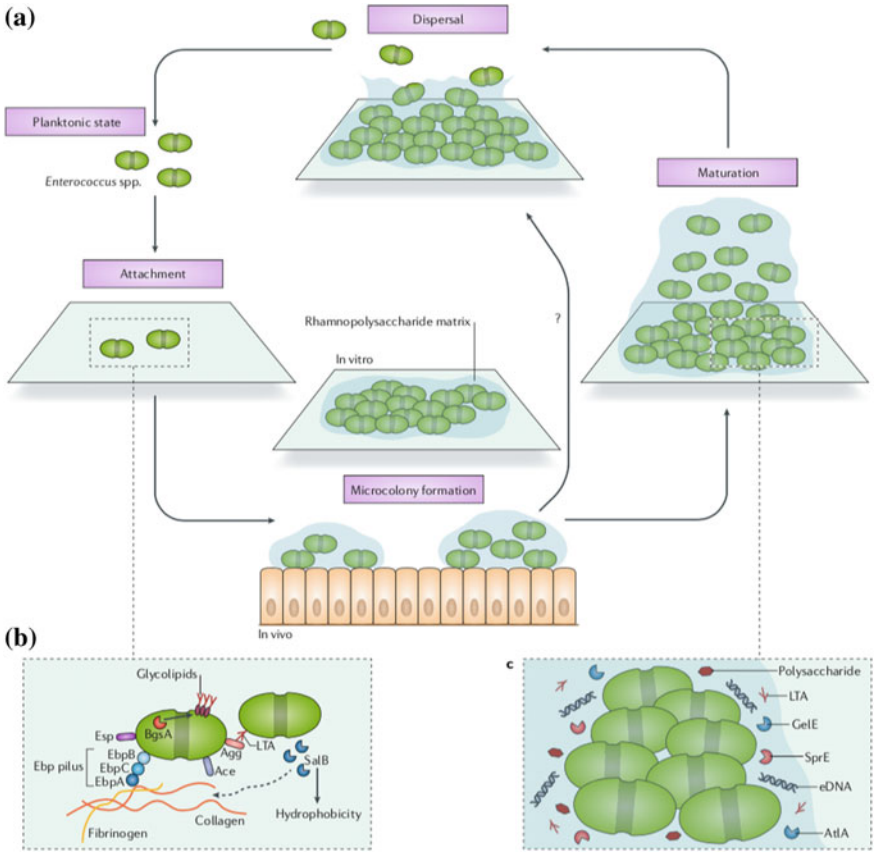

Fig. 13.5 Stages of biofilm development in *Enterococci* (reproduced from Ch'ng et al. (2019)). BgsA-biofilm-associated glycolipid synthesis A; SalB- and SagA-like protein B

regulator of dissemination in *C. albicans* biofilms and is also required for biofilm antifungal drug resistance (Robbins et al. 2011). In addition, Hsp90 was also required for biofilm-mediated antifungal drug resistance (Mayer et al. 2013).

Various transcription factors such as Bcr1, Tec1, and Efg1 control biofilm formation, and defect in any of these factor resulted in defective biofilm formation in vivo rat infection model (Nobile et al. 2012).

Extracellular matrix production is controlled by some other additional factors. The zinc-responsive transcription factor Zap1 regulates β-1,3 glucan negatively, while glucoamylases (Gca1 and Gca2), glucan transferases (Bgl2 and Phr1), and the exoglucanase, Xog1, regulate β-1,3 glucan positively (Nobile et al. 2009; Taff et al. 2012).

C. albicans biofilms are found resistant to killing by neutrophils. Biofilm does not stimulate production of reactive oxygen species (ROS) (Xie et al. 2012). Evidence suggests that β-glucans in the extracellular matrix is responsible for preventing *C.*

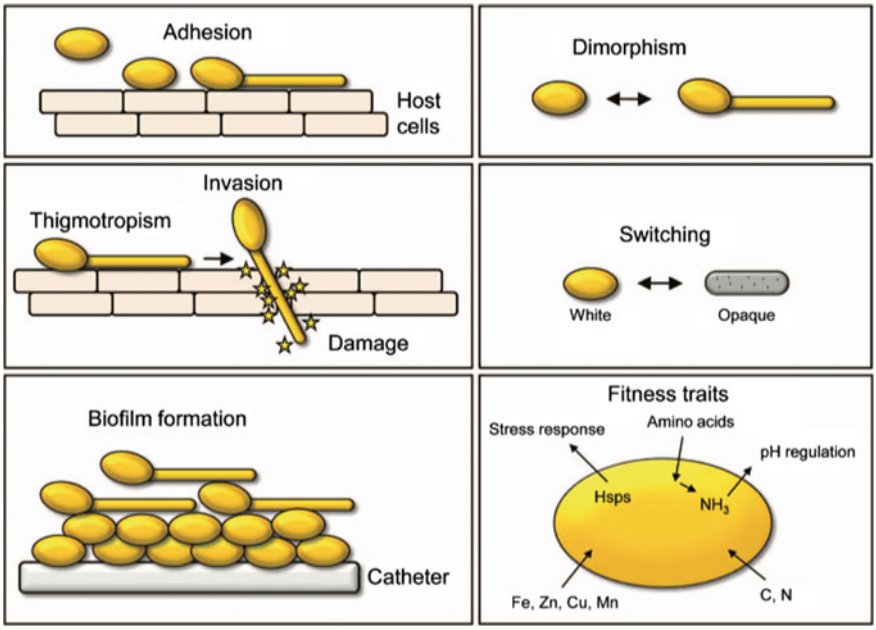

Fig. 13.6 Adhesion of yeast cells to surface (reproduced from Mayer et al. (2013)). Yeast cells adhere to host cells with the help of adhesins. Contact with host cell surface triggers yeast-to-hypha transition. With the help of invasins, fungus penetrates host cells through induce endocytosis. It has also been proposed that fungus can invade host cells by breaking down barriers with the help of adhesion, physical forces, and fungal hydrolases. Attachment of yeast cells to catheter surface (abiotic) or host surface (biotic) may lead to biofilm formation with yeast cells in lower part, and hyphal forms in upper part of biofilm. This phase transition among yeast cells may also influence antigenicity of fungus, thus interfering with host defenses. Apart from these virulence factors, several fitness factors also influence fungal pathogenicity, e.g., heat shock proteins (Hsps)-mediated stress response, auto-induction of hyphae formation due to uptake of amino acids, excretion of ammonia (NH_3), uptake of carbon (C) and nitrogen (N) sources, and uptake of various trace metals, e.g., zinc (Zn), copper (Cu), etc.

albicans from these attacks (Xie et al. 2012). On coming to contact with surface hyphal growth may occur (Brand et al. 2007).

13.4 Treatment and Prevention of Biofilm-Mediated UTIs

13.4.1 Antimicrobial Treatment of Biofilms

As biofilm provides high resistance to antibiotics, treatment with antibiotics becomes difficult. Several studies have recommended for combination therapy rather than

using single antibiotics, with macrolides being one of the first antibiotics taken (Ichimiya et al. 1996).

Macrolides are highly active against biofilm-associated infections caused by Gram-negative bacteria as they inhibit the production of an important component of the matrix, alginate (Ichimiya et al. 1996). More recently, macrolides have been found effective against *Staphylococcus* spp. biofilms (Parra-Ruiz et al. 2012). Roxithromycin plus imipenem have been shown to increase infiltration of neutrophils into biofilm, thus destabilizing the biofilm (Yamasaki et al. 2001).

Another way to use antimicrobials is to impregnate catheters with these antimicrobials agents. This may restrict bacteria to attach themselves to catheter surface and further development of biofilm (Hamill et al. 2007). Silver due to its broad-spectrum antimicrobial activity has been used largely to coat surfaces. Antimicrobial activity of silver compounds depends upon the amount of bioactive silver ion released and its accessibility to interact with microbial cell membrane. It has been experienced that bacterial attachment and capacity to form biofilm reduces by 50% on silver-coated surfaces (Ahearn et al. 2000; Regev-Shoshani et al. 2011). Synthetic cationic peptide variants obtained from natural peptides have also been used to prevent or reduce biofilm formation (de la Fuente et al. 2013).

Substances with antibacterial properties, such as gendine (gentian violet plus chlorhexidine), nitrous oxide, and nitrofurazone (nitrofuran), have also been used to transform the surface of urinary catheters. However, when the antibiotics are used to coat catheter surface, the risk of antimicrobial resistance increases as antibiotics to treat the catheter surface may lead to the development of antimicrobial resistance (Siddiq and Darouiche 2012).

13.4.2 Newer Strategies

Biofilm eradication is difficult due to the high level of antimicrobial resistance shown by these structures. Thus, new therapeutic strategies are being studied continuously, so as to avoid biofilm production as well as to avoid emergence of resistant strains in biofilm.

13.4.2.1 Coating of Catheters with Hydrogels or Antibiotics

Hydrogels are hydrophilic polymers having the ability to trap water. This characteristic can be exploited to increase surface lubrication of catheters so that bacteria could not adhere so efficiently on surface, and it has also shown to reduce catheter encrustation. However, whether these hydrogels could prevent from CAUTIs is not very much clear till now. It was also observed that hydrogel layer increased aggregation of planktonic cells, leading to catheter blockage, which is a negative effect of hydrogel, but this can be suppressed if an active agent is added to hydrogel.

A high number of antimicrobial agents and other chemical compounds along with hydrogels have been used to coat catheters, e.g., silver alloy used in hydrogel-coated urinary catheter decreased up to 45% of CAUTI (Rupp et al. 2004).

13.4.2.2 Nanoparticles

Nanoparticles have the capacity to penetrate into bacterial cells, thereby disrupting their membrane and interacting with chromosomal DNA (Lellouche et al. 2009).

Nanoparticles of MgF were coated on glass surface, and it was observed that they inhibit biofilm formation by both, *E. coli* as well as *S. aureus* (Lellouche et al. 2009). Catheters coated with nanoparticles showed significant reduction in bacterial colonization when observed over a time period of 1 week and compared with uncoated catheter.

13.4.2.3 Iontophoresis

Iontophoresis is a physical process in which electric field is used to diffuse ions in a medium. This method has been shown to enhance the efficacy of anti-biofilm agents "in vitro" (Costerton et al. 1994). It was noticed that electric current improved the activity of tobramycin and biocides against *P. aeruginosa* biofilm. However, this effect was observed only in those antibiotics which were effective against planktonic cells (Jass and Lappin-Scott 1996). Whether this process will be effective "in vivo" is yet to be studied

13.4.2.4 Enzyme Inhibitors

Urease is very well known for catheter encrustation in *P. mirabilis* infection. It was thought that if anyhow activity of urease could be decreased or pH of the medium can be controlled, crystallization of biofilm could be controlled. In this sense, fluorofamide has been a candidate molecule as it can prevent the increase in pH by *P. mirabilis* "in vitro" (Morris and Stickler 1998, 1998). Other natural compounds, such as vanillic acid (Torzewska and Rozalski 2014), natural plum juice (Zhu et al. 2012), and germa-γ-lactones (Amtul et al. 2007), also decreased bacterial growth and catheter encrustation by the inhibition of the urease enzyme.

13.4.2.5 Bacterial Interference

Bacterial interference is related to antagonism among different bacteria during the colonization of surfaces and biofilm formation. It was observed that adherence of a surface with non-pathogenic strain could prevent the attachment of pathogenic bacteria, thereby avoiding infection (Siddiq and Darouiche 2012); e.g., ABU strains

of *E. coli* could reduce urinary catheter colonization by a wide variety of pathogens (Trautner et al. 2002; Trautner et al. 2003). Thus, the *E. coli* HU2117 strain, derived from *E. coli* 83,972, which has the capability of persistent colonization without symptomatic infection (Andersson et al. 1991; Otto et al. 2001; Hull et al. 2002), has been used for coating urinary catheters, and it was observed that it reduced biofilm formation by other pathogens (Trautner et al. 2003).

13.4.2.6 Bacteriophages

Bacteriophages are viruses that infect bacteria. Lytic phages are able to lyse bacterial metabolism, favoring viral replication (Carson et al. 2010). The phage characteristics that allow them to control biofilm are the ability to multiply at the site of infection, lysis of bacteria, the production of enzymes that degrade the EPS of the biofilm (Hughes et al. 1998, 1998; Hanton 2001; Donlan 2009), and their capability to propagate through the biofilm (Doolittle et al. 1996). These phages when used along with hydrogel-coated catheters showed reduction in biofilm formation by *S. epidermidis* and *P. aeruginosa* (Curtin and Donlan 2006; Fu et al. 2010).

13.4.2.7 Quorum Sensing Inhibitors (QSI)

Within biofilm, bacteria communicate by quorum sensing (QS). QS is used to coordinate the signaling which is required for bacterial motility and biofilm formation. If anyhow this signaling could be impeded, then biofilm formation can be reduced.

Several QSI are known till date, many having been isolated from nature (Hentzer and Givskov 2003; Hentzer et al. 2003a, b; Rasmussen et al. 2005a, b). For example, the pyrimidinone compound inhibits biofilm formation; it also disrupts previously formed biofilm. Garlic extract has been found to increase the susceptibility to tobramycin by altering the structure of the bacterial biofilms so that antibiotic can penetrate more efficiently in biofilm (Rasmussen et al. 2005a).

13.4.2.8 Anti-adhesion Agents

The first step for the establishment of infection is the adhesion of microbes to surface. The prophylactic use of anti-adhesive compounds/molecules to prevent UTIs is currently an important objective in clinical research (Rafsanjany et al. 2013). Thus, anti-adhesive compound should be able to bind to adhesins of bacteria so efficiently that it could not bind to host cell (Hensel and Xiao 2009; Löhr et al. 2011).

One such compound is cranberry extract (Jepson et al. 2001). The anti-adherence effect of cranberry against uropathogenic *E. coli* (UPEC) is due to the presence of A-type proanthocyanidin trimmers (anti-adhesin agent) in the cranberry extract (Foo et al. 2000a, b). Other anti-adhesion agents are mannosides, curlicides, and pilicides. These agents inhibit the synthesis of adhesions among bacteria.

Salicylate is a member of a large group of non-steroidal anti-inflammatory substance; it has been observed that it inhibits type 1 fimbriae expression in UPEC.

In conclusion, it can be said that such compound should be searched upon which not only disrupts biofilm but also inhibits its formation and can act upon multidrug-resistant bacteria.

13.5 Future Prospects

Device-related infections are of concern because of increasing resistance to currently available antibiotics. It is also seen that when device-related infections are associated with biofilm, the bacteria can stand up to 1000-fold higher concentration as compared to that required to kill their planktonic cell (Olsen 2015). Even though many techniques have been suggested to prevent biofilm formation, e.g., antimicrobial coating, contact killing, etc., very few are there in use in clinical practice (Grainger et al. 2013).

One of the reasons for this is lack of in vitro model of biofilm, which is so close to reality that it could predict antimicrobial and anti-biofilm activity of device in vivo. Such a laboratory "biofilm model" should imitate the natural (in vivo) situation with focus on some selected relevant factors such as materials, fluid flow, growth media (nutrients), and intercellular interactions.

Most important factor to be kept in mind before designing any biofilm model is to use appropriate use of bacterial strain. In many studies, irrelevant bacterial strains or cultivation conditions have been used, and many antimicrobial substances developed on the basis of these studies which claim to be effective against biofilm remain questionable; e.g., if we have to develop a surface which can reduce the adhesion of bacteria to the surface, we have to use bacteria with same adhering capacity as that of bacteria that grow in biofilm in vivo.

The mutations, for example, fimbriae mutants of *E. coli*, already have reduced capability to form biofilms, which in turn may increase their susceptibility to antimicrobials, and if any coating material is developed by using this mutant strain, then it may not be that much effective in vivo (Olsen 2015).

Also, bacteria usually grow in constraints of nutrients and in the environment of host defense. Nutrient limitations can lead to an altered gene expression, enhanced expression of pathogenic factors so that they can grow in adverse environment. Thus, a growth medium artificially rich in nutrients if used may lead to an unrelated phenotype, making bacteria more sensitive to antimicrobials.

Furthermore, biofilms formed under stagnant growth conditions (i.e., suspension cultures) can have different gene expressions and features than those formed under continuous medium flow as a result of different shear stresses (Dötsch et al. 2012). Therefore, any result of in vitro condition should always be counterchecked in vivo, before declaring that the result will be effective in vivo conditions too.

Ideally, in vitro model should predict the efficacy of antimicrobial material or anti-adhesive material in vivo.

A detailed consideration of biological setting should be explained before designing any model. Factors that may affect antimicrobial activity of any model are such as bacteria-derived, host-derived, and abiotic factors.

Choice of bacterial strain depends on the area for which biofilm model is to be designed and duration of use. Growth media also impose a challenge in establishing model, e.g., in urinary catheters and stent; encrustation takes place when urease-producing bacteria grow since urease activity increases pH leading to precipitation of salts.

Various studies have used human urine for bacterial culture, but when human urine was used, it was seen that there was variation of results among batches of experiments. On the other hand, when artificial urine was used, inactivation of antimicrobial surface took place due to precipitation of salt (struvite and hydroxyapatite). Artificial urine was high in yeast extract, thus providing very high nutrient supply for bacterial cultivation. This was later on improvised by replacing yeast extract with trace element solution (Brooks and Keevil 1997; Dohnt et al. 2011). However, role of iron imitation in urinary tract was not attended so well; thus, it was also seen that presence of iron in media components should be taken into consideration.

Other problem of using human urine was that it contains high amount of antimicrobial proteins and peptides (uromodulin, RNAse 7 and antimicrobial peptide), as a consequence of urinary tract inflammation (Ali et al. 2009). These factors should be taken into consideration while preparing a realistic biofilm model, but it will increase cost of experiment, since purification of these proteins is very costly and challenging. Also the shear stress (flow vs. static condition), osmolarity, and temperature can also influence the result of in vitro biofilm model. It was seen that when experiments were done in static conditions, antimicrobial tends to elute in single burst with subsequent loss of bioactive coating. Continuous medium flow if used could help to design in vitro model which was very closer to reality.

In a biological environment, devices are in contact with host surface such as epithelial cells and/or biological fluids. Cellular debris, extracellular polymeric substance, etc., also form cover which can compromise the performance of antimicrobials and provide extra surface for bacterial attachment (Nuryastuti et al. 2011). To mimic this condition; small amount of serum was added to the medium in some studies (Rosman et al. 2014).

Few in vitro models are proposed for CAUTIs (Cortese et al. 2018):

13.4.1. Bladder model by Stickler et al.,
13.4.2. Urinary tract model by Gaonkar et al.,
13.4.3. CAUTI model by Rosenblatt et al., and
13.4.4. Meatus model by Holland and Fish.

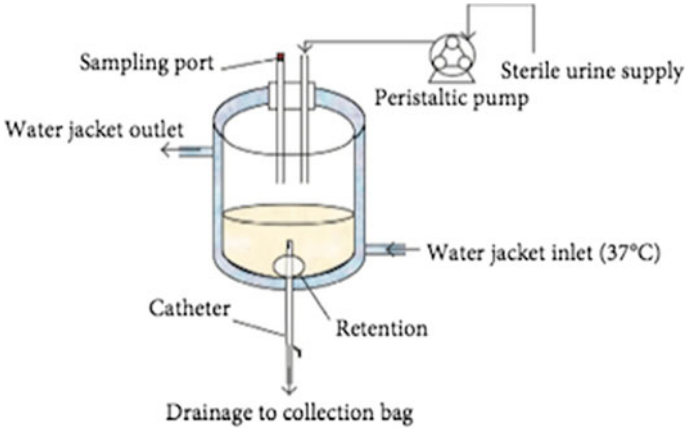

Fig. 13.7 Illustration of in vitro bladder model (reproduced from Cortese et al. (2018))

13.5.1 Bladder Model

Cortese et al. (2018) developed a bladder model in 1999. This model consisted of a glass vessel surrounded by a water jacket maintained at 37 °C. Whole system is sterilized before an indwelling catheter is inserted into a glass outlet tube. Retention balloon is inflated so that catheter remains in its place; then, it is attached to a drainage bag (Fig. 13.7). With the help of peristaltic pump, urine is pumped into model and urine gets drained into the attached drainage bag through the catheter. This model has been used to produce bacterial biofilms on both the surfaces of catheter. This model can also be used to produce encrusted biofilms where blockage of the catheter is very usual.

13.5.2 Urinary Tract Model

Gaonkar et al. developed a model of the urinary tract, to find out how pathogen migrates along the surface of indwelling catheter from meatus to urinary bladder (Cortese et al. 2018). This model consists of two tubes: First tube is an open narrower tube with a cap at one end and a rubber cork with a hole at the other end, and second tube is a larger vessel that is open at one end so that it can connect to first tube and closed at the other end to collect urine. All parts are sterilized with ethylene dioxide before doing experiment. Agar urethra portion is formed by placing a catheter segment aseptically into the top of first tube, and it is protected by pushing through the hole in the rubber cork; then, molten agar is poured along the sides of the catheter, and once solidified, the rubber cork is removed. First tube is then secured on the top of tube 2 for testing (Fig. 13.8).

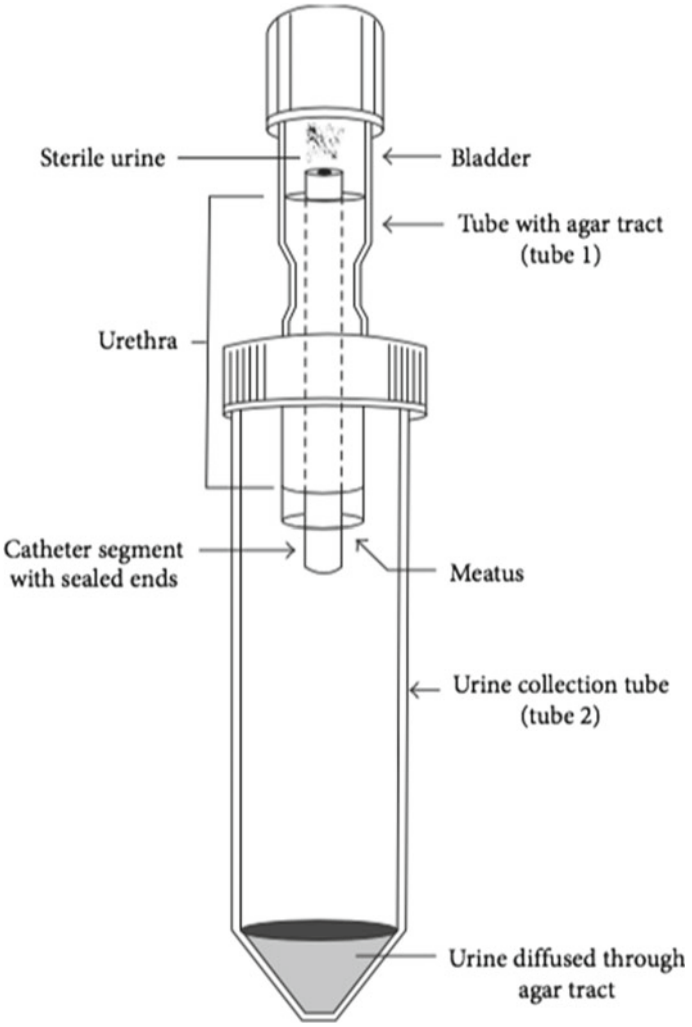

Fig. 13.8 Illustration of in vitro urinary tract model (reproduced from Cortese et al. (2018))

In this model, the "meatus" is inoculated daily with bacteria, and then top of first tube is filled with sterile urine. Samples are taken from the urine at the top of first tube periodically to assess the amount of bacteria migrated up the catheter, if any. This method tests indwelling catheter for a couple of days.

13.5.3 CAUTI Model

It is the modified version of the urinary tract model or CAUTI model proposed by Cortese et al. (2018).

This model consists of only one tube with an upper bulbous end to allow inflation of the retention cuff with a cap at the other end. The agar channel is wider than that of Gaonkar et al. model. This agar channel allows second catheter cuff or "proximal irrigation cuff", inside, and this was the main modification, which was done for testing (Fig. 13.9).

In this model also, meatus was inoculated and periurethral space was then washed via the proximal irrigation cuff. The whole setup was then incubated to promote any growth, after which the catheter was removed, segmented, and bacterial growth, if any was assessed.

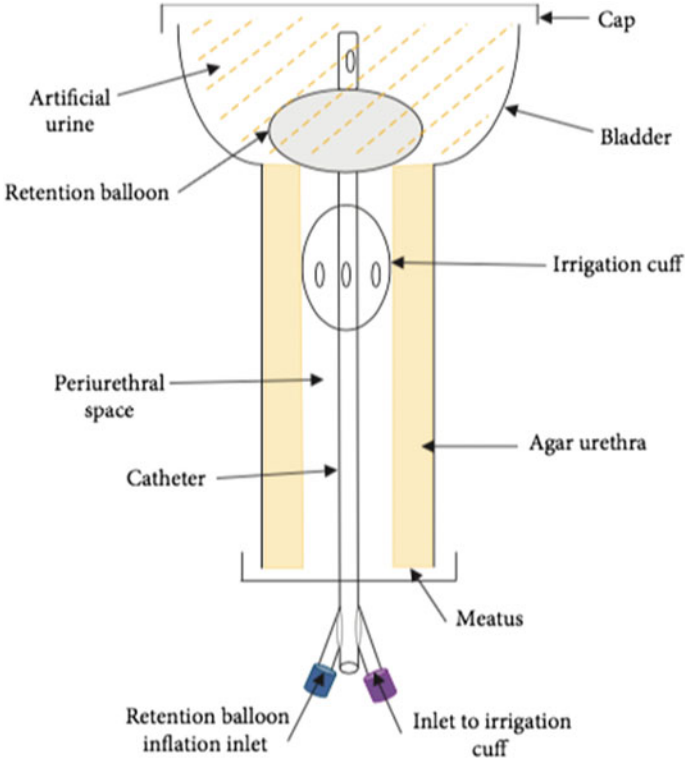

Fig. 13.9 Illustration of in vitro CAUTI model (reproduced from Cortese et al. (2018))

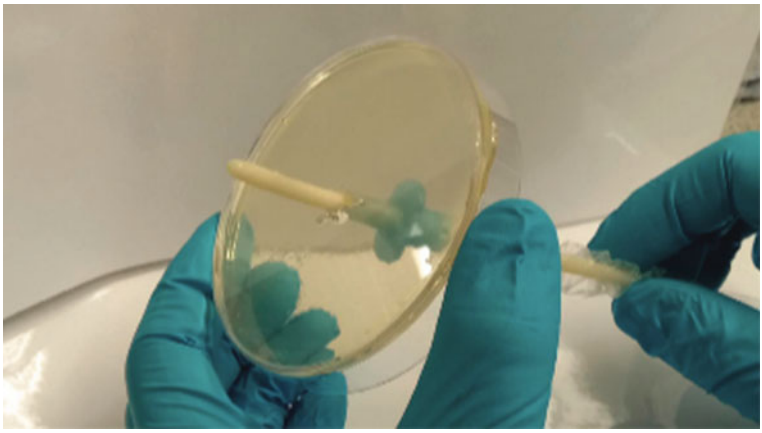

Fig. 13.10 Illustration of in vitro meatus model (reproduced from Cortese et al. (2018))

13.5.4 Meatus Model

This model was developed by Holland and Fish (Cortese et al. 2018) for the testing of intermittent catheters. This model consists of an agar plate with one or more boreholes penetrating both the agar and the plastic petri plate as well. Upper surface of agar acts as outer tissue surrounding urethra, i.e., meatus.

Whole of the agar surface is inoculated with bacteria, and a catheter is then passed through the agar and the boreholes. The portion of the catheter that has passed through the agar is cut off aseptically, and the bacteria is then recovered from the surface of the catheter and bacterial load is calculated (Fig. 13.10).

Thus, CAUTIs are one of the most common nosocomial infections, and few important factors such as type, texture, and chemical properties of material used, growth conditions of material used, bacterial strain used, etc., should be kept in mind while designing in vitro biofilm model.

Such predictive in vitro models may further help in developing material which can be used to minimize bacterial colonization, thereby reducing the occurrence of device-related infections.

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Chapter 14
Biofilm-Mediated Skin Infections

Amresh Kumar Singh, Vivek Gaur and Santosh Kumar Singh

Abstract Approximately 99.9% of microorganisms came in contact with biological and inert surfaces; then, they form biofilm. Skin diseases including atopic dermatitis, various forms of chronic ulcers, paronychia, necrotizing fasciitis, miliaria, cellulitis, erythema nodosum, and erysipelas are usually caused by polymicrobial pathogens. The etiology of these diseases is well studied in recent years and associated with initiation of bacterial accumulation and biofilm formation on the soft tissues or epidermal layer of the skin. Hence, the polymicrobial nature of microorganism can find its way to wound from both exogenous (from water and soil) and endogenous (saliva, urine, skin, and feces) sources. The predominant floras in the skin are *Staphylococcus spp*., *Corynebacterium spp*., and *Propionibacterium spp.,* which cumulatively represent more than 60% of the microbial load in the human skin. It is important to diagnose skin infection for rational treatment decisions, and it provides very useful information for understanding the etiology as well as pathogenesis of the different skin diseases.

Keywords Biofilms · Skin infection · Chronic ulcers · Staphylococcus

14.1 Introduction

The skin is considered as largest organ in human body, and it works as the first protective barrier to detrimental effects of environmental exposure and or changes. The skin also acts as physical barrier and protects our body from the assault of foreign microorganisms and or toxic substances. Moreover, the skin is also colonized by mixture of complex polymicrobial microorganisms that include bacteria, fungi, and viruses. The composition of these microbial communities depends upon various skin

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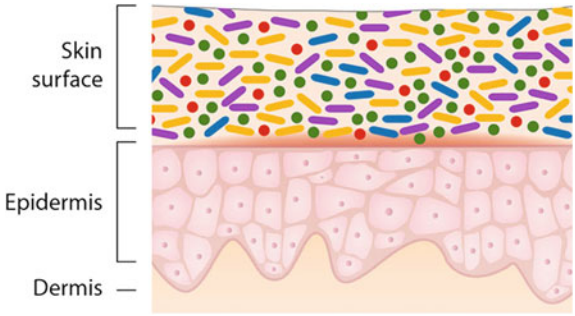

Fig. 14.1 Bacterial biofilms in skin

characteristics such as concentration of sebaceous gland, moisture content, temperature as well as host's own genetics composition and various environmental factors (Picardo and Ottaviani 2014).

Several scientists had already proven the formation of biofilms, both in vitro and in vivo by their experiments using different bacteria isolated from human skin. Previously, bacterial biofilms causing chronic wounds among skin diseases are well understood, but now the presence of biofilms has been associated with wound development, skin infections, and improper wound healing (Brandwein et al. 2016) (Fig. 14.1).

Approximately 99.9% of microorganisms came in contact with variety of surfaces, i.e., biological and inert surfaces; then they form biofilm. When these microorganisms once bind to any biological and inert surfaces, they produce extracellular polymeric substance (EPS) and formation of biofilm starts (Jamal et al. 2015). An estimated 1 million different bacteria, with more than hundreds of different species, inhabit each square centimeter of skin area. Many microbes may cause even various non-infectious pathologies, such as atopic dermatitis, psoriasis, rosacea, and acne in human beings. The complex relationship between host and microorganism not only causes human disease, but also alters in the commensal ecosystem of the different organs. Primary or secondary imbalance is caused due to the changes in host skin; its immunity and this imbalance potentiate epithelial dysfunction, immune dysregulation, or overgrowth of pathogenic microbes (Chen and Tsao 2013).

Biofilm causes a major public health problem due to its resistant nature to most of the commonly used antibiotics and is mainly associated with indwelling urinary catheters and other medical devices (Jamal et al. 2015). Bacteria surviving in a biofilm cannot be killed by routine and normally advised dose of antibiotics. For example, a group of bacteria residing on the outside of a metal screw implanted in a person's leg bone are safe inside their protective biofilm. When the antibiotic comes along, it gets caught up in the sticky extracellular polymeric substances and does not even reach up to the bacterial populations. Some bacteria on the outside of the biofilm may be killed by the antibiotic, but the bacteria at the bottom of the biofilm may be in a dormant mode or "sleeping" state, which makes them resistant to most of the antibiotics. This means, even if antibiotic gets inside a biofilm, it does not always kill all the bacteria at a time. In fact, it would take 1000 times more antibiotic concentration to kill all

Table 14.1 Biofilm-mediated different skin infections (OpenStax Microbiology 2018)

Disease	Pathogen	Symptoms
Acne	*Propionibacterium acnes*	Comedones (whiteheads, blackheads); pustules, papules, nodules, or pseudocyst formation
Cellulitis	*Streptococcus pyogenes*	Localized inflammation of dermis and hypodermis; skin red, warm, and painful to the touch
Erysipelas	*S. pyogenes*	Inflammation, swollen patch of skin, often on face; may be suppurative lesions
Erythema nodosum	*S. pyogenes*	Small red nodules, often on shins
Impetigo	*Staphylococcus aureus*, *S. pyogenes*	Vesicles, pustules, and sometimes bullae around nose and mouth
Necrotizing fasciitis	*S. pyogenes*, *Klebsiella spp.*, *Clostridium spp.*, and others	Infection of fascia and rapidly spreading tissue; can lead to sepsis, shock, and death
Otitis externa	*Pseudomonas aeruginosa*	Itching, redness, earache, progressing to fever, pain, and swelling
Staphylococcal scalded skin syndrome (SSSS)	*S. aureus*	Erythema and severe peeling of skin
Wound infections	*P. aeruginosa*	Formation of biofilm in wound

the bacteria in a biofilm community than is needed to kill non-biofilm-associated bacterial community (Okshevsky and Rikke 2016).

It is well studied and proven that *Haemophilus influenzae* has the ability to form biofilm in body and can escape their self from innate immune system. Biofilm-forming ability has been already observed and reported in different bacteria such as *Staphylococcus epidermidis, Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Enterobacter cloacae,* and *Klebsiella pneumoniae* (Table 14.1). A few bacterial species have described with biofilm-forming ability to cause different kind of infections in human body or organ (Jamal et al. 2015).

14.2 Role of Biofilm in Skin Infection

While doing assessment of chronic wounds from skin microscopically often indicates the presence of biofilm. There are three stages of biofilm microbiota which include contamination, colonization, and infection that are used to describe wound

microbiology. The presence of bacterial species in the wound refers to contamination, whereas the term colonization is used for group of different microorganisms which are multiplying within the wound, but they are not enabling to cause systemic infection. Bacteria that do multiply cannot be considered "infective" until and unless they pose detrimental effects to local or systemic area.

The groups of bacteria which cause wound infection are mostly polymicrobial in nature. Bacteria can find its way to a wound from exogenous (water and soil) and endogenous sources (skin, urine, saliva, and feces). This is particularly in case of *Corynebacterium spp.* and *coagulase-negative staphylococcus,* in which they do not harbor any skin infections (Jamal et al. 2015).

The mass of bacteria in the skin is dominated by few bacteria including *Staphylococcus spp*., *Corynebacterium,* and *Propionibacterium,* which represent more than 60% of the bacterial load in the human skin, which is not as with the intestinal flora. Occurrence of mixed population of bacteria with greater prevalence of b-proteobacteria and flavobacteriales found predominantly at volar forearm, ulnar side of the palm and buttock, where dry skin is present. Sites including the inner elbow, armpit, and inguinal crease rich in moist and are dominated by *Corynebacteria spp*., whereas sebaceous-rich skin such as that of the glabella (between the eyebrows), auditory canal (external), superior part of the sternum and posterior area mostly anchorage *Propionibacteria spp*. and *Staphylococci.*

Microbial diversity of the skin is significantly affected by age of patient and skin pigmentation, which revealed by analysis of the skin microbiota from monozygotic and dizygotic twins and their mothers (Yamazaki et al. 2017).

According to Jamal et al. 2015, after examination of wounds of 22 patients by using fluorescence in situ hybridization using peptide nucleic acid probes (PNA FISH) technique and anti-alginate antibodies, it has been reported the presence of *P. aeruginosa* as biofilm. In 2001, it has been observed that bacteria which colonize chronic wounds may exist as biofilm communities. Later in 2003, specimens from those who were suffering from the skin diseases like atopic dermatitis, bullous impetigo, and pemphigus foliaceus were collected, and various dyes were used like ConA, safranin, and immunofluorescent staining with confocal scanning laser microscope to demonstrate the presence of *S. aureus* in skin biofilm.

14.3 Biofilm Formation and Cell-to-Cell Communication

Biofilm formation is a complicated and is now well-understood process, but according to Jamal et al. (2017), it happens sequentially and always starts with attachment to the biotic or abiotic surfaces. After bacterial attachment to the physical or biological surface, microcolonies become stable which result in formation of different microcolony of biofilms at the site of attachment. Once the bacteria have completed the adhesion process, they enter in the process of colonization in which bacteria synthesize extracellular matrix molecules and the burden of attached bacteria is gradually increased. These additional organisms may be the same or different species as the

already adhered bacterial cells. The colonized cells then continue to grow leading to maturation and formation of dense bacterial aggregates, which is the architecture of the biofilm and finally detachment/dispersion using mechanical or hydrodynamic force occurs. They may subsequently re-adhere to the substratum leading to a spread of the colony of the biofilm (Catherine 2018).

Numerous numbers of bacteria are able to communicate with one another through a now well-understood mechanism called quorum sensing (QS) during biofilm formation. It is a system of expression of certain biofilm-related genes with other cells and response related to the thickness of their microcolonies in local population. There are molecules, which attached to the receptors of new bacteria through QS signaling, and this helps in transcription of genes within particular bacteria as well as between multiple bacterial species. QS enables communication between bacterial interspecies, which helps in biofilm formation, signaling for shortages of nutrition, and detrimental environmental conditions, such as antibiotics, disinfectants, bacterial growth, the identification of provoking species. It also facilitates the inception of normal intestinal flora and protection of harmful intestinal flora. Many clinically significant bacteria utilize QS to control the cumulative production of different virulence factors. QS in Gram-positive bacteria occurs through a series of defined events such as production, detection, and response to auto inducers (AIs) (Sreenivasan et al. 2013). The auto-inducing oligopeptides are detected by membrane-bound two-component signal transduction systems in many Gram-positive bacteria (Jamal et al. 2015).

14.4 Pathogenesis and Types of Skin Infection Caused by Biofilms

In many biofilm-associated skin diseases, microorganisms are well studied in relation to their biofilm-forming capabilities and efforts to reform such biofilm production. Despite that direct linkage between different skin microbes, their biofilm states and infection have been summarized in Table 14.1 (Brandwein et al. 2016).

14.4.1 Rosacea

It is the most common chronic cutaneous disorder (dermatoses) affecting age-group in between 30 and 60 years of age and characterized by papules, pustules, centrofacial persisting erythema, telangiectases, and phymas. Depending upon specific clinical manifestations and morphological characteristics of rosacea, the four different subtypes of this disease are phymatous, ocular, erythema to telangiectatic (ETR), and papulopustular (PPR).

Several provoking factors like sun exposure, dietary agents, and various drugs are responsible for the pathogenesis of rosacea. Ongoing inflammation of cutaneous

vascular system, dermal matrix degeneration, lymphatic system, and abnormalities of the glandulae sebaceae have conjointly been represented as main factors concerned within the pathophysiology of microorganisms like *Helicobacter pylori, Demodex folliculorum, Staphylococcus epidermidis,* and *Chlamydia pneumoniae* which has been known for their role in causation of the disease.

According to recent research, an abnormal signaling of innate immune pattern recognition receptors is found in rosacea patients. Toll-like receptors (TLRs) start the mechanism by that innate immune system activate production of inflammatory cytokines through cascade by the identification of specific microbial products leads to host insult and injury (Takeda et al. 2003). The epidermal layer of the affected person by rosacea expresses rich amount of TLR2 than healthy person, and this one stipulates a possible clarification for the increased inflammatory reaction to external stimulants. Moreover, as a result there is abnormal production of cathelicidin antimicrobial peptides due to increased expression of TLR2 and multiply expression and hyperaction of serine protease kallikrein (KLK5) resulting into clinical manifestation of rosacea.

In addition, recent knowledge indicates the pathogenesis within the development of small intestine's rosacea by bacterial overgrowth (SIBO). Rosacea patients have markedly higher chances of having SIBO ubiquity than healthy individuals, and its destruction ends up in a significant suppression of rosacea lesions. Moreover, rosacea patients, SIBO-negative, do not obtain any improvement after targeted antibiotic treatment (Parodi et al. 2008; Picardo and Ottaviani 2014).

14.4.2 Acne Vulgaris

Acne vulgaris may be a very common and complex disorder of the pilo-sebaceous follicles that involve sebaceous hyperplasia, hyperkeratinization of follicles, hormonal imbalance, bacterial infection, and immune hypersensitivity reactions. Laboratory isolation of *Propionibacterium acnes* from acne lesions in patients establishes the causation and pathophysiology between this skin disorder and native *P. acnes* infection. *P. acnes* influences inflammatory cascade through a large variety of pathways, starting from polymorph chemotaxis by *P. acnes* lipase enzyme to direct induction of TLRs in keratinocytes.

Antibacterial agents like benzoyl peroxide, clindamycin, erythromycin, tetracycline, azelaic acid, triclosan, and different combination of these play an important role for the treatment of acne except keratinolytic and sebo-suppressive agents like retinoids. In case current treatment is not successful or patient's in danger for scarring or pigmentation of the adjacent skin and pigmentary changes, then general antibiotics like tetracycline, doxycycline, minocycline, and erythromycin are used.

The failure of antibiotic treatment in acne vulgaris patients might rather be due to presence of high resistance of sessile *P. acnes* cells. This high resistance to several commonly used antibiotics (including penicillin and clindamycin) and disinfectants (including benzoyl peroxide) was demonstrated in several in vitro studies. It was

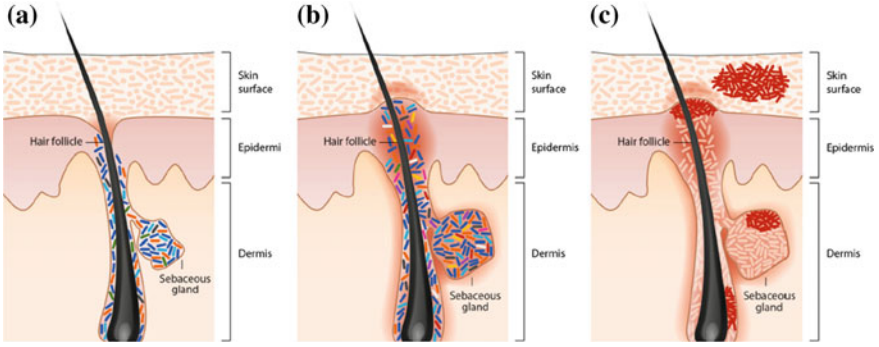

Fig. 14.2 Difference between acne patients (**b**, **c**) and healthy individual (**a**) Relatively *P. acnes* strain abundance in the nose of pilo-sebaceous gland is different between acne patients and healthy individual. **a** Normal relative abundance of dominant *P. acnes* strains in healthy individuals. **b** Acne-induced dysbiosis is characterized by a decrease in the relative number of *P. acnes* strains RT3 and RT6, and an increase in the relative number of strains RT4, RT5, RT7, RT8, RT9, and RT10 (Fitz-Gibbon et al. 2013). **c** The persistent nature of acne vulgaris and its ability to be only partially altered through antibiotics can be due to pockets of biofilm-forming *P. acnes* strains located on different skin appendages, including skin surface, the sebaceous gland, hair follicle and the pore (courtesy by Brandwein et al. 2016)

also demonstrated that sessile biofilm *P. acnes* cells manufacture considerably a lot of enzyme than their being counterparts, which was most pronounced in isolates from patients of acne. Lipase may be an important virulence factor of *P. acnes,* and inhibition of lipase enzyme has been now advised as a correct treatment of acne (Coenye et al. 2008) (Fig. 14.2).

14.4.3 Atopic Dermatitis

Atopic dermatitis (AD) also commonly known as atopic eczema is chronic inflammatory skin disease characterized by intense itching and recurrent eczematic lesions on the skin and significantly affects the quality of life of approximately 10–20% of children in Western countries. AD imposes a significant psychosocial burden on patients and will increase the risk of other allergic manifestations like asthma, allergic rhinitis, food allergy, and other immune-inflammatory-mediated diseases. AD is considered as a childhood disorder that occurs due to an imbalance against a T-helper immune response resulting in increased production of IgE Ab in responses to allergens (Yamazaki et al. 2017).

Two mechanisms are proposed to classify the pathogenesis of the AD, the first one proposes root cause lies in an inherent lacking in function of barrier epithelial cells, which leads to the immune response typical of AD lesions and second an immune defect, which leads to symptoms of AD (Brandwein et al. 2016). The pathophysiology of AD associated with defects in the epidermal layer is more complex that can

be explained by inherited mutation in keratinocyte proteins such as filaggrin that increases the susceptibility to AD (Yamazaki et al. 2017).

In AD*, S. aureus* is responsible to colonize on the skin of almost 90% of patients. Growth of *S. aureus* on skin has been proven to aggravate AD state through several immune-mediated mechanisms, resulting in inflammation and sensitization of skin. Moreover, the overwhelming majority of *S. aureus* obtained from AD lesions were shown to be significantly responsible for biofilm production in vitro, the existence of biofilms on skin is confirmed by Congo red staining method (Brandwein et al. 2016).

Studies uncovered that nearness of explicit IgE Ab against few *S. aureus* Ag was related to serious signs in AD patients. The anti-*S. aureus* IgE Ab reactivity against bacterial antigens was identified in patients of AD, yet not in those patients experiencing different skin issue including unfavorably susceptible bronchial asthma and conjunctivitis. The abundance of the skin commensal bacteria, i.e., *S. epidermidis* also significantly increases during flare-up phase of AD. Further, an expansion in *Streptococcus spp., Propionibacterium*, and *Corynebacterium species* is additionally seen after treatment. These various researches showed that expansion in colonization by *S. aureus* is normal for skin lesion in AD and related to infection flare-up rate. In any case, regardless of whether the role of *S. aureus* is in causation of AD stays to be resolved (Yamazaki et al. 2017).

14.4.4 Cellulitis, Erythema Nosodum, and Erysipelas

Cellulitis, erythema nodosum (EN), and erysipelas are common streptococcal states of the skin. Cellulitis is a disease that progress in the epidermis or hypodermis which displays as a blushed region of the skin that is warm and painful to the touch, caused by *S. pyogens,* which invade the epidermal region through a already cut or scraped area. Despite the fact that cellulitis may else be caused by *Staphylococci spp.*

S. pyogens can cause erysipelas, clinically presents as a large, dreadful inflamed patch often on the legs or face involving the epidermis. These diseases can be presented as suppurative, which resulted in bullous formation, which are a type of erysipelas. Many pathogens including *Streptococcal spp.* may also responsible to cause EN, portrayed by irritation in the adipose tissue of subcutaneous part of the hypodermis. However, different pathogens can likewise cause the same disease. It is not suppurative, however prompts reddish bruise on the skin, most oftentimes on the shins (OpenStax Microbiology 2018) (Fig. 14.3).

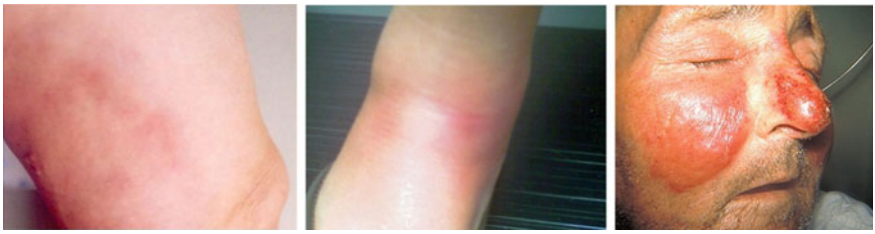

Fig. 14.3 Cellulitis, erythema, and erysipelas (courtesy by OpenStax Microbiology 2018)

14.4.5 Onychomycosis

Onychomycosis is a typical continuous nail contamination caused by *Trichophyton rubrum*, *T. mentagrophytes,* and *Candida spp.* Dermatophytoma is a complication of onychomycosis that conversely influences the chance of complete cure. It is suggested that contagious fungal biofilms leads to resistance of dermatophytomas to commonly used antifungal treatment. This is explanation for the presence of biofilms which is the chronic nature of onychomycosis. The capacity of dermatophytes to frame biofilms has not yet been fully explained, but a few types of yeast have been appeared to shape biofilms. What's more, histopathological examination of nail clippings in onychomycosis cases exhibited resting spores blended and intermixed with effectively developing fungal hyphae. At the point when a base thickness of microscopic organisms/growths collects, qualities associated with biofilm arrangement are incited. Thus, accordingly expanding the chances of effectively setting up a develop biofilm preceding insusceptible identification. This finding is supported with the depiction of fungal biofilms in which many cells exists in a torpid state (Gupta et al. 2018; Vlassova et al. 2011) (Fig. 14.4).

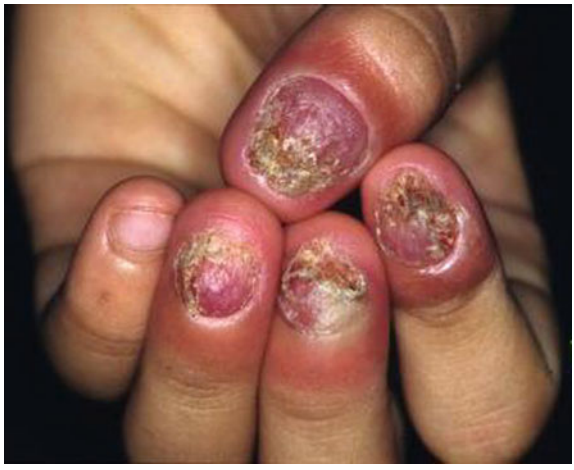

Fig. 14.4 Onychomycosis (courtesy by Tosti 2018)

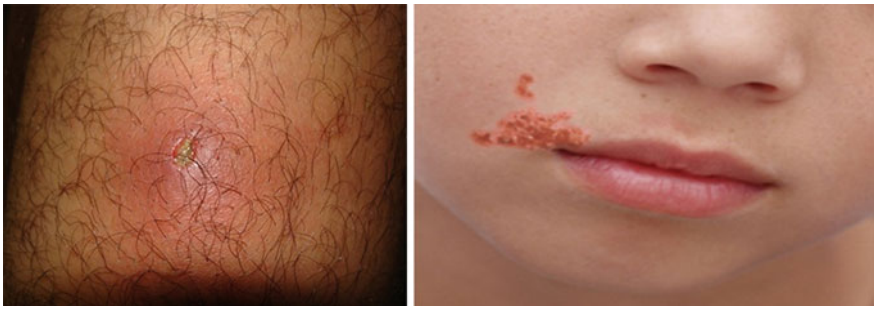

Fig. 14.5 Furuncles and impetigo (courtesy by OpenStax Microbiology 2018)

14.4.6 Furuncles and Impetigo

At long last, furuncles and impetigo are regarded as acute infectious diseases that have been appeared to be related to sessile bacterial colonies formation which leads to biofilm formation. In case of impetigo, it is a superficial contamination of the skin caused by group A *Streptococci or Staphylococcus aureus*. A perifollicular abscess is known as furuncles caused by *S. aureus*. Development of periodic acid–Schiff (PAS)-positive and Ruthenium red (RR)-positive appearance around *S. aureus* segregated from impetigo and furuncle sores in vitro. Two similar groups of examinations later exhibited the arrangement of glycocalyx by *S. aureus* isolated from furuncle and impetigo sores in vivo. It is examined by the researchers that *S. pyogens in nonbullous impetigo* sores in vivo utilizing CLSM and *S. pyogens* cells shaped into microcolonies circled by glycocalyx in the external dividers of the injuries, and these settlements existed freely from microcolonies framed by *S. aureus* (Vlassova et al. 2011) (Fig. 14.5).

14.4.7 Staphylococcal Scalded Skin Syndrome

Staphylococcal scalded skin syndrome (SSSS) is a shallow contamination due to *S. aureus*, mostly found in younger's, particularly infants. Primarily, redness of the skin (erythema) occurs due to secretion of bacterial exotoxins afterward, scalding followed by serious stripping of the skin. It is diagnosed by different characteristics of skin. SSSS is assessed by investigating quality of the skin (skin has come off effortlessly), elevated lymphocyte counts, microbial culturing, and other molecular methods. Fluid management and intravenous antibiotics are used preferably for the treatment of SSSS (OpenStax Microbiology 2018) (Fig. 14.6).

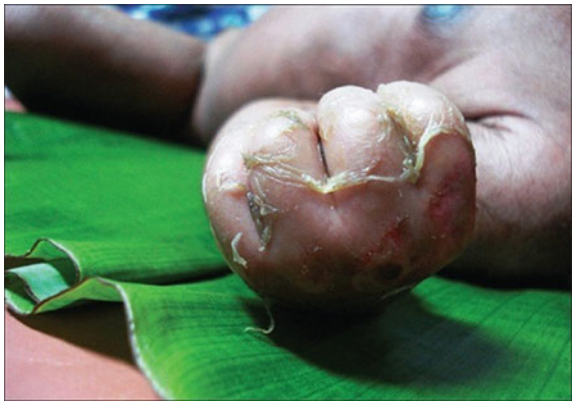

Fig. 14.6 Appearance of staphylococcal scalded skin syndrome

14.4.8 Miliaria

Commonly referred to as roseola caused by barrier of the eccrine ducts, key to sweat retention in several layers of the epidermis. The forearms of healthy people inoculated with many strains of *coagulase-negative Staphylococcus spp.* (*CONs*) under occlusion end up in development of miliaria. All the *CoNS* tested only the EPS-producing strains of *S. epidermidis* evoked miliaria. Given this finding and also the method that the sweat glands were observed to be choked by EPS, it is concluded that *S. epidermidis* has the ability to create biofilm and play a crucial role within the pathogenesis of miliaria (Vlassova et al. 2011).

14.4.9 Necrotizing Fasciitis

Necrotizing fasciitis occurs when the fascia (a thin layer of connective tissue between the skin and muscle) becomes infected. *Streptococcal* infections that start in the epidermal layer of the skin can sometimes spread by the formation of biofilm, may progress into a rare but potentially life-threatening clinical condition called necrotizing fasciitis, also known as flesh-eating bacterial syndrome. Among bacterial pathogens *Streptococcus pyogenes* is a type of bacteria that can cause this uncommon, however, conceivably lethal condition; others incorporated organisms are *Klebsiella spp., Clostridium spp., E. coli, S. aureus*, and *Aeromonas hydrophila*.

It occurs when virulence factors of *S. pyogenes* overcome host defense mechanism that is responsible for adhesion and invasion up to fascia. *Streptococcus pyogenes* invasions enable bacterial cells to adhere to tissues and initiate pathogenesis of infection. Bacterial proteases distinctive to *S. pyogenes* lead to aggressively infiltrate and damages host tissues, inactivate complement system, and inhibit neutrophil migration to the site of infection. This damage spread rapidly and resulted in tissue death,

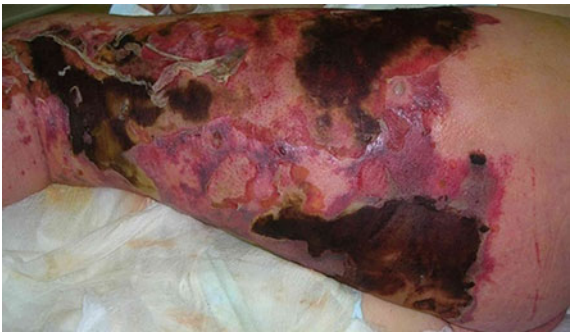

Fig. 14.7 Left leg of this patient shows the necrotizing fasciitis

as a result large areas of skin become detached and patient die. Containment of necrotizing fasciitis includes surgical removal of dead or infected tissue or amputation of infected limbs to terminate the spread of the infection followed by intravenous higher antibiotics and other supportive therapies.

In contrary to this fact, in some cases of necrotizing fasciitis, there is no well-defined cause of entry of such pathogen occurs, so it does not always emerge from a biofilm (OpenStax Microbiology 2018) (Fig. 14.7).

14.4.10 Pseudomonas *Infections of the Skin*

Another important Gram-negative, oxidase-positive, aerobic bacillus which has the ability to form biofilm on a wide range of surfaces generally found in soil and water as well as on human skin as commensal. *Pseudomonas aeruginosa* is a typical reason for entrepreneurial contaminations of superficial wounds and burns cases. It can also cause hot tub rash, a clinical condition portrayed by folliculitis that often trouble swimming pools users or hot tub full of water used for hydrotherapy, relaxation, or pleasure.

It may harm swimmer's ear also, which leads to an auditory canal infection, ie. otitis externa that causes tingling, redness, distress, and can advance to fever, agony, and swelling. Typically treated by antibacterial, or steroids to reduce inflammation, ear drops containing acetic acid also include antifungal drops because sometimes fungi can cause this disease.

Wound infections caused by *Pseudomonas* spp. have a typical odor (grape soda or fresh corn tortillas) due to the 2-aminoacetophenone that is used by *P. aeruginosa* in process of quorum sensing and contributes into pathogenicity. On the basis of this, it may be treated with topical antibiofilm agents that have capacity to disrupt the formation of biofilms (OpenStax Microbiology 2018) (Fig. 14.8).

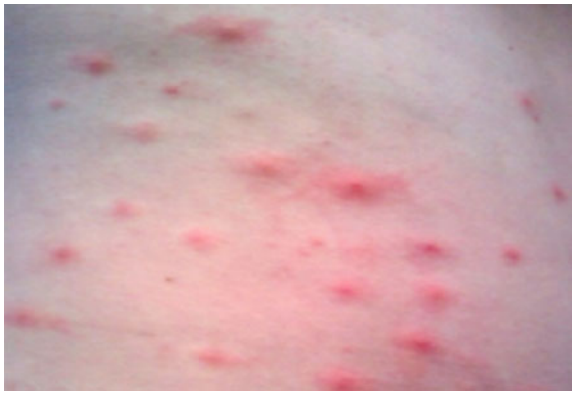

Fig. 14.8 Folliculitis caused by *P. aeruginosa*

14.4.11 Paronychia

Paronychia is a standout among the most widely recognized diseases of the hand due to the formation of biofilm epidermally. Paronychia is limited, shallow contaminations, or abscesses of the perionychium. Non-infectious reasons for paronychia incorporate contact aggravations and intemperate dampness. Clinically, paronychia presents as an acute or chronic course.

Acute paronychia most usually results from nail gnawing, finger sucking, forcefully manicuring, a hang nail or entering injury, with or without retained foreign particles. An artificial fingernail arrangement has likewise been appeared to be related to the advancement of paronychia. *Staphylococcus aureus* is the most common infecting organisms, trailed by *Streptococcus spp.* and *Pseudomonas spp.* Gram-negative microorganisms, dermatophytes, and yeasts have additionally been accounted for as causative agents. Kids are prone to acute paronychia through direct contact of hands with flora from the mouth and secondary to finger sucking and nail gnawing (Leggit 2017) (Fig. 14.9).

Chronic paronychia is a fiery hard-headed turmoil influencing the nail folds. It tends to be characterized as an irritation going on for over about a month and a half and including at least one of the three nail folds (one proximal and two horizontal). The patient presents with redness, delicacy, swelling, liquid under the nail folds, and thick stained nail. Morphologically, it is portrayed by induration and adjusting off of the paronychium, repeating scenes of intense eponychial aggravation and drainage. Nail plate may demonstrate thickening and longitudinal scoring. Onychomadesis, transverse striation, setting, and hypertrophy can be available and are most likely because of irritation of nail lattice. Nail plate may introduce a green staining of its parallel edges because of *P. aeruginosa* growth, and *Candida albicans* is also one of the reasons for chronic paronychia.

Paronychia reacts gradually to treatment and may take half a month or months, but this should not be a deterrent to therapy. On the off chance that the patient is not dealt with, sporadic excruciating scenes of intense irritation might be experienced

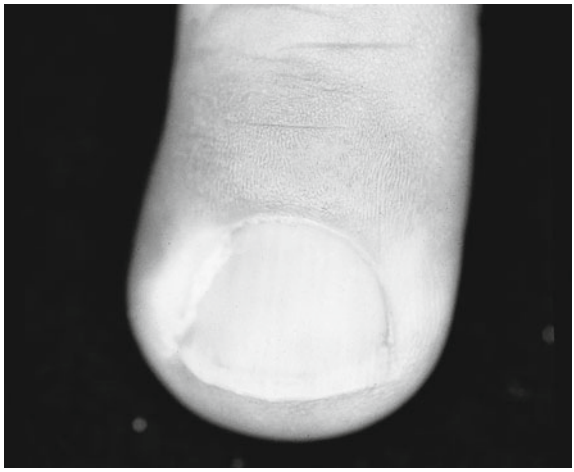

Fig. 14.9 Acute paronychia (courtesy Leggit 2017)

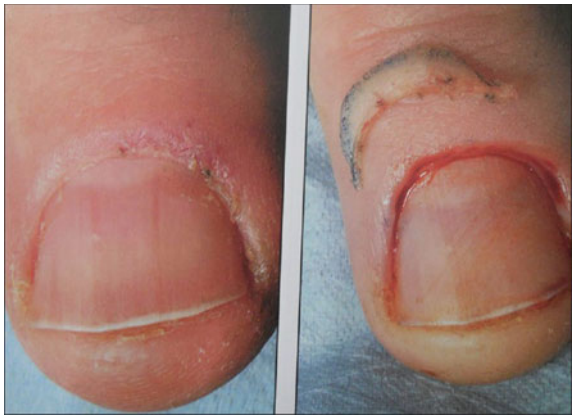

Fig. 14.10 Eponychial marsupialization in case of chronic paronychia

because of non-stop entrance of different pathogens (Relhan et al. 2014; Leggit 2017) (Fig. 14.10).

14.4.12 Chronic Non-healing Ulcers

Chronic or non-recuperating ulcers are characterized as unconstrained or awful injuries, typically found in lower extremities of human body that are inert to initial treatment or that endure regardless of appropriate care and do not proceed toward healing in a fixed time of period with an underlying etiology that might be identified as systemic disease or local disorders. There are numerous kinds of non-healing ulcers that may include venous, arterial, diabetic, pressure, and traumatic ulcers.

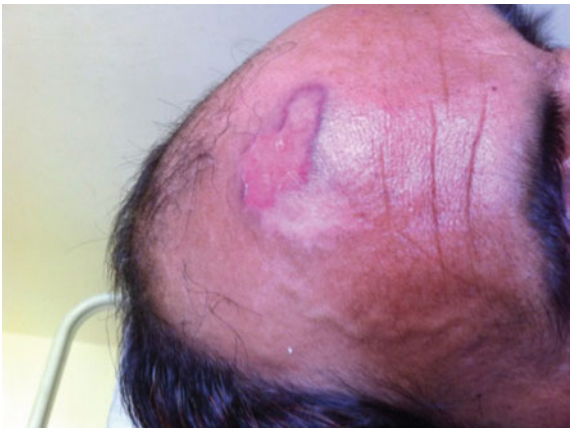

Fig. 14.11 Chronic non-healing ulcer on forehead

The typical injury recuperating process is dynamic and complex having three stages: aggravation, tissue arrangement, and tissue renovating. However, if the normal healing process is slow or incomplete, an ulcer can become chronic in nature due to absence of growth factors and cytokines which delay the healing process (Suthar et al. 2017) (Fig. 14.11).

According to Iqbal et al. (2017) chronic non-healing caused due to colonization of anaerobic bacteria such as *Bacteroides*, *Clostridium,* and *Streptococcus* ulcer and is a major health issue which may be active at levels of endodermis, insulated from the healing influence of oxygen. Anaerobic microorganism responsible for several devastating infections leads to gangrene of organ or tissue. Aerobic bacteria are more closely identified with superficial layers of skin but may also be involved in infective processes through the formation of biofilm and include *Staphylococcus epidermis*, *Corynebacteria spp.,* and *Propionibacteria.*

Lower extremity ulcers contain generous extent of interminable ulcers, particularly those attributed to venous sickness, diabetes, or blood vessel infection. Chronic no-healing ulcer is a serious health issue and its prevalence calculated approximately from 1.9 to 13.1% in the different parts of world. It is accounted that almost 10% of the population would develop a chronic wound in their lifetime, with wound-related mortality rate of 2.5%. These types of chronic non-healing ulcers not only affect the comfort and productivity of the patient but also become a substantial financial burden for the patient and the healthcare system (Suthar et al. 2017).

14.4.13 Other Biofilm-Related Skin Infections

There are several reports on skin-related biofilm formation by different microorganisms, and these are frequently based on the minute observation of microcolonies on skin. In most of the cases, attention has been paid to *S. aureus,* and few reports

have shown microcolony development and creation of an extracellular grid in skin lesions both in vivo and in vitro. Arrangement of microcolony by *S. pyogenes* in infected skin has likewise been illustrated, again recommending that biofilm development may assume a role in skin disease by this organism. Likewise, in vitro biofilm arrangement has been exhibited for different individuals from the common vegetation of the human skin, including *S. epidermidis* and different *Corynebacteria spp*. (Coenye et al. 2008).

14.5 Conclusion

In human, bacterial biofilms can be presented on many surfaces of body such as the skin, teeth, and mucosa. Most bacteria are capable of forming biofilms leads to many human diseases. In addition to plaque-forming bacteria, *Streptococci, Staphylococci, and Lactobacilli* also frequently form biofilm (Catherine 2018). In biofilm, bacteria perform different metabolic and physiological reaction to become more virulent and resistance to normally used antibiotics. This is the situation where biofilms may be involved in the etiology and aggravation of various cutaneous and skin disorders. Studies based on combination with culture-independent sequencing techniques are now developed to expose the complexity of the skin microbiome, and its function leads to common skin disorders. Subsequently, by invent of newer methods of diagnosis of skin disorders that regarded as non-infectious contamination of skin may prove to include as infectious component, or to be associated with more than one microbial agents (Brandwein et al. 2016).

Skin-related biofilms bacteria have expand to grow on specific skin niches, and their mechanism of attachment, survival, and propagation on skin is only partially understood (Percival et al. 2012). Future studies should include measuring the expression of biofilm-related genes in different skin disease lesions, and/or using proteomics methods to categorize microbial biofilms on the skin. At last, inducing biofilm growth in vivo skin models and eventually profiling the physiological and molecular adaptations are undertaken by both the organ and microbe, which can help further to understand the skin–biofilm relationship and potentially lead to the development of novel therapeutics to control such infections.

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Chapter 15
Approaches Towards Microbial Biofilm Disruption by Natural Bioactive Agents

Rolee Sharma, Preeti Bajpai, Uzma Sayyed and Iffat Zareen Ahmad

Abstract Biofilms formed by microbes are the aggregates of bacterial masses that are fixed in the matrix produced by itself comprising of extracellular polymeric substances (EPS). Microbial biofilms pose serious threat to the hospital-based infections as well as other types of infections. This is because biofilm provides highly enhanced protection and tolerance to the pathogens towards antimicrobial compounds. Moreover, the pathogen also survives the immune response of the host. This leads to extremely intractable, prolonged infections resulting in high tolls of morbidity and mortality. The fact that around 80% of human diseases are biofilm-based; the scientists have started to explore effective remedies to precisely aim at the disruption of biofilm, thus, diffusing the cells of microbes into their more susceptible planktonic type of life. With the advent of the significance of biofilm disruption to combat serious infections, various antibiofilm agents have been investigated for their efficacy. This includes some primary metabolites including complex carbohydrates, peptides and fats and various categories of secondary metabolites. Many enzymatic biofilm dispersal agents have also attracted the attention of those working in the given area. Other dispersal compounds include anti-matrix molecules, dispersal signals and sequestration molecules. These antibiofilm agents have shown high effectiveness in inhibiting clinically relevant pathogens. These biofilm dispersal agents will pave a way for a new approach towards future drug development for the treatment of clinically severe infections.

Keywords Biofilm · Pathogens · Quorum sensing · Dispersal agent · Enzymatic · Natural compounds

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15.1 Introduction

Microbial biofilms are the masses of cells which are fixed in a matrix produced by itself in extracellular polymeric substances (EPS). These biofilms pose serious damage to human health by causing various lethal infections which are strong and show resistance to antibiotics and immune system of host. This makes the treatments of pathogens challenging and expensive.

In the environment under natural conditions, microorganisms frequently grow on various biotic and abiotic planes in the form of multi-cellular populations which are recognized as biofilms. Microbes can form biofilms by their capability of attachment to household items like showers, taps and water tanks besides living in the form of biofilms in nature (Mullis and Falkinham 2013; Rozej et al. 2014; Xu et al. 2014). *Pseudomonas aeruginosa* is the most common organism which forms biofilm in the household system (Mullis and Falkinham 2013). Bacterial biofilm populations can comprise of multiple cell layers and also in the form of mushroom-like assemblies as in case of *Staphylococcus epidermidis* and various *Pseudomonas* species. The morphology of biofilms vary from dry, flat and wrinkled colonies on agar plates as in case of *Bacillus subtilis* and *Vibrio cholerae* (Bester et al. 2010; Lopez et al. 2010; Seper et al. 2014) to small yellow air balloons as in *Myxococcus xanthus* (Dubey 2003; Jiyoung et al. 2009). *Anoxybacillus flavithermus* is capable of forming biofilms in silica and is a danger in processing of food (Saw et al. 2008). But the common character of all microbial biofilms is a matrix surrounding the cells, a slimy layer on the surface of each bacterial cell that provides defence to the cells and provides food and water to bacteria (Bester et al. 2010; Lopez et al. 2010). The matrix comprises of complex polysaccharides, proteins, phospholipides and extracellular DNA (Christner et al. 2010, 2012; Linnes et al. 2013; Reichhardt et al. 2014; Becker et al. 2014). Occasionally, the matrix appears in different colours (pink, brown or blackish) so as to prevent DNA impairment by solar radiations (Xu et al. 2014). Nutrients and other materials can be pooled amongst the microbial cells by passive diffusion through the porous matrix. In this manner, they work as cell communication machineries, and this phenomenon is known as quorum sensing (Banat et al. 2014). The most significant consequence of the matrix is the defence against external stimuli and mechanical destruction. Moreover, the matrix also guards the cells from chemical compounds for instance antibiotics, antimicrobial agents and disinfectants. Additionally, the biofilm also safeguards bacterial cells against shearing forces, physical and chemical forces and inadequate availability of nutrient (Taylor et al. 2014; Banat et al. 2014). The cells present in the matrix share mutual benefit from one another and help in each other's progression and survival (Xu et al. 2014; Bester et al. 2010).

15.2 Development of Biofilms as a Threat to Human Health

In the early 1940s, antibiotics have been introduced to human medicine and now emerged as a major threat to public health at an alarming rate. This problem is amplified by pathogenic bacteria existing most commonly in biofilm form, creating additional bacterial tolerance to antimicrobial agents and has been considered as primary cause of chronic infection, transforming bacteria into antibiotic-resistant form in biofilm formulation (Bowler 2018). Biofilm-associated bacteria are 1000 times more resistant to antibiotics than their planktonic counterparts and are often insensitive towards host immune system (Olson et al. 2002). The resistant mechanism attributed by biofilm bacteria due to resistant capsules, enzyme-mediated resistance, heterogeneity in metabolism and growth rate, metabolic state of the organisms in the biofilm, genetic adaptation and most important effective quorum sensing and other membrane modification (Singh et al. 2017).

The mono-species of bacteria which have the capacity to synthesize a biofilm in clinical surroundings are accountable for biofilm-related diseases, in both animals and humans (Dubey 2003). These are most frequently seen not only close to oral areas like dental caries and periodontitis, or in infections of respiratory tract in cystic fibrosis patients (Lambiase et al. 2009; Hall-Stoodley et al. 2004), but also found on the exteriors of implanted medical devices. Amongst clinical pathogens, the most dangerous are gram-positive, coagulase-negative staphylococci (e.g. *S. epidermidis*) and coagulase-positive *S. aureus* that make biofilms on abiotic surfaces (Rohde et al. 2006; Mack et al. 2006; Moretro et al. 2003; Rupp and Archer 1994; Mack et al. 1992). Microbes have enhanced pathogenicity in the form of biofilm and frequently cause infections on artificial biomedical parts such as surgical pins or hip joints (McCann et al. 2008). Infections can occur in cardiac pacemakers with a trailing endocarditis and intravenous catheters because of extracellular matrix of human and coating of the implant by serum being rich in nutrient and provide environment for the growth (Hall-Stoodley et al. 2004; McCann et al. 2008). This is the reason that the disease and death of hospital acquired, nosocomial infections are increasing each year (Rohde et al. 2006; Rupp and Archer 1994). Majority of the cells associated with material-associated infections are due to *S. epidermidis* cells, as they are skin inhabitant where wounds and implants are easily accessible (Gotz 2002). Another deleterious effect of biofilm synthesis on abiotic planes is the infection of edible items during production of food and its processing. Meat and milk can be contaminated through multi-resistant staphylococcal species on coming in contact with the biofilm-coated surface (Moretro et al. 2003; Mettler and Carpentier 1998).

The approaches for the inhibition and elimination of these biofilms are very restricted. This is because of the increasing antibiotic resistance of microbial species, but also because of the increased resistance of biofilm-based structure of microbes against antimicrobial agents and disinfectants (Mack et al. 2006; Gotz 2002; Ganeshnarayan et al. 2008). The extracellular polymeric compounds of the matrix are responsible for providing the protection to the microbes against external forces which comprise of polysaccharides, proteins, lipids, nucleic acids and humic acids. These matrix

components also result in the characteristic mushroom-like biofilm assembly (Flemming and Wingender 2010). Considering the complicated structural organization and regulatory network resulting in the formation of biofilm, the understanding of the complexity of the matrix in detail and also the innovative strategies are essential to disperse established microbial biofilms and prevent their occurrence on abiotic surfaces to improve the management of medical patient and food safety. Up to now, novel targets for the screening of antimicrobial agents and vaccine development for the prevention of biofilms are promising (Gotz 2004). The present chapter reviews the latest developments in the area of biofilm disruption taking into consideration various agents which have shown potential towards this.

Biofilms can be developed on almost every moist surface which is most often unwanted as they cause serious complications in various sectors, including the food division. They are recognized as the preferential microbial lifestyle due to the numerous advantages offered by them for the embedded cells. Biofilm cells show strong resistance to stress conditions, mainly to antimicrobials, since their multifarious and compact structure hinders the permeation of antimicrobials and the contact with the cells present deep in the biofilm. The increased resistance to the presently employed control strategies accentuates the urgent requirement of new alternative and/or complementary eradication approaches. To this direction, the use of enzymes is an interesting alternative antibiofilm approach due to their capability to degrade crucial components of the biofilm matrix, cause cell lysis, promote biofilm disruption and interrupt the cell-to-cell signalling events monitoring biofilm formation and maintenance. This review provides an overview of the enzymes used for biofilm control, their targets and examples of effective applications (Meireles et al. 2016).

15.3 Natural Antibiofilm Agents

15.3.1 Fatty Acids as Antibiofilm Agent

15.3.1.1 Myriads Role of Fatty Acid Inhibitor (FAI) in Biofilm Formulation

The mode of growth of microorganisms could be manipulated either by preventing foaming of biofilms or by disrupting the existing ones. In this context, extensive literature are available that have detailed signal (extracellular) accountable for biofilm scattering coupled with an array of factors that have been shown to arouse biofilm disturbance. For example, addition of chemicals, rapid reduction in oxygen and increased concentration of organic carbon resulted in cell cluster disaggregation in *P. aeruginosa* (Chen and Stewart 2000). In this section, emphasis will be primarily upon the different fatty acids as inhibitor molecules to augment the vulnerability of biofilm cells by weakening the normal biological processes that maintain biofilm integrity,

and also critically discussed process in controlling bacterial virulence, alteration in cellular phenotype and promote biofilm tolerance (Table 15.1).

15.3.1.2 FAI Interactions with Quorum Sensing (QS)

Owing to its importance in nutrition, fatty acids (FA) are representative of quorum sensing chemicals capable of modulating virulence-associated behaviour of bacterial population. Bacterial pathogen component actively up-regulated the expression of QS system to induce virulence factors that promote biofilm formation and infectious diseases. Recently, it was documented that chains of monounsaturated fatty acids such as palmitoleic acid and myristoleic acids significantly diminished biofilm synthesis of *Acinetobacter baumannii* with drastically reduced motility (Nicol et al. 2018). This might be due to fatty acids involvement in down-regulation of LuxIR-type quorum sensing (QS) communication system, thus consequently reduced the *N*-acyl-homoserine lactone production (AHL). Another medium-chain fatty acid derivative, *cis*-2 decenoic acid (C2DA), showed lethality towards multiple bacterial strains, including gram-positive, gram-negative and yeast strains by inducing dispersion of biofilms, although this biofilm inhibition was only demonstrated in *P. aeruginosa* (Stoodley et al. 2011). Furthermore, combination of antibiotic (linezolid) with C2DA resulted in 16% inhibition of biofilm either individually or in combination with daptomycin and vancomycin. The easy incorporation of linezolid and C2DA into the plasma membrane of bacterial cells and also due to cis-conformation increased membrane permeability are the fundamental mechanisms for enhancement of antibiofilm activity of these compounds (Jennings et al. 2012). Global gene expression analysis lyngbyoic acid (LA)-treated *P. aeruginosa* revealed that LA down-regulates gene controlled by quorum sensing (Kwan et al. 2011). Another study conducted by Zhen Cai et al. clearly revealed that fatty acid diffusible signal factor (DSF) binds and allosterically activates histidine kinase RpfC of phytopathogenic bacterium *Xanthomonas campestris* to regulate quorum sensing and virulence. The role of fatty acid or its derivative is directly or indirectly involved in inhibitor of biofilm formulation as well as virulence, but précised mode is doubtful in various studies hence extensive effort is required for better understanding.

15.3.1.3 Fatty Acid as a Signalling Molecule

In pathogenicity research, fatty acid molecules have already been identified as signalling as well as inducer molecules, ranging from yeast to gram-positive and gram-negative bacteria which regulate a wide range of cellular functions (Marques et al. 2014). In addition to this, signals generated by fatty acid are engrossed in intra-species, inter-species and cross-kingdom communication. These signals are known to regulate motility, virulence, polymer production, biofilm development, biofilm dispersion, bacterial growth and persistence. The different fatty acids as signalling molecules in various organisms have been summarized in Table 15.1. More than 50

Table 15.1 List of fatty acid signalling molecules reported in various microorganisms with their known functions

S. no	Compound name	Bacterial species	Function	References
1	cis-11-methyl-2-dodecenoic acid (DSF)	*Xanthomonas campestris, Xanthomonas oryzae, Stenotrophomonas maltophilia, Burkholderia multivorans*	Virulence, biofilm formation, floc Disaggregation, microcolony formation, tolerance to antibiotics, detoxi fication, hyphal growth inhibition	Wang et al. (2004), Deng et al. (2010), Tang et al. (1991), Barber et al. (1997), He et al. (2010), Huang and Wong (2007)
2	cis-2-dodecenoic acid (BDSF)	*Burkholderia cenocepacia, Burkholderia lata Burkholderia stabilis Burkholderia vietnamiensis Burkholderia dolorosa Burkholderia ambifaria Burkholderia anthina Burkholderia pyrrocinia B. multivorans, X. oryzae*	Virulence, hyphal growth inhibition	Deng et al. (2010), He et al. (2010), Boon et al. (2008)
3	cis-2-decenoic acid (cis-DA)	*Pseudomonas aeruginosa*	Biofilm formation, biofilm dispersion, persister cell formation, persister cell awakening, tolerance to antimicrobials	Davies and Marques (2009), Marques et al. (2014), Sepehr et al. (2014), Jennings et al. (2012), Rahmani-Badi et al. (2014)
4	cis-2-tetradecenoic acid	*Xylella fastidiosa*	Virulence and aggregation	Beaulieu et al. (2013)
5	trans-2-decenoic acid (SDSF)	*Streptococcus mutans*	Hyphal growth inhibition	Vílchez et al. (2010)
6	cis-11-methyldodeca-2,5-dienoic acid (CDSF)	*B. multivorans, B. stabilis B. anthina, B. pyrrocinia, X. oryzae*	Hyphal growth inhibition	Deng et al. (2010), He et al. (2010)

(continued)

Table 15.1 (continued)

S. no	Compound name	Bacterial species	Function	References
7	12-methyltetradecanoic acid	*Xylella fastidiosa*	Virulence, biofilm formation, motility	Colnaghi Simionato et al. (2007), Chatterjee et al. (2008)
8	3-hydroxypalmitic acid	*Ralstonia solanacearum*	Virulence	Flavier et al. (1997)
9	3-(3-hydroxy alkanoyloxy) alkanoic acids (HAAs), and phospholipids (e.g. phos-phatidylethanolamine)	*P. aeruginosa*	Biosurfactant	Desai and Banat (1997), Lang and Wullbrandt (1999)

molecules including autoinducer-1 (AI-1) also known as *N*-acylhomoserine lactones (*N*-AHL), autoinducer-2 (AI-2) a furanosyl borate, PQS, oligopeptides (5–10 amino acid cyclic thiolactone) known as autoinducer peptides (AIP) and short-chain fatty acids which are typically unsaturated at the number 2 carbon in a *cis* configuration have been identified till date (Parsek and Greenberg 2005; Ryan and Dow 2011; Kalia et al. 2015). Signals of fatty acids are known to increase aggregative behaviour and biofilm formation capability. For example, isolation from *Xylella fastidiosa,* i.e. 12-Me-C14, has been reported to eradicate swarming motility and to subside biofilm formation by capability of *X. fastidiosa* and in *P. aeruginosa*. Apart from bacteria, fungi like *C. albicans* are also documented for the induction of fatty acid signals which reported the production of farnesoic acid that inhibits the formation of germ tube by regulating the morphological transition from a yeast-form to a hyphal-form (Estrela and Abraham 2010).

Biofilm dispersion autoinducers have been reported the experience of involvement of the fatty acid. Like in *P. aeruginosa* commonly known fatty acid signal, *cis*-2-decenoic acid (C10:Δ2) is identified and has been established as a biofilm dispersion auto inducer (Davies and Marques 2009). Moreover, *Propionibacterium acnes*, *Actimomyces naeslundii*, *Lactobacillus casei* and *Streptococcus mutans* when cultured as single or mixed species are seen to be susceptive towards *cis*-DA which induces biofilm dispersion in them. However, an entire comprehensive signalling mechanism of the *cis*-DA system is yet to be elucidated.

15.3.1.4 Fatty Acid Regulation in Biofilm Dispersion

Studies in the past decades related to the effect of fatty acids and its derivatives on the biofilm resulted in concluding on the intracellular mechanisms involved in some species of bacteria. *P. aeruginosa* produces C*is*-2-decenoic acid in batch cultures, and biofilm cultures induce a dispersion response in biofilms formed by a range

of gram-negative and gram-positive bacteria and yeast, as well as in *P. aeruginosa* (Huang and Wong 2007; Chatterjee et al. 2008). It is also well cited that DSF has also played roles for the regulation of pathogenicity in *X. campestris* (Barber et al. 1997) together with sysnthesis of extracellular proteases and exopolysaccharide production, flagellum synthesis, aggregative behaviour, biofilm formation and resistance to toxins. Along with *cis*-2-decenoic acid and DSF, small chain-monounsaturated fatty acids and BDSF have activity across a wide array of bacteria as extracellular signals. If microorganisms depend on the degradation of extracellular polymers produced by neighbouring microorganisms of other species as well as their own species will relieve the cells from the biofilm matrix during a dispersion response. Cross-kingdom activity has been proposed previously for fatty acid messengers from evidence that DSF is recognized by *C. albicans* binding to the receptor of farnesoic acid, leading to an arrest in filamentation. The application of a dispersion inducer mainly fatty acid prior to, or in combination with, treatment by antimicrobial agents provides a novel mechanism for enhancing the activity of these treatments through the disruption of existing biofilms; in this context, broad-spectrum activity of *cis*-2-decenoic acid suggests that this and other short-chain *cis*-2-monounsaturated fatty acids likely have deep evolutionary roots. It is interesting that fatty acid communication has been found to be present in many plant and animal species, and the connection to cell dispersion in these systems may be an interesting area for future investigation.

15.3.2 Enzymes as Antibiofilm Agent

15.3.2.1 Amylases and Cellulases

Amylase and cellulase enzyme complex was produced from *Penicillium janthinellum*, a mutant EU2D-21 under submerged fermentation. Good specific enzyme activities were found after eight days of incubation at 30 °C. This enzyme complex was evaluated for its capability to target and disrupt the biofilms of different bacteria. It was seen that it disrupted biofilms of *Escherichia coli* (85.5%), *Salmonella enterica* (79.72%), *P. aeruginosa* (88.76%) and *Staphyloccus aureus* (87.42%) within 1 h of incubation at 50 °C. The exopolysaccharide matrix of the biofilm and bacteria from the cell surface were detached by the enzyme complex as shown by the scanning electron microscopy (SEM), quantitative analysis of biofilm removal assay and crystal violet assay (Nagraj and Gokhale 2018).

15.3.2.2 Proteases

The cell surface proteins in *Staphylococcus aureus* strains promote the development of biofilm. Proteinase-mediated biofilm dispersion was investigated in the present study in different isolates of *S. aureus*. It was shown by microtitre plate-based biofilm assay demonstrated that Proteinase K (2 μg/mL) markedly checked the development

of biofilm in *bap*-positive *S. aureus* as well as other *S. aureus* strains but not in weak biofilm-producing strains, that is, *bap*-mutant M556 and SA392. However, there was no effect of Proteinase K treatment on the planktonic growth of *S. aureus*. It was indicated by the results of the study that Bap might also play role in eDNA retention in the matrix of biofilm that supports biofilm stability. A synergistic response in antibiotic efficiency was seen against all biofilm forming *S. aureus* strains when a combination of Proteinase K was applied in combination with antibiotics (Mukherji et al. 2015; Shukla and Rao 2017).

The treatment of biofilms with broadly specific proteases, such as Proteinase K and trypsin results into biofilm disassembly (Boles and Horswill 2008; Mootz et al. 2013). The serine proteases Proteinase K (from *Tritirachium album*) and trypsin have often been utilized as efficient biofilm disruption agents that hamper bacterial adherence and biofilm formation in *S. aureus* (Gilan and Sivan 2013) presumably through degradation of surface structures (Boles and Horswill 2008; Gilan and Sivan 2013; Loughran et al. 2014). Shukla and Rao (2013) also reported that biofilms formed by *S. aureus* with the help of Bap proteins were vulnerable to Proteinase K-mediated detachment and dispersion. Biofilm assays done in 96-well-plates showed that Proteinase K obstructed both biofilm adherence and progression in Bap expressing *S. aureus* cultures.

A number of proteases have been recognized as an antibiofilm disrupting agents, with varying degrees of success (Loughran et al. 2014; Craik et al. 2011). Serine proteases, in particular, have been effective at disrupting the matrix. This is not entirely surprising, as serine proteases have been produced number of biofilm-forming microbes which likely aid in active dispersal and biofilm structural arrangement (Loughran et al. 2014; Marti et al. 2010; Chen et al. 2013).

15.3.2.3 Hydrolase

Glycoside hydrolases were evaluated for potential therapeutic effect on *P. aeruginosa,* and it was showed that glycoside hydrolases specifically target and degrade the exopolysaccharide constituent of the biofilm matrix. Bacterial biofilms present a significant clinical challenge because they are recalcitrant to existing therapeutic regimes. The main part of biofilm production in the opportunistic human pathogen *P. aeruginosa* is the biosynthesis of the exopolysaccharides Pel and Psl, which are responsible for the formation and maintenance of the structural biofilm scaffold and defence against antimicrobials and host defenses. Knowing that the glycoside hydrolases PelAh and PslGh encoded in the Pel and Psl biosynthetic operons, respectively, are utilized for in vivo exopolysaccharide processing, it was anticipated that these would provide specificity to target *P. aeruginosa* biofilms. Evaluating these enzymes as potential therapeutics, it was demonstrated that these glycoside hydrolases selectively target and degrade the exopolysaccharide component of the biofilm matrix. PelAh and PslGh restrict biofilm synthesis over a 24-h period with a half-maximal effective concentration (EC50) of 69.3 ± 1.2 and 4.1 ± 1.1 nM, respectively, and are capable of disrupting pre-existing biofilms in 1 h with EC50 of 35.7 ± 1.1 and

12.9 ± 1.1 nM, respectively. This treatment was effective against clinical and environmental isolates of *P. aeruginosa* and reduced biofilm biomass by 58–94%. These noncytotoxic enzymes potentiated antibiotics because the addition of either enzyme to a sublethal concentration of colistin reduced viable bacterial counts by 2.5 orders of magnitude when used either prophylactically or on established 24-h biofilms. In addition, PelAh was able to enhance neutrophil killing by ~50%. This work illustrates the feasibility and benefits of using bacterial exopolysaccharide biosynthetic glycoside hydrolases to develop novel antibiofilm therapeutics (Baker et al. 2016).

Aspergillus fumigatus and *P. aeruginosa* produce galactosaminogalactan and Pel, respectively, which are cationic heteropolysaccharides. These exopolysaccharides both contain 1,4-linked N-acetyl-D-galactosamine and play a vital function in biofilm formation by these microorganisms. Proteins comprising glycoside hydrolase domains have been identified recently as a part of the anabolic pathway of each exopolysaccharide. Recombinant hydrolase domains from these proteins degrade their respective polysaccharides under in vitro conditions. These glycoside hydrolases were shown to exhibit antibiofilm activity against varied microorganisms and may be useful as novel therapeutic agents for the degradation of biofilms and reducing virulence (Snarr et al. 2017).

In another study, a Psl-specific glycoside hydrolase (PslG) was covalently bound to numerous, chemically different planes using amine functionalization (APTMS) and glutaraldehyde (GDA) linking. Since bacterial colonization and biofilm synthesis on surfaces are typically facilitated by the accumulation of exopolysaccharides and conditioning protein layers. *P. aeruginosa* is a nosocomial opportunistic pathogen that employs strain-specific exopolysaccharides such as Psl, Pel or alginate for both initial surface attachment and biofilm formation. To generate surfaces that resist *P. aeruginosa* colonization, in situ quartz crystal microbalance (QCM) experiments and fluorescence microscopy confirmed a complete lack of Psl adsorption on the PslG-bound surfaces. Covalently bound PslG was also seen to markedly reduce *P. aeruginosa* surface adherence and biofilm formation over-extended growth periods (8 days). The PslG surfaces showed a ∼99.9% (∼3 − log) reduction in surface-associated bacteria compared to control surfaces or those treated with inactive enzyme. This work showed a non-eluting 'bioactive' surface that specifically targets a mechanism of cell adhesion, and that surface-bound glycoside hydrolase can significantly reduce surface colonization of bacteria through local, continuous enzymatic degradation of exopolysaccharide (Psl). These results have significant implications for the surface design of medical devices to keep bacteria in a planktonic state, and therefore, susceptible to antibiotics and antimicrobials (Asker et al. 2018).

Poly-β(1,6)-*N*-acetyl-D-glucosamine (PNAG) is a main constituent of biofilm of many pathogenic bacteria. The synthesis, modification and export of PNAG in *E. coli* and *Bordetella* species need the protein products encoded by the *pgaABCD* operon. PgaB is a two-domain periplasmic protein that contains an N-terminal deacetylase domain and a C-terminal PNAG-binding domain that is crucial for export. In the current study, it was shown that the C-terminal domains of *Bordetella bronchiseptica* PgaB (PgaB) and *E. coli* PgaB (PgaB) work as glycoside hydrolases. These enzymes

hydrolyze purified deacetylated PNAG (dPNAG) from *S. aureus*, degrade PNAG-dependent biofilms formed by *Bordetella pertussis*, *Staphylococcus carnosus*, *S. epidermidis* and *E. coli*, and potentiate bacterial killing by gentamicin. Furthermore, it was seen that PgaB was only able to hydrolyze PNAG produced in situ by the *E. coli* PgaCD synthase complex when an active deacetylase domain was present. Mass spectrometry analysis of the PgaB-hydrolyzed dPNAG substrate showed a GlcN-GlcNAc-GlcNAc motif at the new reducing end of detected fragments. This work magnifies the role of PgaB within the PNAG biosynthesis apparatus, defines a new glycoside hydrolase family GH153 and identifies PgaB as a possible therapeutic agent for treating PNAG-dependent biofilm infections. The work provides further insight into the mechanism of periplasmic PNAG modification, and suggests PgaB could be utilized as a therapeutic agent to eliminate biofilms (Little et al. 2018).

15.3.2.4 Lactonase

The tenacity of bacterial infection is often linked to quorum sensing-mediated biofilm synthesis. Thus, the interruption of this signalling circuit presents an attractive anti-virulence strategy. Quorum sensing is an important aspect of biofilm formation. Quorum-quenching lactonases have been shown to be effective in disrupting of quorum sensing circuits. The present study pronounces a method to degrade biofilm in a clinically significant *A. baumannii* S1 strain by the application of an engineered quorum-quenching lactonase. This treatment by engineered lactonase attained significant reduction in *A. baumannii* S1 biofilm (Tay et al. 2016).

15.3.2.5 Dispersin B

An artificial gene encoding dispersin B of *Aggregatibacter actinomycetemcomitans* was cloned and expressed in *E. coli* cells. Procedure for purification of recombinant dispersin B was established, and its in vitro activity was determined. The enzyme was used in experiments on disruption of the biofilms formed by various microorganisms. It exhibited high activity against *S. epidermidis* biofilms. The biofilms formed by *Burkholderia cenocepacia* and *Achromobacter xylosoxidans* were more resistant to the recombinant enzyme (Dobrynina et al. 2015).

15.3.2.6 Papain

Considering the proteolytic nature of papain and the biopolymer matrix structure of bacterial biofilms, the present study was aimed to evaluate the ability of papain to act as an inhibitor of biofilms in different concentrations. The effect of different concentrations of papain in biofilm production by several methicillin-resistant *S. epidermidis* (MRSE) and methicillin-resistant *Staphylococcus haemolyticus* (MRSHa)

isolates was explored. When papain was mixed into the culture, the biofilm formation by MRSE was restricted. However, the enzymatic action of papain exhibited more efficiency for MRSHa isolates. The experiment suggested that papain is able to affect the ability of cells to form biofilm, thus affecting the bacterial attachment (de Oliveira et al. 2014).

15.3.2.7 DNAses

DNases have been applied for degradation of mucous in cystic fibrosis patients, demonstrating its viable potential for human use as a therapy (Shak 1995; Sawicki et al. 2015; Shak et al. 1990). In vivo studies utilizing DNase have shown that degradation of eDNA can disrupt biofilms present in a mammalian host (Hymes et al. 2013; Conover et al. 2011).

15.3.2.8 Polysaccharide Depolymerase

In the current work, bacterial exopolysaccharide was degraded by a heat-stable polysaccharide depolymerase which was prepared from the phage infecting *Klebsiella*. Treatment at 75 °C for 10 min led to the complete inactivate of phage. However, there was no loss of phage enzyme activity after the treatment. The colony counting showed the phage enzyme could rapidly decrease the number of biofilm-associated bacteria. The rate of inhibition reached the maximum (80%) after 4 h of treatment. Enzyme pre-treatment could also enhance the fumigation effect of chlorine dioxide. Approximately, 92% of the BF bacteria were eliminated after treatment with the phage enzyme followed by 30 min of treatment with chlorine dioxide. According to the results of colonies counting and scanning electron microscopy, the phage enzyme could effectively decrease the bacterial adherence as well as the adhesion of extracellular polymeric substances in the BF. This study has demonstrated that the phage-borne polysaccharide depolymerase enzyme is valuable for eradicating the bacterial BF (Chai et al. 2014).

15.3.3 Inhibitors of Quorum Sensing

15.3.3.1 Implication of Quorum Sensing in Biofilm Development

The interactions and communication amongst bacteria done collaboratively for biofilm formation are known as quorum sensing (QS). This mechanism was elucidated initially by Professor Greenberg where he proposed different ways to intervene with pathogenic microflora and to moderate microbiome for better health approaches (Fuqua et al. 1994). Many species of bacteria are inhibitors of biofilm. This communication system includes induction of a specific set of bacterial genes that hold

potential of response to expanded volume of cell population (Platt and Fuqua 2010). Today, targeting the inter-bacterial interactions has become the foremost in healthcare researches, especially with multi-drug resistance amongst the pathogens (Haque et al. 2018; Golberg et al. 2013; Saurav et al. 2016).

Recently, the use of natural products to interfere with pathogenic bacterial quorum-sensing systems (Jamal et al. 2018; Hirakawa and Tomita 2013; Brackman and Coenye 2015; Rémy et al. 2018) has been proposed for development of new antimicrobial agents. Since this strategy does not require bacterial killing, it is proposed to reduce the development of resistant strains (Tang and Zhang 2014).

Autoinducers like acyl homoserine lactones (AHL) are synthesized and secreted by the phenomenon of quorum sensing (Newton and Fray 2004). Gram-positive bacteria secrete quorum-sensing peptides as a regulatory system consisting of two components, a membrane-bound histidine kinase receptor and an intracellular response regulator (Platt and Fuqua 2010). Besides these, gram-negative as well as gram-positive bacteria can use a common entity, borate furanosyl, an autoinducer-2 (IA-2) and (IA-3). The mechanism of quorum sensing is also reported for the control of growth of biofilm (Abee et al. 2011).

15.3.3.2 Bacterial QS Inhibitors

Quorum sensing affects the structure of biofilm, and lack of quorum sensing is related with development of thin biofilm as well as increased susceptibility to antibiotics (Shih and Huang 2002).

In aeromonads, the QS regulates biofilm formation, motility, multicellular synchronized social life and virulence (Talagrand-Reboul et al. 2017).

Numerous bacteria produce certain substances (Table 15.2) on their surface that prevents biofilm formation by others, such as **extracellular polysaccharides**, which are crucial in formation of biofilm in bacteria. They also prevent biofilm formation in adjacent cells (Rendueles et al. 2013).

Actinobacillus pleuropneumoniae serotype 5 which exhibits antibiofilm activity has extracellular polysaccharide which prevents intra- and inter-cellular communications amongst microbes, thereby exhibiting an example of natural anti-biolfilm phenomena (Karwacki et al. 2013).

Another study of Schertzer et al. exhibited similar activity of glycolipid and glycoprotein structure and QS signals to sense and stop external positive-charged compounds (Schertzer et al. 2009).

Similarly, the extracts of coral-associated bacteria have been shown to induce a decrement in *S. aureus* and *Serratia marcescens* biofilm development. Ethyl acetate extracts of ***Bacillus firmus***—a coral-associated bacterium–show antibiofilm activity against multi-drug resistant (MDR) *S. aureus* (Gowrishankar et al. 2012). A new natural compound, **4-phenylbutanoic acid**, extracted from *Bacillus pumilus,* demonstrated antagonistic activity against biofilms (Nithya et al. 2011).

Table 15.2 Bacterial antibiofilm agents

S. no.	Bacteria inhibiting biofilm formation in other bacteria	Bacteria inhibited	Mechanism of biofilm inhibition (antibiofilm agents)	Reference
1	*Actinobacillus pleuropneumoniae* serotype 5 EPS	Broad-spectrum, non-biocidal antibiofilm activity	Extracellular polysaccharides (EPS)	Karwacki et al. (2013)
2	*Bacillus pumilus* (marine bacteria)	Antagonistic activity against biofilms from a broad range of bacteria	4-phenylbutanoic acid	Nithya et al. (2011)
3	Ethyl acetate extract of *Bacillus firmus* (coral-associated bacteria)	Antibiofilm activity against MDR *S. aureus*		Gowrishankar et al. (2012)
4	*Streptococcus salivarius*, a harmless, non-biofilm inhabitant of human mouth	Inhibit dental biofilms (plaque) formation	Uses two enzymes—fructo-syltransferase (FTF) and exo-β-D-fructosidase (FruA), to inhibit EPS production and dental plaque formation	Ogawa et al. (2011)

Another example is seen with ***Streptococcus salivarius***, that exhibit antibiofilm activity by use of two enzymes, viz. fructosyltransferase (FTF) and exo-β-D-fructosidase (FruA), that decrease EPS production (Ogawa et al. 2011). However, excess production of FruA may have an involvement in development of sucrose-dependent biofilm in the oral cavity.

15.3.4 Inhibition of QS by Phytochemicals

Phytochemicals from fruits and vegetables hold the potential to hinder quorum sensing in human pathogens (Vattem et al. 2007). This is appealing, since the regular intake of such anti-QS associated food may be of therapeutic value in preventing the invasion of intestinal pathogens.

15.3.4.1 Polyphenols or Phenolic Compounds

Range from simple molecules to polymerized tannins, and have one aromatic ring with six carbons. These secondary metabolites affect biofilm development in microbes (Huber et al. 2003; Sarabhai et al. 2013).

15.3.4.2 Phenolic Acids

Are organic compounds which have one carboxylic functional group and phenolic hydroxyl group. In plants, the acid phenols are mostly in the form of esters of aliphatic alcohol or of quininic acid, glycosides or rosmarinic acid. Hydroxylation of the phenolic group has exhibited direct effect upon their antimicrobial activity. The mechanism for this phenolic damage involves inhibition of enzymes by oxidized compounds (Mason and Wasserman 1987).

15.3.4.3 Flavonoids

Belong to the polyphenol family. They have a structure with 15 carbon atoms, constituting two aromatic units, two C_6 rings joined by a C_3 chain (Heim et al. 2002); flavonoids are the compounds which give colours to flowers, fruits as well as leaves.

Flavanones, found enormously in citrus spps, show interference with quorum sensing and also affect other physiological process (Truchado et al. 2012). **Naringenin, quercetin** and **apigenin** are inhibitors of HAI-1 or Al-2-mediated bioluminescence production in *Vibrio harveyi*. Naringenin and **taxifolin** decrease the synthesis of pyocyanin and elastase in *P. aeruginosa* where growth of bacteria was unaffected. Naringenin and taxifolin also hindered the quorum sensing by affecting the gene expression in *P. aeruginosa* PAO1. Naringenin holds the potential of hindering the synthesis of quorum-sensing mediators such as N-(3-oxododecanoyl), lactone-1-homoserine (3-oxo-C12-HSL), acylhomoserine lactone and N-butanoyl-1-homoserine lactone (C4-HSL) (Vandeputte et al. 2011).

15.3.4.4 Quercetin, Sinensetin, Apigenin

Also inhibit the development of biofilms in *V. harveyi* BB120 as well as *E. coli* O157:H7 (Truchado et al. 2012; Vikram et al. 2010). In *P. aeruginosa* PAO1, flavonoids such as the catechin decrease virulence factors (pyocyanin and elastase) influenced by quorum signalling, leading to inhibition of biofilm development.

The HLA molecules are deteriorated by legume products including alfalfa, clover, lotus, peas and yam beans (Delalande et al. 2005; Gotz et al. 2007).

15.3.4.5 Terpenes

Are natural compounds having hydrocarbons of either cyclic or open-chain skeleton. The basic molecule is isoprene, i.e. C_5H_8. The term terpenoid refers to terpene skeleton substances with one or more functional groups (alcohol, aldehyde, ketone, acid, lactone, etc.). The terpenoid classification is based on the number of repetitions of the isoprene base unit: hemiterpens (C_{30}), tetratepens (C_{40}) and polyterpens.

Essential oils are plant extracts that mainly contain mono- and sesqui-terpenes. Essential oil from medicinal plants such as *Citrus reticulate* (Luciardi et al. 2016), *Eucalyptus radiate*, *Eucalyptus globulus* (Luis et al. 2016) and *Thymus vulgare* (Myszka et al. 2016) have shown anti-QS effects.

In addition, compounds such as vanillin in *Vanilla planifolia* (Ponnusamy et al. 2009), eriodictyol in *Eriodictyon californicum* (Vandeputte et al. 2011), methyl eugenol in *Cuminum cyminum* (Packiavathy et al. 2012), erucin in *Brassica oleracea* (Ganin et al. 2013), ajoene in *Allium sativum* (Jakobsen et al. 2012a) and naringin, naringenin, kaempferol, quercetin, rutin, neoeriocitrin in *Citrus sinensis* (Vikram et al. 2011) were found to cease quorum sensing.

15.3.5 Medicinal Plants as QS Inhibitors

Different parts and organs of various plants have been reported to possess medicinal values. Examples include *Glycyrrhiza glabra* (Bhargava et al. 2015), *Psoralea corylifolia* (Husain et al. 2018), *Piper bredemeyeri* (Olivero et al. 2011), *Bauhinia acuruana*, *Pityrocarpa moniliformis*, *Commiphora leptophloeos* (Trentina et al. 2011), *Cocos nucifera* (Viju et al. 2013) and *Terminalia catappa* (Taganna et al. 2011).

Rubus rosaefolius (Oliveira et al. 2016), *Centella asiatica* (Vasavi et al. 2016), *Areca catechu* (Koh and Tham 2011) and *Sclerocarya birrea* (Sarkar et al. 2014) have also been reported to have an inhibitory effect against QS signalling.

15.3.6 Plants as a Source of Anti-QS Drugs: Mechanism of Action

Anti-quorum sensing role of **essential oils** and their major constituents: amongst the essential oils, lavender, clove and rosemary have shown anti-QS activity. Recently, Luciardi et al. revealed essential oil from *Citrus reticulata* to possess antibiofilm and anti-quorum sensing activity against *P. aeruginosa*. At 0.1 mg/mL, this compound showed 41% decrease in biofilm cell viability and a 33% decrement in AHL production (Luciardi et al. 2016).

Thymus vulgare and its derivatives carvacrol and thymol exhibited antibiofilm and anti-quorum sensing activity against *Pseudomonas fluorescens* KM121, thus

revealing a tremendous decrease in AHL production and biofilms (Myszka et al. 2016).

Organic compounds extracted from the medicinal plants also act as inhibitory agents of quorum sensing (Thakur et al. 2016). **Organic compounds** from the medicinal plants (Table 15.3) have same chemical skeleton to those of quorum sensing signals or possess the potential to deteriote signalling receptor (LuxR/LasR) (Vattem et al. 2007; Al-Hussaini and Mahasneh 2009).

- Indeed, GABA (gaminobutyric acid), synthesised by some plants, promotes signal degradation by OHC8HSL HLA lactonase (ATTM) in *Agrobacterium tumefaciens*, thus hindering the process of quorum sensing-dependent infection (Chevrot et al. 2006).
- *Medicago truncatula* modulating AhyR, CVIR and LuxR activities in various microorganisms (Gao et al. 2003) and quorum sensing in *P. aeruginosa* and *Sinorhizobium meliloti* (Mathesius et al. 2003).
- *Curcuma longa* produces curcumin which hinders the expression of the virulence genes of *P. aeruginosa* PA01.
- Polyphenols like hydroxycinnamic acids, rutin and epicatechin found in the extracts of apples and its derivatives have shown a significant role in anti-quorum sensing activity; they also show synergistic activity against *Chromobacterium violaceum* (Fratianni et al. 2011, 2012).

The anti-quorum sensing activity has also been exhibited by various other medicinal plants like *Laurus nobilis*, *Rosmarinus officinalis* and *Pityriasis alba*. These plants had the potential to decrease the synthesis of violacein (Vattem et al. 2007; Al-Hussaini and Mahasneh 2009).

The **hydroalcoholic extracts** of *Berberi saristata* and *Camellia sinensis* exhibited anti-quorum sensing activity against *E. coli* (Thakur et al. 2016). In addition, the in silico studies affirm the anti-quorum activity of the phytomolecules present in these extracts (flavonoids, alkaloids and tannins) through the antagonistic activity of LuxS.

The extract fractionation permits screening of chemical molecules and moieties for a significant outcome. Vasavi et al. showed that the flavonoids-rich ethyl acetate fraction of *C. asiatica* decreased pyocyanin synthesis and biofilms development by inhibition of elastolitic and proteolytic activity in these bacteria (Vasavi et al. 2016).

The ethanol and ethyl acetate extracts from *Hypericum connatum* have been shown to possess an anti-quorum sensing effect against *C. violaceum*, which hinders in the synthesis of violacein (Fratianni et al. 2013).

Gallic acid containing polyphenolics such as epigallocatechin gallate, ellagic acid and tannic acid from medicinal plants, hold the potential of interfering and inhibiting the interaction amongst bacterial cells via AHL mediated signalling (Sarabhai et al. 2013). Similarly, grenades and berries are rich in ellagitannins such as punicalagin and ellagic acid (Larrosa et al. 2010). In the intestinal flora, the conversion of ellagitanins to ellagic acid is done by the micro-gut flora and then metabolized to form urolithin A and urolithin B. The resulting metabolites get easily assimilated in the human intestine, playing their vital roles. In recent studies, it has also been revealed

Table 15.3 Anti-quorum sensing activity of some medicinal plants extracts and essential oils

Plant species	Products	Major compound	Strains tested	Effects	Reference
Thymus vulgare	Essential oils	Carvacrol thymol	*Pseudomonas fluorescens* KM121	Significant diminution of AHL synthesis in 72 h. Its action suppresses bacterial motility and lowers the mRNA flagella gene expression	Myszka et al. (2016)
Piper bredemeyeri	Essential oil	ND	*Chromobacterium violaceum*	It inhibits violacein production	Olivero et al. (2011)
Citrus reticulate	Essential oil	Limonene	*Pseudomonas aeruginosa*	At 0.1 mg/ml concentration, inhibits biofilm cell viability (41%) and AHL production	Luciardi et al. (2016)
Eucalyptus radiate	Essential oil	Limonene; a-terpineol; a-terpinyl acetate; a-pinene	*Acinetobacter baumannii*	Inhibits quorum sensing-regulated bacterial pigment violacein	Luis et al. (2016)

(continued)

Table 15.3 (continued)

Plant species	Products	Major compound	Strains tested	Effects	Reference
Centella asiatica	Flavonoid-rich fraction	*Pseudomonas aeruginosa* PAO1 and *Chromobacterium violaceum* ATCC12472	ND	Hinders synthesis of violacein in *C. violaceum* at 400 mg/mL. Inhibits quorum sensing-regulated phenotypes such as pyocyanin production, elastolytic and proteolytic activities, swarming motility and biofilms formation in PAO1	Vasavi et al. (2016)
Cocos nucifera	Extracts from fibres	ND	*Pseudomonas sp., Alteromonas sp.,* and *Gallionella* sp.	Inhibits biofilm formation and EPS production	Viju et al. (2013)
Eucalyptus globules	Essential oils	1,8-cineole (eucalyptol); a-pinene; Aromadendrene; p-cymene	*Acinetobacter baumannii*	Inhibits QS-regulated bacterial pigment violacein	Myszka et al. (2016)
Terminalia chebula	Fruit extract	Ellagic acid (benzoic acid)	*Burkholderia cepacia*	Inhibits biofilm formation	Huber et al. (2003)
Rubus rosaefolius	Phenolic extracts	ND	*Chromobacterium violaceum, Aeromona hydrophila* and *Serratia marcescens*	Inhibits violacein production, swarming motility and biofilms formation	Oliveira et al. (2016)
Glycyrrhiza glabra	Methanol extract	Flavonoids	*Acinetobacter baumanni*	Decreases biofilm formation and motility. Reduction of virulence factors promoting QS	Bhargava et al. (2015)

that urolithin A and B hinder quorum-sensing activity and also lower the levels of AHL produced by entherocolitica entheropathogen.

15.3.7 Anti-QS Action of Isolated Molecules from Medicinal Plants

With the application of bio-guided fractional approach, numerous secondary metabolites extracted from medicinal value plants have been obtained which exhibit anti-quorum sensing activities. For example, furanones have been exhibited to inhibit AHL processes (Manefield et al. 2002). Numerous plant secondary metabolites imitate AHL of bacteria, hence influencing quorum sensing and biofilm development in bacteria (Teplitski et al. 2000).

Cinnamaldehyde (major constituent in essential oils) and their derivatives have been estimated to hinder quorum sensing and biofilm development (Brackman et al. 2008; Niu et al. 2006). Cinnamaldehyde has been shown to inhibit bioluminescence in *V. harveyi* BB170 and eugenol in *P. aeruginosa* and *C. violaceum* via inhibition of virulence factor production such as violacein, elastase, pyocyanin and biofilm (Zhou et al. 2013).

Iso-limonic acid and ichangin (molecules contained in bigaradier seed extracts) include **limonoids** that inhibit the growth of *V. harveyi* at a very low concentration and these mechanisms are related to the inhibition of HAI- and AI-2-mediated bioluminescence (Vikram et al. 2011).

Curcumin, obtained from *C. longa*, has anti-quorum sensing activity. This molecule can attenuate the QS dependent factors such as EPS production of several pathogenic strains such as *E. coli*, *P. aeruginosa*, *Proteus mirabilis* and *S. marcescens*. In addition, curcumin has been reported to reduce a few phenotypes concerned with QS inhibition including swimming, swarming and motility. Curcumin also inhibits biofilms and pyoacin formation in *P. aeruginosa* via modulation at gene expression related to QS signalling pathways (Rudrappa and Bais 2008).

Usnic acid, a lichen metabolite, has anatagonistic activity against biofilm of fungus and bacteria. QS inhibitors can increase the susceptibility of biofilms to antibiotics and do not pose threat to humans (Sun et al. 2013).

Garlic inhibits quorum sensing at the gene expression level. Ajoene is obtained after crushing the garlic, and it has sulphur. Ajoene also shows antagonistic activity against synthesis of rhamnolipid, which protects biofilms from white blood cells. The combination of ajoene and the antibiotic tobramycin has been shown to kill ~90% of biofilm bacteria. Garlic possesses anti-viral, anti-fungal and anti-protozoal properties and is useful for the cardiovascular and immune systems (Jakobsen et al. 2012b). The only drawback with these sulphur-containing compounds is that their activity is lost as soon as they get in contact with oxygen.

Hamamelitannin extracts from the willow bark, also hinders quorum sensing (Morgan 2015).

Bacterial extracts with anti-quorum sensing activity are also analyzed using a mass spectrometry-based metabolomics Global Natural Products Social Molecular Networking platform (GNPS; https://gnps.ucsd.edu/) for compound dereplication (Allard et al. 2016). In addition, an integrated biological, genomic and metabolomic approach using 16SrRNA sequences is being lately employed to discover anti-quorum sensing molecules from bacterial strains (Ong et al. 2019).

15.3.8 Conclusion

Biofilm-associated bacteria are less sensitive to antibiotics than free-living (planktonic) cells. Furthermore, with variations in the concentration of antibiotics throughout a biofilm, microbial cells are often exposed to levels below inhibitory concentrations and may develop resistance. This, as well as the irresponsible use of antibiotics, leads to the selection of pathogens that are difficult to eradicate. Biofilms comprising of multicellular, surface-adherent communities help the microorganisms to survive in various stress conditions which include antibiotics, lack of nutrient, heat shock and immune responses. In view of the increment in the numbers of old patients who require artificial medical devices such as knees and hips, there will be an extended need for novel agents and strategies to treat biofilm-related infections. Some strategies have been described recently which appears to play an important role in future antibiofilm therapies. Both established and novel experimental treatments targeted at various stages of wound healing that are specifically aimed at reducing and eliminating biofilm bacteria. Importantly, the highly tolerant nature of these bacterial communities suggests that most singular approaches could be circumvented and a multifaceted, combinatorial approach will be the most effective strategy for treating these complicated infections. The usage of enzyme complex as antibiofilm therapeutics to eradicate biofilms is feasible and beneficial. This can also be used as a promising strategy to improve treatment of multidrug-resistant bacterial infections. The biofilm-forming microorganisms pose great threat to frequent infections in immune-compromised patients and is problematic to eliminate from medical devices. There are several classes of antibiofilm agents which need proper investigation in terms of their efficiency in inhibiting the microbial growth, understanding of the complex matrix organization in biofilms to deduce the mechanism of their action. The potential biofilm inhibiting compounds include natural bioactive compounds, enzymes that disturb the biofilm structure and other nonenzymatic molecules (Fig. 15.1).

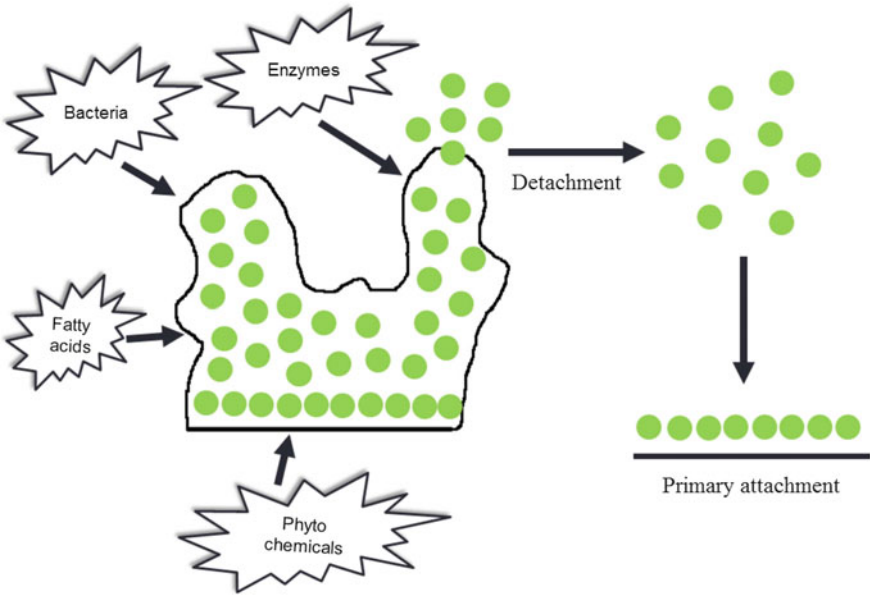

Fig. 15.1 Disruption of microbial biofilms by various antibiofilm agents

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Chapter 16
Probiotics and Biofilms

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Abstract Probiotics are live microorganisms which when administered in adequate amounts confer health benefit on the host. Biofilm is a microbially derived sessile community in which the bacteria are attached to a substratum or interface or to each other and are embedded in a matrix of extracellular polymeric substances that they have produced. Probiotics can be used for the treatment of biofilm-forming pathogens in various organ systems of the body. Lactobacilli and Bifidobacteria have been found to help in the treatment of dental caries. In the gastrointestinal tract, probiotics have been used to treat disorders like antibiotic-associated diarrhea, inflammatory bowel disease, and irritable bowel syndrome wherein by biofilm formation, they alter the pathogenic milieu. For better delivery of the probiotics to the intestine and to prevent degradation by gastric acid, probiotics have been capsulated with chitosan and alginate. Probiotics containing Lactobacillus in combination with antimicrobials have been found to be effective in the treatment of recurrent urinary tract infection and bacterial vaginosis. In nonhealing wound infections caused by biofilms formed by *Staphylococcus aureus* and Pseudomonas, probiotics appear as a promising tool because when topically applied, they helped in the treatment. Recently, novel treatment strategies like coadministration of antibiotics and biofilm inspired encapsulated probiotics have been used to treat chronic wound infections while also avoiding emergence of antimicrobial resistance.

Keywords Probiotics · Biofilms · Antibiotics

16.1 Introduction

Bacteria are ubiquitous in the environment. They are seen as either free-floating forms (planktons) or as biofilm. In nature, 90% of bacteria exist in biofilms. The definition of a biofilm is "a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced,

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and exhibit an altered phenotype with respect to growth rate and gene transcription" (Donlan and Costerton 2002).

Biofilms are composed of the extracellular polymer matrix and during development can assume differentiated forms such as mushroom-like microcolonies and filamentous streamers (Cheow et al. 2014). Interstitial spaces separate the vertical structures of biofilm. They aid the biofilm in acquiring nutrients easily and rapidly from the surrounding medium as well as moving the toxic by-products away (Secinti et al. 2011). Bacteria have the ability to produce extracellular matrix, and it helps them to adhere to each other (Bucior et al. 2012). The inhabited bacteria get embedded and secured in the matrix during the course of biofilm maturation. Three main processes regulate formation of biofilm in gram-negative bacteria, they are: attachment, maturation, and dispersion (Laverty et al. 2014). Biofilm assumes clinical importance in relation to certain resistant nosocomial bacterial infection like Pseudomonas. Infections on medical devices like prosthetic joints, intravascular catheters, cardiac devices, shunts, and prosthetic vascular grafts can be caused by biofilms.

Antibiotics may fail to penetrate beyond the surface of biofilm leading to antibiotic resistance and antibiotic action may be antagonised in zones of depleted nutrition or waste product accumulation. Probiotics are defined as live microorganism which when administered in adequate amounts confers health benefit on the host (FAO/WHO 2002). Probiotics confer health benefits by competing with pathogenic microorganism for energy substrates, space, by secreting antimicrobial substances. Probiotics have been postulated to modulate immunity by enhancing barrier, increasing mucus secretion, epithelial membrane integrity, and by counteracting pro-inflammatory cytokines. There is evolving interest in utilizing probiotics to counter and treat multidrug-resistant microorganisms.

Skin, oral cavity, gastrointestinal mucosa, and genitourinary mucosa host plenty of commensal bacteria. When there is alteration in the microbiota due to immunological changes, pathogenic bacteria proliferate and result in disease. Dental plaque, bacterial vaginosis, nonhealing skin wounds, infective and inflammatory bowel diseases are some common diseases in which dysbiosis plays an important role in pathogenesis. There has been growing interest in harnessing the therapeutic potential of probiotics to treat these disorders.

16.2 Interaction of Probiotics and Oral Biofilms

Dental caries and periodontal disease are common dental diseases in which dental plaque plays a major role in pathogenesis. Dental plaque is a well-defined multispecies biofilm constituted by a complex microbial community. When strictly anaerobic gram-negative bacteria get accumulated within the biofilm, it results in microbial imbalance, thus predisposing to the onset of periodontal diseases and transforming the dental plaque into a difficult to treat "pathogenic" biofilm.

Bacteriocin producing Lactobacilli and Bifidobacteria has been found to counteract pathogenic bacteria and to restore the microbial balance. In several studies,

probiotics have been found to have the ability to co-aggregate with caries-associated strains and thus resulting in reduction in the number of cariogenic bacteria, especially *Streptococcus mutans*, within the dental plaque (Twetman et al. 2009; Lang et al. 2010).

Lactococcus lactis NCC2211 was able to mimic a dental plaque by incorporating itself into a biofilm and was shown to modulate the growth of the cariogenic *Streptococcus sobrinus* OMZ176 (Comelli et al. 2002). Teanpaisan et al. (2011) had shown that Lactobacillus SD1–SD6 exhibited a strong inhibitory effect against gram-negative periodontal pathogen *Porphyromonas gingivali* and *Aggregatibacter actinomycetemcomitans*. Vuotto et al. (2013) reported inhibitory effect of *Lactobacillus brevis* over periodontal pathogen *Prevotella melaninogenica*.

The oral bacterium S11, which was isolated from the saliva of young children without dental caries and found to have a 99.5% similarity with *L. fermentum*, was demonstrated to inhibit the ability of *S. mutans* Ingbritt (a laboratory reference strain) to adhere on cuvette walls and to synthesize extracellular glucans. *L. fermentum*-derived biosurfactant was demonstrated to greatly reduce the ability of *S. mutans* to produce sucrose using glucosyltransferases (GTs) and to grow as biofilm, thus having an antibiotic effect (Tahmourespour et al. 2011). It has been reported that streptococcal adhesion on saliva-coated hydroxyapatite can be reduced by Lactobacilli (Marttinen et al. 2013).

16.3 Competition and Interference of Probiotics with Intestinal Biofilm

Probiotics have been used extensively in treatment of variety of gastrointestinal disorders like antibiotic-associated diarrhea, inflammatory bowel disease. The administration of viable probiotic microorganisms to intestine is hindered by acidic milieu in stomach. Probiotic bacteria have limited survival during food storage or in the gastrointestinal tract when taken in the form of dairy products such as yogurt or milk (Candela et al. 2008). To improve the bioavailability of probiotics in intestine, probiotics are encapsulated. The definition of encapsulation is that it is a process which may be physicochemical or mechanic in order to trap substances in a certain material and hence produces particles with small diameters in the order of nanometers to millimeters. It has multiple advantages in the form of sustained and instant liberation of the substance to the specific target site, tolerance to harsh environments like extremes of temperatures, and gastrointestinal juice (Guandalini et al. 2000; Miyazaki et al. 2010; Reid and Habash 1998; Grin et al. 2013).

Materials used to encapsulate:

- Alginate: Alginate is a polysaccharide which is naturally derived from various algae species. It is composed of β-D-mannuronic and α-L-guluronic acids. Since it is nontoxic, biocompatible, and of low cost, it is the most used material to microencapsulate probiotics. Its very low resistance to the gastric environment is

the disadvantage of alginate (Mortazavian et al. 2008). To overcome this problem, double coating with locust gum and chitosan is done (Cheow et al. 2014). Lactobacillus which is coated with alginate has been demonstrated to be able to resist stressful environments, thus reducing the loss of viability and maintaining the activity of strains against *H. pylori* (Khalil et al. 2015). It was also observed that *L. rhamnosus* strain as biofilm with double coating was more resistant than the double-coated planktonic strain.

- Chitosan: It is a linear polysaccharide which is composed of glucosamine units able to polymerize in a reticulating agent in the presence of anions. For the gastrointestinal tract, chitosan-coated alginate capsules are the most widely used capsules for probiotics as they provide resistance to stressful environments and increase probiotic viability in gastric juice.

Alginate–chitosan capsules maintain their ionic bonds to beads at low pH, making the bead matrix material to remain intact. In the intestine at a neutral pH, the anionic alginate in the Ca–alginate–chitosan complex is displaced by hydroxyl ions (Anal et al. 2003) thus leading to the release of therapeutic molecules by polymer degradation (Arora and Budhiraja 2012; Du et al. 2006). As a result, the complex dissociates, the matrix erodes, and the protein is released in the surrounding fluid (Krasaekoopt et al. 2003). Based on the previous report, in the case of bacteria too, it can be postulated that the capsule would behave in a similar manner, liberating them in the intestine.

Probiotics have shown clinical benefit in the following conditions involving the GI tract-intestinal infection, inflammatory bowel disease, and irritable bowel syndrome. They exert their effect by transiently modulating the composition and function of intestinal microbiota (O'Toole and Cooney 2008; Floch et al. 2008).

Combinations of specific probiotics strains appear to be able to enhance the inhibition percentages of pathogens adhering to intestinal mucus when compared to single probiotic strain (Collado et al. 2006). On the contrary, Candela et al. have shown single strains (*L. acidophilus* Bar13, *L. plantarum* Bar10, *Bifidobacterium longum* Bar33, and *B. lactis* Bar30) were found to be effective in displacing enteropathogens *Salmonella typhimurium* and *Escherichia coli* H10407 from a Caco-2 cell layer (Candela et al. 2008). *L. acidophilus* A4 was able to drastically decrease enterohemorrhagic *E. coli* biofilms by affecting genes related to curli production (crl, csgA, and csgB) and chemotaxis (cheY) (Kim et al. 2009). Probiotics have been conclusively shown to reduce overall duration of acute infectious diarrhea on the basis of meta-analysis of randomised controlled trials. Probiotics are of proven benefit in treatment of and prevention of antibiotic-associated diarrhea, Clostridium difficile diarrhea.

VSL#3, a commercially available capsule which contains eight different strains, has proven to be effective in the primary prevention (Gionchetti et al. 2003) and maintenance of remission among patients with pouchitis.

16.4 Competition and Interference of Probiotics with Biofilms of Genitourinary Tract

Illnesses including urinary tract infections and bacterial vaginosis can be prevented by Lactobacilli inhabiting the genitourinary environment (Reid and Habash 1998; Grin et al. 2013; Kumar et al. 2013; Klebanoff et al. 1991).

Probiotics have been conclusively proven to be useful in treating bacterial vaginosis with high cure rates (Parent et al. 1996; Anukam et al. 2006a; Mastromarino et al. 2009). Probiotics have also been shown to reduce recurrence rates when used following antibiotic treatment (Anukam et al. 2006b; Petricevic and Witt 2008; Larsson et al. 2008).

Clinical trials suggested that the amount of Lactobacilli could also play an important role in the effectiveness of the probiotic product (Mastromarino et al. 2013). The notion that hydrogen peroxide (H_2O_2) production by Lactobacilli is a key factor in resisting bacterial vaginosis is now supported by clear evidence, and these strains that produce H_2O_2 are found in 61% of pregnant women with normal flora and only in 5% of women with bacterial vaginosis (Hillier et al. 1993) Lactobacilli also have the ability to co-aggregate with some urinary pathogens thus allowing them to block the ability of pathogens to adhere and by the production of antimicrobial substances, it kills the pathogen (Reid et al. 1990).

The combination of *Lactobacillus gasseri* 335, *L. brevis* CD2 and *L. salivarius* FV2 in a vaginal tablet reduced the infection caused by *G. vaginalis* by coaggregating with it (Kaewnopparat et al. 2013; Mastromarino et al. 2002).

Recent experiments suggest that probiotics alone or in combination with antibiotic therapy may have a place in the treatment of bacterial vaginosis (Senok et al. 2009; Verstraelen and Swidsinski 2013). Additionally, probiotics, such as *L. reuteri* RC-14 producing low amounts of H_2O_2, are able to largely displace *G. vaginalis*, and changes in the structure and viability of the biofilms with loss of dense *G. vaginalis* biofilm pods can be seen by deconvolution microscopy (Saunders et al. 2007).

In vitro studies have shown that the use of *Pediococcus pentosaceus* SB83 as a vaginal probiotic has the ability to prevent *Listeria monocytogenes* colonization in pregnant women (Borges et al. 2013).

With regard to the ability of specific probiotics to enhance the antibiotic activity against *G. vaginalis* biofilm, it was found that metronidazole could produce holes within the biofilm without eradicating bacteria, whereas *L. rhamnosus* GR-1 and *L. reuteri* RC-14 infiltrated bacterial vaginosis biofilms leading to a higher bacterial cell death (McMillan et al. 2011).

In children, recurrences of urinary tract infections caused by pathogenic bacteria could be diminished by a combination of fluoroquinolone and probiotics as demonstrated by a study done by Madden-Fuentes et al. (2015). These findings provided evidence that probiotics could eradicate pathogenic biofilms, alone or combination with antimicrobials.

16.5 Competition and Interference of Probiotics with Wounds Biofilm

Alterations in skin microbiota could lead to chronic wound pathology as indicated by recent studies. Thus, probiotics could be promising tool to topically prevent and treat nonhealing wounds (Wong et al. 2013).

Probiotic organism *L. plantarum* has the ability to inhibit the pathogenic activity of *Pseudomonas aeruginosa*. It was demonstrated by Valdez and colleagues (2005) that probiotic whole cultures of *L. plantarum* as well as culture filtrates were able to in vitro inhibit *P. aeruginosa* elastase and biofilm by affecting the production of the quorum-sensing signal molecules, acyl-homoserine-lactones. Taking these promising results into consideration, local treatment of *P. aeruginosa* burn infections can be done by *L. plantarum* and/or its metabolites.

Surfactants obtained from probiotics like *L. acidophilus* strains reduced the bacterial deposition rate and biofilm development caused by pathogens like *S. aureus* and *S. epidermidis* by influencing the staphylococcal cell surface hydrophobicity (Walencka et al. 2008).

Furthermore, biofilm formation by staphylococcal species could be inhibited by cell-free supernatants of *L. acidophilus* H-1 was shown in the study done by Sadowska and coworkers (2010). Growth and biofilm formation of several *S. aureus* and *P. aeruginosa* strains could be inhibited by *L. fermentum*-secreted compound(s) (Varma et al. 2011). Finally, topically applied *L. plantarum* has been demonstrated to be effective in preventing skin wound infections in mice (Sikorska and Smoragiewicz 2013) caused by *P. aeruginosa* (Valdez et al. 2005) and *S. aureus*.

16.6 Coadministration of Antibiotics and Encapsulated Probiotics

One of the major healthcare challenges encountered in modern times is the emergence of antimicrobial resistance. Probiotics can be potential tools to counter emergence of antimicrobial resistance. However, coadministration of probiotics and antibiotics leads to decreased effectiveness of probiotics. Thus, in order to protect probiotics from coadministered antibiotics, attempts are being made to harness the property of biofilm.

In studies, it has been found that encapsulation of probiotics with alginate can confer protection from antibiotics like tobramycin in a way that growth and metabolic activity of encapsulated probiotic remained unaffected. It has also been demonstrated that encapsulated probiotic containing three Lactobacillus strains, *Lactobacillus acidophilus* CL125, *L. casei* LBC80R, and *L. rhamnosus* CLR2 combined tobramycin has the ability to completely eradicate methicillin-resistant *S. aureus* and *P. aeruginosa*, the two most commonly implicated bacteria in chronic wounds.

16.7 Conclusion

Bacteria can exist in nature as free-living planktons or more commonly as part of biofilms. Biofilm formation provides survival advantage to bacteria by providing easy access to nutrition, excretion of toxic by-products, and decreased permeability to antibiotics. Certain species of bacteria used as probiotics also exhibit biofilm formation which enhances the therapeutic effects. This article has reviewed utility of such probiotics in treatment of wound infection, dental plaques, gastrointestinal diseases, and bacterial vaginosis. Novel treatment strategies like coadministration of antibiotics and biofilm inspired encapsulated probiotics have been used to treat chronic wound infections while also avoiding emergence of antimicrobial resistance.

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Chapter 17
Probiotics to Counteract Biofilm-Associated Infections

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Abstract Eradication of pathogenic microorganisms without disturbing useful microenvironment is the primary goal in the treatment of biofilm-based infections. Although conventional treatment delivering antibody or or antibody-like molecules suppress the infection but fails to accomplish complete removal of infection. Novel treatments using probiotics proved to be more beneficial in the disruption of biofilm-related infections and become an emerging field of investigation. This novel treatment is still in preliminary stage and further research should be focused on viability, functionality and efficacy of the probiotic strains. To know the complete mechanism through which it exerts its effect, detailed molecular level study is also needed. Further studies on probiotics will fill the gaps in the current information and prove its potential in removing biofilm-associated infections.

Keywords Microbes · Biofilm · Infection · Probiotics · Therapy

17.1 Introduction

Biofilm development plays a vital role in the progression of many subacute and prolonged bacterial infections. The infections such as cystic fibrosis lung infection, chronic as well as recurrent otitis media, chronic wounds and catheter-associated infections are a considerable reason for morbidity and mortality. Infections caused due to biofilm are difficult to eliminate as it shows considerable resistance toward conventional antibiotic therapy due to limited diffusion, heterogeneity in growth, alternation in membrane formation, etc. So to overcome these limitations, an alternative approach which must be safe, host receptive and cost-effective is needed. The term probiotic is a complex word composed of Latin word pro and the Greek word

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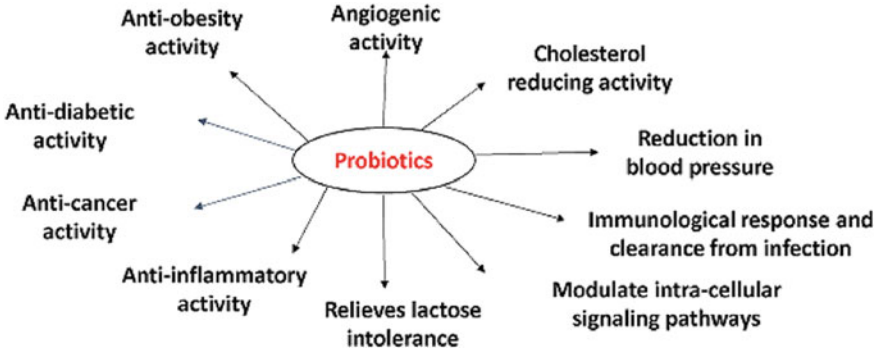

Fig. 17.1 Applications of probiotics

bios, and it factually means for life (Saxami et al. 2016). According to the World Health Organization (WHO), probiotics are defined as 'live microorganisms which deliberate a health benefit on the host, when administrated in acceptable amounts' (Sikorska and Smoragiewicz 2013). Probiotic therapy is interesting due to its effectiveness and noninvasive low-cost approach, which aims to reconstruct natural flora rather than its disruption (Sorokulova et al. 2008). Probiotics contain live beneficial microorganisms which offer variety of health benefits to the host (Fig. 17.1). It has shown outstanding outcomes against various pathogenic bacteria in the gut. This chapter focuses on the role of probiotics in treating biofilm-related infections.

17.2 Probiotics in Treating Microbial-Associated Biofilm Infection

Probiotics not only prevent diarrhea and other side effects of antibiotics but in some cases also improves cure through disruption of biofilms, enhancing host cell junction integrity, priming immune defenses and dismantling pathogenic virulence factors (Reid 2017). Various non-pharmaceutical approaches such as silver nanoparticles and photodynamic therapy have also shown satisfactory activity against biofilm formation and maturation. Especially, probiotics are likely to be realism, once there is an acceptable clinical database to support their use (Bandara et al. 2017). A list of microorganisms showing potential activity against biofilm infection is listed in Table 17.1.

An antimicrobial agent, reuterin released by the probiotic bacterium, *Lactobacillus reuteri*, has a wide range **of** action against a variety of pathogens, including bacteria, fungi, protozoa and viruses (Spinler et al. 2008). Probiotics not only overwhelm the growth of foodborne pathogens but also lessen biofilm development by protecting infection caused due to pathogens by releasing antimicrobial compounds. Probiotic lactic acid bacteria create an acidic environment, lower the risk of pathogen

Table 17.1 List of probiotics strains showing beneficial effect against microorganisms

Strain	Result	Mode of study	Reference
Milk containing *Lactobacillus rhamnosus*	Reduction in *S. mutans after 7 months of treatment*	Clinical	Nase et al. (2001)
Tablets of *L. reuteri*	Decrease in salivary *S. mutans* after 3-week oral intake	Clinical	Caglar et al. (2006)
Ice cream containing *Bifidobacterium lactis Bb-12*, *L. acidophilus La-5*	After 7 days, reduction in *S. mutans* was observed	Clinical	Ashwin et al. (2015)
L. plantarum, *L. paracasei*, *L. rhamnosus GG*, *L. acidophilus*, *L. reuteri*	Inhibited *Candida* growth in the presence of probiotics	In vitro	Hasslof et al. (2010)
Commercial probiotic drinks containing *L. casei*	Decreased *Candida* occurrence after 30 days with increment of sIgA level	Clinical	Mendonca et al. (2012)
L. reuteri	Improved *Candida* score	Clinical	Kraft-Bodi et al. (2015)
L. plantarum, *L. fermentum*, *L. paracasei*, *L. casei*	Inhibition of *S. mutans* biofilm by probiotic strains	In vitro	Kojima et al. (2016)
L. reuteri strains	Inhibition of *S. mutans* biofilm through release of hydrogen peroxide and bacteriocin-like compound	In vitro	Kang et al. (2011)

colonization, create more favorable environment for the resident microbiota and the reduced pH in the gut by releasing organic acids (mainly lactic and acetic acids) which is inhibitory to the pathogens (Servin 2004).

Detoxification of inhibitory molecules and oxygen-scavenging compounds such as amines or nitrates, one of the metabolic mechanisms shown by probiotic strains, forms a favorable anaerobic ecosystem in the gut for the resident microbiota. These metabolic activities and survival capability throughout the gut are commonly relied on the selected probiotic strains (Chaucheyras-Durand et al. 2008). The exopolysaccharides secreted by probiotic strains can prevent the formation of biofilms by pathogens (Kim et al. 2009). The biofilms formed within and on surface of various food-processing plants can be reduced with the help of probiotic microorganisms. Biofilms present in the floor drains of a ready-to-eat poultry products processing plant formed by *Listeria monocytogenes* reduced by two LAB probiotic strains *Enterococcus durans* strain 152 and *Lactococcus lactis* subsp. *Lactis* strain C-1-92 (Zhao et al.

2013). *L. monocytogenes* biofilms within a meat-processing plant on abiotic surfaces could be inhibited by *Lactobacillus* spp. (Ibarreche et al. 2014). According to another investigation, probiotic *Lactobacillus* strains (*L. acidophilus* KACC 12419, *L. paracasei* KACC 12427, *L. casei* KACC 12413 and *L. rhamnosus* KACC 11953) can efficiently diminish biofilm formation by the foodborne pathogens *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *L. monocytogenes* through competition, exclusion and displacement mechanisms (Woo and Ahn 2013).

L. monocytogenes biofilms present on stainless steel surfaces, widely used for equipment in food-processing plants can be controlled by spray-dried crude bacteriocin fermentate (CBF) from *L. lactis UQ2*, *or L. lactis UQ2 cells* (García-Almendárez et al. 2008). As per the investigation, *L. lactis* UQ2 can reduce more than 5 log cycles of *L. monocytogenes* planktonic and sessile cells on stainless steel chips. Biofilm formation by *L. monocytogenes* on stainless steel surfaces can also reduce by *L. sakei 1* and its bacteriocin (Winkelströter et al. 2011). Probiotic biofilms can be used as substitute to lessen the formation of pathogenic biofilms in the food industry (Gomez et al. 2016). From the investigation, it was found that LAB probiotic strains from foods were able to control biofilm formation by *Salmonella enterica subsp. enterica serovar Typhimurium*, *Escherichia coli O157*:*H7* and *L. monocytogenes* foodborne pathogens in food-processing facilities, without deliberating any risk to consumers (Guerrieri et al. 2009).

An antimicrobial exopolysaccharide (r-EPS) compound produced by probiotic agent *L. acidophilus A4* can lessen biofilm development by enterohemorrhagic *E. coli* O157:H7 on 96-well microplates (87%) and on polystyrene and polyvinyl chloride surfaces (94%) (Kim et al. 2009). In another study, culture supernatant (CS) of probiotic strain *Lactobacillus* spp. inhibited the biofilm formation in *Vibrio cholera* by more than 90%. But the biofilm dispersive action of CS is found to be strain specific and has pronounced therapeutic potential at high pH (Kaur et al. 2013). Different strains of probiotic *Lactobacillus* spp. inhibited biofilm formation in *Streptococcus mutans* by reducing expression of genes involved in EPS production, acid tolerance and quorum sensing (Wasfi et al. 2018). Three lactic acid bacteria (LAB) were evaluated for anti-bacterial and anti-biofilm activities against oral bacteria isolated from barley, traditional dried meat and fermented olive. The tested LAB were γ-hemolytic and vulnerable to four antibiotics. All the strains were resistant to low pH, bile salt, pepsin and pancreatin which strongly recommends the use of probiotic strain in the prevention of oral disease (Ben Taheur et al. 2016).

Cell-free spent media (CFSM) of six probiotics belonging to the genus *Bifidobacterium* and *Lactobacillus* which were grown in Man–Rogosa–Sharpe (MRS) broth have shown strong anti-bacterial activity against all *E. coli* isolates. The CFSM of MRS fermented by all probiotics also resulted in inhibition of biofilm formation supporting its effective use to eliminate biofilms formation by multidrug-resistant *E. coli* (Abdelhamid et al. 2018). Fungal biofilms possessing different characteristic such as increased resistance to the immune defense and anti-mycotic agents as compared to their planktonic cells counterpart also inhibited by probiotics strains. Probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 were shown to completely inhibit vulvovaginal candidiasis (VVC)-causing *Candida glabrata* biofilms. The inhibition

occurs by partially hindering the adherence of yeast cells and the outcome might be contributed by the secretory compounds formed by these probiotic lactobacilli strains (Chew et al. 2015). In another experiment, single and mixed non-albicans *Candida* species biofilm formed in the 96-well microplate and on the surfaces of medical-grade silicone were inhibited by cell-free supernatants of probiotic strains of *L. gasseri* and *L. rhamnosus*. *Lactobacilli* supernatants were added 24 h after biofilm initiation, which disrupt mature biofilm formation. The inhibition of the mixed biofilms and damage to the cells were confirmed with confocal laser scanning microscopy and scanning electron microscopy (Tan et al. 2018).

In another study, probiotics have shown better result as compared to conventional anti-fungals in reducing experimental oral candidiasis in a murine model (Matsubara et al. 2012). 2-Hydroxyisocaproic acid (HICA) produced by *Lactobacillus* species reduces biofilms of *C. albicans* and inhibits acetaldehyde production at acidic pH in in vitro (Nieminen et al. 2014). In the presence of probiotics containing *L. salivarius*, the biofilm mass and the number of colonies of *S. mutans*, *C. albicans* and *S. mutans* with *C. albicans* were reduced. The intermediate secreted by *L. salivarius* inhibits fungal growth which weakens the pathogenic potential of *C. albicans* (Krzysciak et al. 2017).

In eliminating biofilms, the introduction of probiotics will likely to be one of the more promising approaches. However, before administration these probiotic organisms need to possess a very high safety margin and optimization of their treatments for long-lasting effect (Bandara et al. 2017).

17.3 Probiotics in Treating Biofilm-Mediated Dental Diseases

Various studies have proven that in vitro growth of two important cariogenic streptococci, *S. mutans* and *S. sobrinus* inhibited by one strain of *L. rhamnosus* and the species *L. casei*. In children of 3–4 years of age, consuming milk containing probiotic has shown significantly fewer dental caries and lower salivary counts of *S. mutans* than control (Bonifait et al. 2009).

Regular consumption of probiotic yoghurt has shown to reduce number of cariogenic streptococci in the oral cavity (Bizzini et al. 2012; Ruiz-Martinez et al. 2015). In another study, *L. rhamnosus* strongly inhibited formation of cariogenic biofilm by reducing production of glucan in *S. mutans* (Lee and Kim 2014). Co-aggregation of probiotic lactobacilli with *S. mutans* and other strains associated with caries—inhibit the growth of *S. mutans* (Hasslof et al. 2010; Lodi et al. 2010). The production of antimicrobial agents such as organic acids, hydrogen peroxide and anti-fungal compounds such as fatty acids and bacteriocins may responsible for the bactericidal and bacteriostatic effects of probiotic lactobacilli (Twetman et al. 2009).

The beneficial effects of probiotics exerted by improving gut health (normalization of intestinal microbiota), production of antimicrobial compounds (organic

acids, hydrogen peroxide and bacteriocins) leading to antagonizing pathogens, competing for pathogen-binding sites, available nutrients, growth factors and modulate the immune response and metabolic effects (Mahasneh and Mahasneh 2017; Lin et al. 2017).

Recently studied clinical investigation proved the efficacy of probiotics in relieving mucosal *Candida* infections oral infections (Ishikawa et al. 2015; Kraft-Bodi et al. 2015; Li et al. 2014; Mendonca et al. 2012; Hatakka et al. 2007). In a large clinical study in children, *L. rhamnosus* strain GG significantly lower the incidence of dental caries as well as lower the numbers of *S. mutans* at the end of exposure (Nase et al. 2001). In another clinical study, *Lactobacillus*-containing tablets reduced periodontal pathogen counts in 57 subjects (Ishikawa et al. 2003). Administration of mixture of *L. sporogens*, *L. bifidum*, *L. bulgaricus*, *L. thermophilus*, *L. acidophilus*, *L. casei* and *L. rhamnosus* in both capsule and liquid forms significantly increased salivary *Lactobacillus* counts in a clinical study consisting of 35 subjects (Montalto et al. 2004). *L. reuteri* ATCC 55730 reduced *S. mutans* counts in both directly or by straw delivery of probiotics in a clinical study involving 200 healthy young adults (Caglar et al. 2006).

17.4 Probiotics in Treating Biofilm-Mediated Urinary Tract Diseases

Bacterial vaginosis (BV) contributes more than 60% of all vulvovaginal infections and become the leading vaginal disorder in women during her childbearing age (Sobel 2000). Various probiotic strains were found to be effective against BV.

In a study, probiotic strain *L. reuteri* RC-14 and *L. rhamnosus* GR-1 incorporated into pathogenic biofilms associated with BV in in vitro decrease cell density due to increased cell death (Mcmillan et al. 2011). In another study, probiotic strains *L. rhamnosus* GR-1 and *L. fermentum* were suspended in skim milk and given to women with a history of BV, twice daily for 14 days. After 14 days, these two strains were recovered from vagina, which demonstrates its potential in restoration of urogenital flora in affected women (Reid et al. 2001). *L. rhamnosus* GR-1 also reduce the colonization of the vagina by gram −ve pathogens (Bruce and Reid 1988).

L. plantarum and *L. fermentum* isolated from the vagina of healthy women have shown probiotic potential. Both species produce a huge amount of biosurfactants as well as H_2O_2 which inhibit the growth of intestinal and urogenital pathogens (Anukam and Reid 2007). Probiotic *L. fermentum* inhibits the growth of urogenital pathogens either by inhibiting the adhesion of pathogens or by producing compounds such as hydrogen peroxide, bacteriocins and bio-tensioactives (Kaur et al. 2013). In another investigation, *L. casei* isolated from the urethra of healthy women have shown to prevent the onset of urinary tract infection (UTI) in 84% of tested female rats (Reid et al. 1985). The growth and inflammatory responses of *E. coli* causing UTI were inhibited by intra-urethral administration of *L. casei* Shirota in female mice

(Asahara et al. 2001). Co-administration of probiotics can reduce the side effects of antibiotics used for the treatment of BV. Probiotics will help in restoration of intestinal homeostasis by reducing the production of pro-inflammatory cytokines as well as prevention of epithelial cell apoptosis.

L. rhamnosus GR-1 and *L. reuteri* RC-14 (previously called *L. fermentum* RC-14) appeared to be the most effective among the examined lactobacilli for the prevention of urinary tract infections (UTIs). In specific studies, *L. casei Shirota* and *L. crispatus* CTV-05 have also shown effectiveness (Falagas et al. 2006).

Recently studied clinical investigation also proved the efficacy of probiotics in relieving mucosal *Candida* infections of urogenital (Kovachev and Vatcheva-Dobrevska 2015; Hu et al. 2013; Vicariotto et al. 2012; Martinez et al. 2009). In a randomized clinical trial, probiotics have shown a beneficial effect on adult women who are suffering from BV (Huang et al. 2014).

17.5 Probiotics in Treating Biofilm-Mediated Gastrointestinal (GI) Diseases

Probiotics restore normal bacterial microflora and prove their efficacy in treating various GI disorders (Verna and Lucak 2010).

Administration of *L. rhamnosus* GG moderately increased treatment success in school-aged children suffering from functional abdominal pain disorders (Gawronska et al. 2007). Two probiotic strains *E. faecium* RM11 and *L. fermentum* RM28 have triggered anti-proliferation of colon cancer cells at the rate of 29%. Both culture medium and live whole cells from these two probiotic strains were effective in anti-proliferation of colon cancer cells as compared to the control group (Thirabunyanon et al. 2009).

In a study, probiotic bifidobacteria strain which is effective against ulcerative colitis (UC), in bifidobacteria fermented milk (BFM), raises the production of IL-10 in peripheral blood mononuclear cells (PBMNC) which inhibit the secretion of IL-8 in intestinal epithelial cells (Imaoka et al. 2008). In another investigation, probiotics reduce the Streptococcus count and increase the absolute count of probiotic bacteria by modifying fermentation pattern in the large intestine (Hunter et al. 1996).

Some clinical investigation also proved the efficacy of probiotics in relieving mucosal *Candida* infections of gastrointestinal (Roy et al. 2014; Manzoni et al. 2006; Romeo et al. 2011). In a randomized controlled trial, consumption of *L. casei* strain Shirota (Yakult) reduces small intestinal bacterial overgrowth (SIBO) (Barrett et al. 2008). The normal function of GI tract affected due to alternation in the gene expression by intestinal microbes. In a human study, it was demonstrated that the presence of probiotics in the GI tract can alter the gene expression (van Baarlen et al. 2010).

17.6 Probiotics in Treating Biofilm-Mediated Skin Infections

Probiotics have shown antimicrobial property against chronic inflammatory skin conditions such as acne and atopic dermatitis (AD) through production of bacteriocin-like inhibitory substances (Mottin and Suyenaga 2018). Anti-bacterial protein, bacteriocin-like inhibitory substance (BLIS), produced by probiotics *S. salivarius* and *E. faecalis* causes significant inhibition in the growth of *Propionibacterium acnes* in in vitro condition (Bowe et al. 2006). A lotion produced with probiotics *E. faecalis* SL-5 isolated from feces of a healthy Korean adult has shown anti *P. acnes* effect (Kang et al. 2009). In a clinical study involving *L. plantarum*, a reduction in light acne lesions was observed with erythema reduction and barrier reconstruction (Muizzuddin et al. 2012). In another study, whole cultures, culture filtrates of probiotic organism *L. plantarum* have shown improved tissue repair and decrease apoptosis in a burned-mouse model infected with *Pseudomonas aeruginosa* (Valdez et al. 2005). Probiotics *Bacillus coagulans* RK-02 produce extracellular polysaccharides in in vitro, which have shown significant antioxidant and free radical scavenging properties (Kishk and Al-Sayed 2007; Kodali and Sen 2008). This study recommend role of probiotics in slow down of aging of the skin by reestablish the balance between free radical scavengers and the free radical production (Kober and Bowe 2015).

17.7 Probiotics in Treating Biofilm-Mediated Numerous Diseases

Probiotics also play a significant role in treating biofilm-mediated diseases of the eye, ear, nose, throat, heart and lungs.

The combination of probiotics *L. acidophilus* and *B. lactis* prevent the pollen-induced infiltration of eosinophils into the nasal mucosa and reduce nasal symptoms in children suffering from allergic rhinitis (Ouwehand et al. 2009). Infections of the ears, nose and throat (ENT) caused by biofilms form on moist biotic and abiotic surfaces and difficult to eliminate (Kramer and Heath 2014). Beneficial effects on ENT biofilms were observed by probiotics such as *L. casei* (West et al. 2014). The suppression of asthmatic response with attenuation of Th17 cell development was shown by oral administration of *E. faecalis FK-23* (Zhang et al. 2012).

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Chapter 18
Biofilm and Antimicrobial Resistance

Vineeta Mittal

Abstract Biofilm-forming bacteria cause severe health problems in patients with implanted devices by attachment of cells to surface matrix. Antibiotics can act on planktonic bacteria more easily than biofilm bacteria. Biofilm bacteria have several mechanisms for combatting antibiotic action on them. Poor penetration of antibiotics, exopolysaccharide, eDNA in matrix degradation has a role in antibiotic resistance. Limited nutrient, slow growth, the response of adaptive stress and persister cell formation also cause multilevel protections for antibiotic resistance. Genetically horizontal gene transfer and higher mutation frequency also show a pivotal role in antimicrobial resistance in biofilm bacteria.

Keywords Biofilm · Antibiotic resistance · Polysaccharide · Extracellular DNA · Persister cells · Quorum sensing · Efflux pump

18.1 Introduction

Manifestations of bacterial-based infection have been the cause of various human ailments, and it has remained a challenge in spite of the availability and progresses in advance antimicrobial agents which could act on the multitude of the bacterial infection and sterile it.

Bacteria persist in two basic forms, first as free-living planktonic replicating cells, which are only 1% and other as biofilm bacteria found in nature, as well as the industrial and clinical environment. Bacterial biofilm causes chronic infections such as periodontitis, cystic fibrous pneumonia, and numerous device associated infections such as the catheter, heart valves, and prosthesis. New challenges have emerged due to progressive resistance developed by the bacterial infection to the antibiotic.

This is clearly understood that traditional antibiotic resistance of planktonic bacteria causes enzymatic inactivation of antimicrobial agents, alteration in normal binding protein, membrane permeability, and bypass of metabolic block initiated by antimicrobial agents, antibiotics modifying enzymes such as β lactamases.

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The mechanism of bacterial biofilm is to protect the activity of bacteria from antibiotics by forming a non-permeable barrier for the antimicrobial agents to penetrate.

18.2 Biofilm and Antimicrobial Resistance

Planktonic bacteria are susceptible to antibiotics by antibodies or phagocytes. When bacteria make biofilm and change into sessile communities, they become resistant to antibiotics, antibodies, or phagocytes. Biofilm producing bacteria have 100–1000 increased minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of antibiotics in respect of planktonic bacteria and 150–3000 times more resistant to disinfectants.

Pseudomonas aeruginosa biofilm, which can grow on urinary catheters, has ~10^3 times increased resistance for tobramycin as compared to planktonic cells. Biofilm-mediated antibiotic resistant and tolerance are dependent on various factors such as:

- Particular antimicrobial agent
- Strains and species of bacteria
- Biofilm age
- Biofilm development stage
- Growth conditions of biofilm.

18.3 Mechanism of Antimicrobial Resistance in Biofilm

18.3.1 Biofilm Matrix

Around 97% part of biofilm is water and solutes. The size of antibiotics diffuses readily in the biofilm matrix. Biofilm has a self-produced biopolymer matrix. The matrix contains exopolysaccharides, proteins, and DNA. Antibiotics have to penetrate this layer to reach targets. The penetration of antibiotics inside the biofilm does not confirm by only the physical movement of antibiotics in biofilm.

18.3.2 Factors on Which Antimicrobial Penetration into a Biofilm Matrix Depends

I. Biofilm thickness
II. Effective diffusivity of the agent in the biofilm

III. Reactivity of the agent in the biofilm
IV. The sorptive capacity of the biofilm for the agent
V. Dose concentration and dose duration
VI. External mass transfer properties.

18.3.3 Antibiotic Penetration of Biofilm

Antibiotic delivery is retarded to the depth of film due to either deactivated antibiotic by consequent reactions or sequestration of antibiotics by binding as it diffuses into the biofilm.

A biofilm formed by a beta-lactamase positive bacterium has a sufficient reaction-diffusion interaction to prevent penetration of a penicillin antibiotic (Stewart 2002) (Fig. 18.1). In uropathogenic *Escherichia coli* (UPEC) biofilms, tetracycline penetrated all cells within ten minutes of contact deprived of disturbing viability of cell and MIC of ampicillin (in the absence of matrix β-lactamase) and ciprofloxacin is much more than MICs of planktonic bacteria so that they well permeated into *Klebsiella pneumoniae* biofilms. In contrast, in *Staphylococcus aureus* and *S. epidermidis* biofilms, diffusion capacity of oxacillin, cefotaxime, and vancomycin through biofilm was restricted; this may show that some antibiotics have a penetration barrier for decreased susceptibility of biofilms (Clayton and Mah 2017).

Penetration of antibiotic in the biofilm and the bacterial colony has a complex matrix, and it depends on the time of infusion, its periodicity, dose and duration of the antibiotic agents. These aspects have been studied by Jefferson et al. (2005), Stewart et al. (2015), Singh et al. (2010, 2016), and they have published its mechanism in detail.

The penetration times range from a fraction of a minute to almost a full day. There is an association between the dose duration and penetration time. Penetration time of 30 min could be fast if the dose duration is 8 h and slow if the dose duration is 3 min.

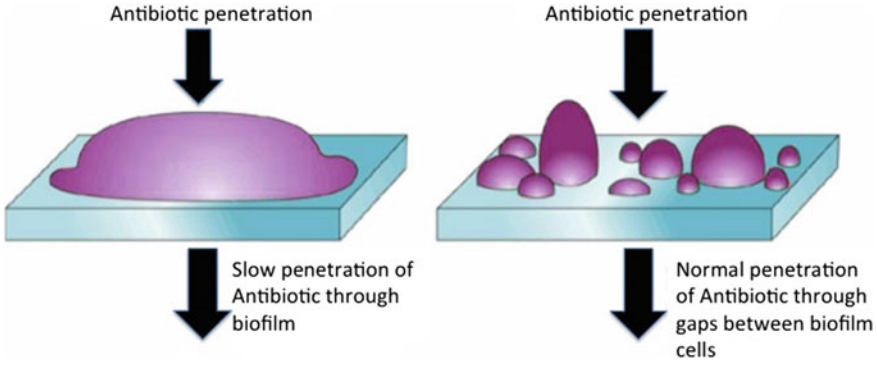

Fig. 18.1 Antibiotic penetration of antibiotic through biofilm

The molecular weight of antimicrobial drugs cannot affect on their penetration times as large antibiotics or antimicrobial peptides can take only a few minutes to penetrate a biofilm such as vancomycin take a half minute, daptomycin one and a half minute and nisin four to ten minutes. Higher molecular weight chemicals are mostly cationic molecules including quaternary ammonium compounds, such as cetylpyridinium chloride and benzalkonium chloride, and an aminoglycoside antibiotic. The retarded penetration of these agents into the biofilm derives from the reaction or sorption of the agent in the biofilm as it diffuses. Halogens react with uncharacterized components of biomass and are neutralized. Hydrogen peroxide is destroyed by the action of catalase. Agents with a positive charge likely bind to negatively charged polymers or cell surfaces are delaying penetration. When considering agents that are subject to reaction or sorption in the biofilm, it is anticipated that the rate of penetration will depend on the applied concentration. This analysis reveals that agents such as chlorine, peracetic acid, and tobramycin penetrate a given biofilm faster as the applied concentration is increased. One of the examples is tobramycin and *P. aeruginosa* biofilm. In *P. aeruginosa* biofilms, there is decreased tobramycin diffusion. If we will add cations in the growth medium, the positively charged tobramycin molecule interacts with eDNA and bacteriophage particles and tolerance of *P. aeruginosa* biofilms will be increased to tobramycin. It is worth mentioning that if time increases, antibiotics would penetrate the biofilm (Clayton and Mah 2017).

18.3.4 Polysaccharide (PS)

The polysaccharide element of the matrix gives several various edges to the cells within the biofilm. This also generates adhesion, protection, and structures integrity. The mechanism of action is that aggregative polysaccharides function as molecular glue. This results in protective action, which facilitates the microorganism cells to stick to every alternative available for binding to the surfaces. The action generated to create adhesion enables the colonization of both biotic and abiotic surfaces. This results in assisting the bacteria to resist physical stresses forced by the fluid movement, which might have delinked the separate, the cells from a nutrient source, and its survival would have been susceptible. Polysaccharides have the potential to initiate protection from a variety of stresses. These may be due to dehydration, immune modifiers, and predators like phagocytic cells and amoebae.

18.3.5 Pseudomonas aeruginosa *Pel*

18.3.5.1 Significance, Structure, and Regulation

Pel is an aggregative PS produced by *P. aeruginosa*. It derives its name from the thick pellicle observed in strains overexpressing the *pel* operon. The primary function of Pel is to help the structural integrity of biofilm. The secondary function of Pel is its

role in resistance to aminoglycoside antibiotics in a biofilm. Mutant *pel* biofilms, deprived of pel, were found to be easily annihilated by the antibiotic compared to those, which were wild type and organized to stop the penetrations of antibiotics (Colvin et al. 2011).

18.3.6 Pseudomonas aeruginosa *Psl*

Biofilm formation is accelerated essentially due to the synthesis of a locus (psl), which is mediated by *P. aeruginosa* PS. It is predicted that this typical polysaccharide is responsible to encode an exopolysaccharide, which is critical for biofilm formation. The *psl* operon consists of 15 genes (*pslA-O*), which are co-transcribed. Mutagenesis studies revealed that 11 of these genes (*pslACDEFGHIJKL*) are essential for Psl production and surface attachment. Psl may essentially exist in two subtypes, which appears to be generated from a cell. It may be a high molecular weight cell-associated form or a low molecular weight form. Psl consists of D-mannose, D-glucose, and L-rhamnose in a 3:1:1 ratio, respectively. The structure of cell-associated Psl is unknown but is believed to be a polymer of mannose, glucose, and rhamnose and possibly galactose. Psl is produced primarily in nonmucoid strains because the expression of the *psl* operon is repressed in mucoid strains (Billings et al. 2013).

Role of Psl:

- Biofilm structural integrity
- Generation of resistance to polysorbate 80 (a non-ionic surfactant, which inhibits biofilm formation)
- Resistance to cationic antibiotics like colistin, polymyxin B, tobramycin and anionic antibiotics like ciprofloxacin.

18.4 Antibiotic-Modifying Enzymes in the Matrix

Enzymes such as secreted β-lactamases are present in the matrix of biofilm. These enzymes can degrade antimicrobials so that they cannot reach the cellular target. Anderl et al. (2000) have established that effective degradation and prevention of ampicillin to reach cells within the biofilm are done by secreted β-lactamase produced by *K. pneumoniae* biofilms. Bowler et al. (2012) described that the amount of β-lactamase is higher in mature cells than nascent cells in the matrix so ceftazidime and meropenem are more resistant for mature *P. aeruginosa* biofilms.

Giwercman et al. (1991) showed the secretion of chromosomally encoded AmpC β-lactamase which presence and secretion, into the matrix of *P. aeruginosa* biofilms, works as basic factor for developing resistance to an antibiotic in these biofilms.

18.5 Extracellular DNA (eDNA)

In biofilm formation and its development extracellular DNA has a pivotal role with high concentrations in the extracellular matrix. It can safely be said that eDNA is a significant constituent of the bacterial biofilm matrix. Its role in horizontal gene transfer via natural transformation is also important. There are two types of eDNA—endogenous or exogenous. Biofilm resistance increases to some antimicrobial agents by eDNA.

Mechanisms of eDNA in biofilm bacterial resistance are:

- Alteration of the extracellular environment by chelating cations like magnesium ions.
- Spermidine synthesis due to decreased Mg^{2+} in the environment.

Spermidine is a polyamine, which selectively concentrates, in the outer membrane. It is believed that selectively enhanced concentration of polyamine helps in protecting the cell. The mechanism of protective action is facilitated by decreasing the outer membrane permeability for aminoglycosides and other positively charged antimicrobial agents (Johnson et al. 2012).

18.6 Bacteriophages

The bacteriophage is naturally occurring viruses, which infects bacteria.

Mechanism:

- Produce enzymes that degrade extracellular matrix of biofilm so that release of eDNA
- Produce antibiotic tolerant small colony variants within biofilms
- Produce liquid crystalline matrix in *P. aeruginosa* biofilm, which acts as tolerant to an antibiotic.

18.7 Establishment of Microenvironment Within the Biofilm

Reduction in supply of nutrients and oxygen inside biofilms induces changes in the metabolic activity and the bacterial growth is also retarded. Several studies have suggested that oxygen concentration may be differentially distributed which may be high at the surface but low in the center of the biofilm. This leads to the creation of an anaerobic environment in the center. Usually, antimicrobials are acted efficiently in killing rapidly growing cells. So, biofilm resistance is contributed by the slow growth

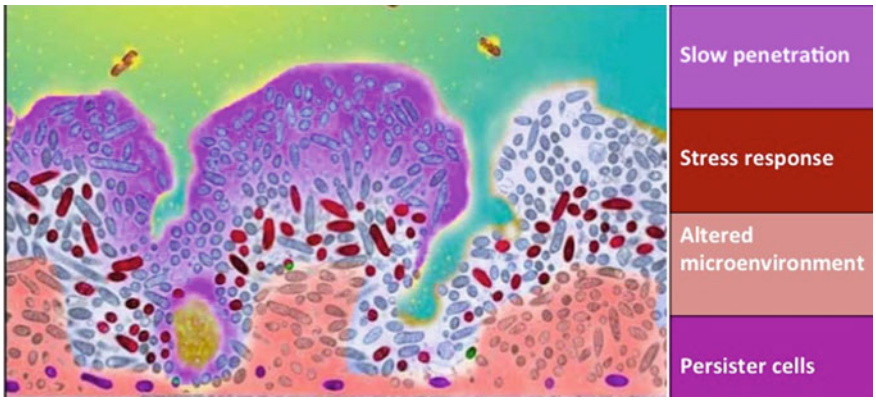

Fig. 18.2 Factors of antimicrobial resistance in biofilm

of bacteria (Paraje 2011). Exceptionally, *P. aeruginosa* cells can sustain metabolic activity by denitrification or by fermenting arginine under anaerobic conditions. Kolpen et al. (2016) showed that *P. aeruginosa* biofilms had increased susceptibility to colistin under anaerobic conditions (Fig. 18.2).

18.8 Persister Cells

Persister cells are a small group of planktonic culture cells in biofilm, which are dormant, non-growing, and persist despite antibiotic treatment (Lewis 2001) (Fig. 18.3).

Characteristics of persister cells:

I. Persister cells are the normal cell at a particular stage of the cell cycle
II. Persister cells are non-growing or slow-growing cells
III. Persister cells are not mutant cells
IV. Cells within biofilm that neither grow nor die in the presence of antibiotics
V. Formation of persister cells depends upon the metabolic activities of bacteria
VI. The incidence of persister cells is much more in bacterial biofilm than planktonic cells
VII. Persister cells directly not acted on the bactericidal agent but they compete for an antibiotic target for the production of multidrug-resistant (MDR) protein.

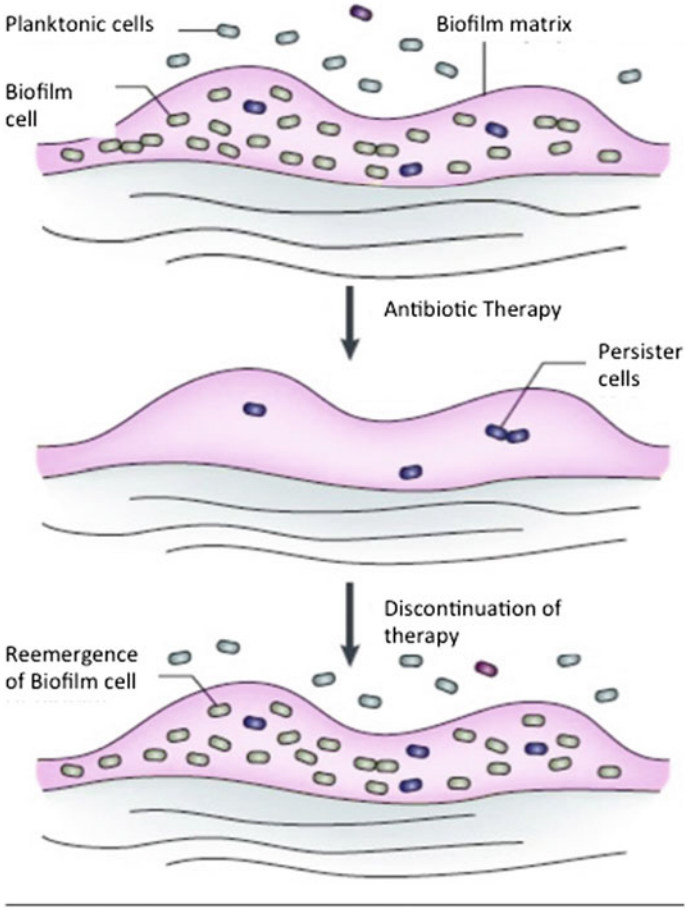

Fig. 18.3 Persister cells formation in biofilm cells

18.9 Oxidative Stress

It is prudent to mention that oxidative stress is essentially caused by a mismatch between the generation of oxidants and the level of antioxidative defense. The oxidative stress is caused by the presence of free radicals, peroxide and nitric oxide. This imbalance causes facilitated damage to the cellular components, including the matrix, DNA, proteins, and lipids (Paraje 2011). The generation of endogenous oxidative stress in biofilms is one of the important causative factors for promoting antibiotic resistance. Thus, the addition of antioxidants in the local environment reduced the occurrence of diversity and overall resistivity to antimicrobial elements in biofilms (Hoiby et al. 2010).

Factors for stress induction:

- Nutrient deprivation caused by stationary part microorganism growth
- High or low temperature
- High osmolality
- Acidic pH.

18.10 dltABCD

The *dltABCD* operon had an important role in biofilm-specific gentamicin tolerance in infective endocarditis caused by *Streptococcus mutans*. Nilsson et al. (2016) have published their findings that dltA mutant did not affect biofilm formation, but gentamicin resistance was eight times in this as compared to wild type. Another role of this gene in *S. aureus* and *Enterococcus faecalis* was studied. Planktonic resistance to vancomycin was decreased by deletion of *S. aureus dltA* gene, and planktonic resistance to colistin and polymyxin B was decreased by *E. faecalis dltA* mutant gene (Gross et al. 2001; Fabretti et al. 2006).

18.11 Glycosyltransferases

18.11.1 ndvB

The genetic study to establish causes for resistance to antibiotic penetration in the biofilm has, through a light in understanding the factors related to genetic ingredients. It was found that *ndvB* gene is an antibiotic resistance gene embedded in *P. aeruginosa* biofilm. It was found that *ndvB* gene plays a critical role in the formation of highly glycerol phosphorylated beta (1→3)-glucans, which binds aminoglycosides. ndvB mutant has characteristics of lowered biofilm resistance to tobramycin. Such as ndvB mutant, biofilms are 16 times less resistant to tobramycin and eight times less resistant to both gentamycin and ciprofloxacin wild-type biofilms (Clayton and Mah 2017).

18.11.2 epaI/epaOX

epaI and *epaOX* genes encoded glycosyltransferases for antibiotic resistance in *E. faecalis* biofilm. Studies by Dale et al. (2015) showed that the *epaI* mutant was less resistant to daptomycin, and the *epaOX* strain was more susceptible to gentamicin when growing as a biofilm than wild type (Clayton and Mah 2017).

18.12 Transcriptional Regulators

18.12.1 brlR *(PA4878)*

P. aeruginosa evolves a specific biofilm, which has antibiotic tolerance. It is found that BrlR (*b*iofilm *r*esistance *l*ocus *r*egulator) works explicitly as a transcriptional activator to enhance biofilm-specific antibiotic tolerance to *P. aeruginosa*. BrlR protein was found in biofilms but was not found in the planktonic proteome.

Tobramycin was as susceptible to isogenic *brlR* deletion mutant in planktonic bacteria compared to wild-type *P. aeruginosa*. Liao and Sauer (2012) studied that tobramycin resistance was fourfold increased by *brlR* forming biofilms than wild-type biofilms. The resistance of norfloxacin, trimethoprim, tetracycline, kanamycin, and chloramphenicol was also increased in a *brlR* mutant forming biofilm. Colistin, a cationic antimicrobial peptide have susceptibility toward planktonic and biofilm *brlR* cells, whereas this may not be the case for other antibiotics used for the purpose (Clayton and Mah 2017).

18.12.2 PA0756-0757

Zhang et al. (2013) showed a two-component response regulator system encoded by PA0756 in *P. aeruginosa*, PA0757 functions as an associated cognate histidine sensor kinase. The omission of the two-component system had far-reaching effects and when it was deleted, resistance to tobramycin decreases 4- to 8-times and resistance to gentamicin decreases 2- to 4-times in biofilms (Clayton and Mah 2017).

18.12.3 rapA

RapA is homolog to the family of SWI2/SNF2 and is closely linked as a superfamily of helicase-like proteins. Transcription of sequestered recycling RNA polymerase enzymes activated by rapA associated with RNA polymerase (Sukhodolets et al. 2001; Nechaev and Severinov 2008).

Mechanisms of biofilm-specific antibiotic resistance by RapA:

I. Diminished transcription of 22 genes in the absence of RapA with the inclusion of *yhcQ*, which encodes a multidrug efflux pump.
II. Less exopolysaccharide was produced by the uropathogenic *E. coli* (UPEC) *rapA* mutant biofilms than wild-type, by this antibiotic penetration was speedier into lower layers of the biofilm.

The *rapA* mutant biofilms were less tolerant of penicillin G, gentamicin, norfloxacin, and chloramphenicol as compared to wild-type biofilms (Lynch et al. 2007).

18.13 Efflux Pumps

Bacteria have specialized membrane-associated proteins for the expulsion of a large number of compounds from the cytoplasm to outside. The ability of bacteria to synthesize such specific tools ensures that cytoplasmic concentrations of certain antimicrobial compounds should remain below a critical threshold to inhibit the antibacterial function. The resistance of biofilms emboldened by antimicrobial efflux pumps, which enables resistance to cells by transferring and diluting the antimicrobial agents away and inhibiting action to neutralize intracellular targets. Thus, the antimicrobial agents get confined to extracellular space and its action stand annulled (Clayton and Mah 2017).

P. aeruginosa has numerous multidrug efflux pumps, for example:

I. **MexAB-OprM**: Planktonic resistance to antibiotics was mainly occurring by this (Poole 2011). MexAB-OprM efflux pump in *P. aeruginosa* biofilm resistance was responsible for low concentrations of ofloxacin.
II. **PA1875-1877 biofilm-specific multidrug efflux pump**: PA1874-1877 is a four-gene operon, which is ten times predominantly demonstrated in *P. aeruginosa* biofilms as compared to planktonic cells.
III. **MexAB-OprM or the MexCD-OprJ pumps**: These two genes encode a multidrug efflux pump in *P. aeruginosa* biofilms. MexAB-OprM or the MexCD-OprJ pumps cause resistance for azithromycin, a macrolide antibiotic by induction of mexC expression in biofilm cells on the exposure of azithromycin. The complexity of the influx pump could be a reason for colistin tolerance in metabolically active cells in *P. aeruginosa* biofilms. This has the propensity to induce negative and prohibitive action on multidrug efflux pumps.
IV. **RND family pumps**: The function of multidrug efflux pumps is very pertinent in understanding the biofilms resistance produced by several other bacterial species. For example, RND efflux pumps BCAM0925-0927 (RND-8) and BCAM1945-1947 (RND-9) cause the *Burkholderia cepacia* complex biofilms resistance to tobramycin although the BCAL1672-1676 (RND-3) pump was essential for the resistance of biofilm to both tobramycin and ciprofloxacin.

18.14 Quorum Sensing

The infliction of bacteria is generally influenced and regulated by quantum sensing (QS) by which bacteria engulfs and spreads by cell-to-cell interactions. The spread is also directly linked to the molecular signals produced by extracellular exhibits, its detections, production, and its capacity to automate the detections. The unique ability of the bacteria to induce sensing of specific genes, which are empowered to respond to the enhanced cellular population (Singh et al. 2017). Though the role of quorum sensing (QS) in biofilm resistance is not completely clear, the use of

QS inhibitors (QSI) has been suggested as a likely anti-biofilm strategy. Quorum sensing has been associated with biofilm resistance to antimicrobial agents. Such as, *lasRrhlR* strain of *P. aeruginosa* is lacking in quorum sensing so biofilms formed in this strain were found to have increased vulnerability to tobramycin as compared to wild-type biofilms (Bjarnsholt et al. 2005). Recently, Chua et al. (2016) have reported *P. aeruginosa* biofilms had a colistin-tolerant subpopulation of cells, which were based on quorum sensing.

It is significant to note that, by enhancing the proportion of quorum-sensing mutants comparative to wild type in mixed genotype *P. aeruginosa* biofilms resulted in reduced resistance to tobramycin (Popat et al. 2012). Moreover, in *E. faecalis*, the *fsr* quorum-sensing system and the quorum-regulated *gel E* protease were a prerequisite for biofilm, but the same was not required for antimicrobial actions to planktonic. The resistance to gentamicin, daptomycin, and linezolid is decreased in biofilms due to the presence of quorum-sensing mutants (Dale et al. 2015).

18.15 Genetic Diversity

18.15.1 Horizontal Gene Transfer

One more method for antibiotic resistance in biofilm bacteria is a horizontal transfer which may occur by transfer of plasmid between cells of biofilm via conjugation study, showed that frequency of gene transfer via conjugation of multidrug resistance plasmid in *S. aureus* was 10^4 times greater in biofilm as compared to planktonic culture.

18.15.2 Mutation Frequency

It is well established that biofilm resistance is phenotype, and the property they exhibit is temporary genetic changes. Mandsberg et al. (2009) reported that in biofilm cells, mutations are accumulated at higher rates than planktonic cells. It was suggested that *P. aeruginosa* with the hypermutable frequency with imperfect methyl mismatch repair or DNA oxidative repair often characterized in cystic fibrosis patients was found to be less susceptible to antibiotics than those with intact DNA repair mechanism. The biofilm lifestyle seems to contribute to a higher mutation rate, which may result in the appearance of permanently hypermutable strains. It is established that ciprofloxacin-resistant mutant of *P. aeruginosa* and *Campylobacter jejuni* biofilm cells have greater mutation frequency as compared to planktonic cells (Hoiby et al. 2010). It may be postulated that cells developed in biofilms are inherently prone to spontaneous mutation because endogenous oxidation stress is augmented. This may

also contribute to DNA damage. The possibility that an increase in several antibiotic resistance strains may attributable to mutation ensues in biofilm lands, and it is imperative to develop therapies that may eradicate biofilm.

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Chapter 19
Management of Inflammatory Bowel Disease (IBD) by Probiotics Biofilms

Alok Kumar, Swasti Tiwari and Amit Goel

Abstract Inflammatory bowel disease (IBD) is an inflammatory condition of small and large intestine. The inflammation in IBD is of autoimmune nature which is incited by several possible agents in a genetically predisposed host. Gut microbes are one of the agents known to cause IBD and their role in pathogenesis of IBD is supported by several evidences. Gut bacteria in colon are segregated in two compartments, namely luminal bacteria in feces and mucosal bacteria embedded in mucus coating of colonic mucosa in the form of biofilm. This biofilm represents a highly active ecosystem. The biofilm bacteria are in extreme proximity of host-mucosal immune system and live in symbiosis with host. Bacterial dysbiosis in biofilm could induce autoimmunity and mucosal inflammation as in IBD. Probiotics are the living microorganisms and have shown benefits in several immune-mediated conditions. Probiotics could rebalance the bacterial dysbiosis in mucosal biofilm and subside the immune dysregulation.

Keywords Inflammatory bowel disease · Gut microbiota · Gut flora · Microbiome

19.1 Human Gut Microbiome

Human gut is an excellent example of a complex ecosystem. It hosts trillions of microorganisms such as bacteria, viruses, fungi, and occasionally unicellular parasites. They are collectively called as gut flora or gut microbiome. Metagenomic sequencing of 16S ribosomal (16S rRNA) gene of the gut microbiome revealed approximately 1000–1200 bacterial species which accounts for over 99% of microbes in adult human gut; the remaining genome is contributed by archaea, viruses, and prokaryotes. The majority of the gut microbes are obligate anaerobes which outnumber the facultative anaerobes and aerobes by 100–1000 times in number (Qin et al.

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2010). The density and the composition of bacterial flora are not uniform and homogeneous along the entire length of the bowel but vary greatly. The density of microbes increases along the bowel length from esophagus to colon. In addition, aerobic and facultative aerobes are primarily restricted to proximal bowel, whereas anaerobe dominates in distal bowel.

At present, our understanding and knowledge about the human microbiome are primarily limited to bacteria. We have limited information and insight about human virome and fungiome. In current decade, the gut bacteria have been widely explored in healthy population as well as an etiological agent for various diseases related to gastrointestinal system, liver, cardiovascular system, respiratory tract disorders, and skin.

Most of the bacterial species, present in adult human stool, belongs to a very few taxonomic phylum, i.e., Bacteroidetes, Proteobacteria, Firmicutes, and Actinobacteria, with a very little representation from other phyla (Eckburg et al. 2005). Though the dominance of these phyla remains constant, their relative proportions and the dominant species vary between the individuals. Based upon the presence of dominant species, the existence of three distinct enterotypes is conceptualized with each enterotype characterized by a relatively higher representation of the genera Bacteroides, Prevotella, or Ruminococcus, respectively (Arumugam et al. 2011).

19.2 Luminal and Mucosal Bacterial Microbiome

The gut microbiota, particularly in colon, is compartmentalized into lumen microbes, i.e., the microorganisms present in the lumen admixed with fecal matters which are excreted in the form of stool (fecal flora); and mucosal microbes, i.e., microbes residing in the mucus coating of the gut mucosa. The bacterial composition of stool flora is different from that of mucosal flora. The inter-individual variation of mucosal microbes is relatively lower than luminal microbes. In an individual, mucosal flora seems to represent a subset of luminal flora. The fecal flora contains bacterial colonies from peeled off mucosal layer and a unique subset of non-adherent luminal population (Eckburg et al. 2005). The two microbial habitats, the gut lumen and the mucus coated mucosal lining, represent distinct microbial ecosystem and differ markedly in microbial diversity and composition. As compared to mucosal bacteria, fecal bacterial communities are more tightly clustered and are also more diverse (Ringel et al. 2015).

Though, the dominant bacterial phyla remained the same between fecal and mucosal niches, the relative abundances of Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria are different (Arumugam et al. 2011). Between the two microbial habitats, the abundances of Firmicutes and Actinobacteria are significantly higher, whereas those of Bacteroidetes and Proteobacteria are significantly lower in fecal flora (Ringel et al. 2015). In addition, both the niches also have few unique groups of bacteria (Zhao et al. 2017).

These differences in composition of luminal and fecal microbial communities may reflect either a consequence of difference in micro-environment such as oxygen tension, antimicrobial factors present in mucus, or a unique subset of microbes to accomplish different physiological functions in maintaining mucosal and luminal homeostasis.

19.3 Mucus Lining of the Colon

Epithelial lining of the colon in gastrointestinal tract is coated with a ~400 μm-thick uninterrupted mucus layer which is secreted by goblet cells present in mucosal layer. The mucus layer is organized in two layers—the outer layer on luminal side and inner layer lying close to mucosa. The inner mucus layer is renewed by fresh mucus secreted by goblet cells and pushes the older layer upwards to shed finally in the lumen. The inner mucus payer is relatively thinner, dense, firmly adherent with epithelial lining, resistant to penetration by luminal bacteria and hence is free of bacteria. The inner mucus layer cannot be removed with simple aspiration. On the contrary, the outer layer is thicker, penetrable by bacteria, and can be removed with aspiration; further, the outer mucus layer provides a safe habitat to the bacteria. These resident bacteria cause the proteolysis of the mucus and make the layer relatively looser.

Biochemically, mucus is composed of mucins, a highly glycosylated proteins, which contain approximately 20% protein and 80% glycan (carbohydrate). Mucin is synthesized and stored into the goblet cells. Before being released into the epithelial surface, mucins are folded by a calcium-ion-mediated mechanism and stored in granules inside the goblet cells. Once the mucins are released on cell surface, the calcium ion is chelated rendering the folding mechanism ineffective. Mucin has the characteristics to bind with a lot of water. Thus, the unfolded mucin is converted into an umbrella of gel with volume expanded over 1000-folds.

The mucus layer, present on the surface of colonic epithelium, serves several important function in humans; first, it forms a layer of protective gel and serves as an elastic barrier against bacterial adhesion and invasion, bacterial toxin-mediated mucosal injury, digestive enzymes, and other damaging agents present in food; second, the gel-like layer of mucus lubricates the gastrointestinal tract which helps in smooth and frictionless propulsion of luminal content; third, it provides a safe habitat to the resident bacteria; fourth, the carbohydrate component of mucins serves as a source of energy for resident mucosal bacteria; fifth, it provides a space, to the bacteria, very close to the epithelial layer so that they could prime the immune cells lying in submucosal layer of the bowel mucosa; sixth, bacteria embedded in mucus layer produces short-chain fatty acids (SCFA) which are directly delivered to the underlying epithelial cells for being used as an energy source.

Bacteria, indwelling in mucus layer, are distributed uniformly, though they are usually not found in crypts. Most of the mucosal bacteria are live, particularly those

lying close to the mucosal surface, and are replicating in the mucus layer; their presence in mucus layer is not merely because of fecal contamination.

19.4 Biofilm Present on Gut Mucosa

Mucosa adherent bacterial communities, together with the sea of mucus in which they are embedded, form a 'biofilm'. Bacterial composition of the biofilm is relatively host specific and constant along the entire colonic length (Zoetendal et al. 2002).

The microbial composition of biofilm is shaped under the influence of several factors and the hosts' immune system plays a pivotal role in it. Defensins and other antimicrobial peptides, secreted by the colonic epithelial cells, have prohibitive effects against a range of microorganisms such as viruses, fungi, and bacteria present in gut lumen and mucosa. In addition, the rate of mucus synthesis and its chemical composition, rate of epithelial cell layer turnover, host diet, availability of bacterial adhesion sites in mucus layer, lysozyme production, pancreatic endopeptidases, colonization resistance mediated by the normal commensal microbiota and gut motility are the other important factors which affect the biofilm community.

Biofilm bacteria and the host live in a state of symbiosis with each other. The bacterial communities, housed in mucus layer, enjoy the benefits of space to stay, not been exposed to the risk of excretion with stool because of their adhesion to the mucus layer, easy access to a constant source of energy from the substances present in mucus, and proximity to the colonic mucosa. This unique and safe location of biofilm bacteria is reflected in their differential proliferation and resource utilization compared with the corresponding species in the intestinal lumen. In return, the close proximity of biofilm bacteria with mucosa directs the host's tolerance to the self-microbial pathogens, the activation or destruction of genotoxins and mutagens, and modulation of the immune system (Macfarlane and Macfarlane 2006).

The intrinsic mucus-resident bacteria inhibit the physical contact between luminal bacteria and the colonic wall. Disbalances in numbers or the types of gut bacteria, present in stool as well as in gut biofilm, are implicated in several gastrointestinal (Young 2017) and liver disease (Goel et al. 2014). Inflammatory bowel disease (IBD), a poorly understood gastrointestinal disease, is one of the conditions in which gut flora is proposed to play a vital role in pathogenesis, treatment response, and prognosis.

19.5 Inflammatory Bowel Disease (IBD)

IBD is a chronic inflammatory condition of small and large intestine which is characterized by recurrent episodes of bleeding from gastrointestinal tract and abdominal pain. Clinical spectrum of IBD ranges from Crohn's Disease, through indeterminate colitis to Ulcerative colitis. Histopathological examination of resected bowel specimen reveals bowel wall edema, inflammation, mucosal ulcerations, and fibrosis. In

Ulcerative colitis, there is diffuse and continuous mucosal inflammation that extends proximally to a variable extent from the rectum; on the contrary, Crohn's disease may involve any site of the gastrointestinal tract from esophagus to anus, though the terminal ileum is affected most commonly. In Crohn's disease, the pathological involvement is usually patchy and segmental and involves all the layers of bowel wall, i.e., transmural inflammation. IBD is a result of unregulated immune response, of a genetically predisposed host, against either self- or unrecognised external antigens present in diet, bowel lumen or environment (Xavier and Podolsky 2007).

19.6 Evidences Supporting the Role of Gut Microbiome/Biofilm in Inflammatory Bowel Disease

Gut bacteria are widely implicated in pathogenesis of IBD. They are supposed to bring their pathogenic effect by either damaging the commensal biofilm or formation of pathogenic bacteria biofilm (Mohammadi et al. 2015; Srivastava et al. 2017). Though the pivotal role of the gut microbiota in IBD has long been postulated, the definitive mechanistic relationships remained elusive. The role of gut bacteria in IBD pathogenesis is supported by several lines of evidences as described below:

(i) Ileum and rectum are the distal most part of small and large bowel, respectively; these sites are the parts of bowel which are exposed to the maximum concentration of bacteria and bacterial stasis in small and large bowels; these parts are also the bowel sites which are most frequently involved in Crohn's disease and UC, respectively; this predisposition of bacterial laden anatomical regions to develop the disease indicates a possible link between the them.

(ii) Strains of *Escherichia coli*, which have adherent–invasive properties, have been frequently isolated from the ileal mucosa in IBD patients.

(iii) *Fusobacterium nucleatum* is a commensal gut flora in healthy human; its certain species, with capabilities to invade the gut mucosa, has been isolated from Ulcerative colitis patients.

(iv) Bacterial dysbiosis, characterized by imbalance between helpful commensal and potentially pathogenic microorganisms, is commonly seen in patients with IBD.

(v) Bowel inflammation and colitis like picture has been successfully inducted with gut bacteria in experimental murine models.

(vi) Administration to the antibiotic which is effective against gut bacteria to the patients with active disease shows beneficial effects, though it is partial at times.

(vii) Probiotics are the preparations which contain bacteria and their administration are shown to result in amelioration of inflammation and symptoms in patients with IBD.

(viii) Fecal diversion surgery, which avoids the exposure of the inflamed mucosa of the affected bowel in IBD to the microbial load present in stool, is shown to have therapeutic effects.

(ix) The severe forms of acid peptic disease, which manifest as gastric and duodenal ulcers, were very common few decades ago, and their ulcers have many similarities with those seen in patients with IBD; later on these gastric and duodenal ulcers were proven to be caused by bacterium *Helicobacter pylori*. It will not be astonishing if IBD, later on, is proven to be caused by gut bacteria.

(x) IBD in animals can be initiated by *Helicobacter spp*. infection.

(xi) Proctitis, secondary to *H. cinaedi* and *H. fennelliae* infection in homosexual men, looks similar to IBD.

(xii) DNA of the family Campylobacteraceae bacteria is detected in the lymph nodes of patients with Crohn's disease.

(xiii) The critical role of *Campylobacter concisus* in IBD pathogenesis is supported by several evidences: identification of bacterial proteins involved in chemotaxis, signal transduction, flagellar motility, surface binding, and membrane protein assembly in affected tissue in Crohn's disease patients; high prevalence of bacteria in adult IBD patients; preferential colonization of this bacteria in descending colon and rectum in IBD patients; and their association with oral mucosal inflammation.

(xiv) The prevalence of *Pseudomonas species* was significantly higher in biopsy specimens obtained from Crohn's disease patients than specimens from non-IBD patients.

(xv) Fungal dysbiosis is commonly seen in IBD and is characterized by increased Basidiomycota/Ascomycota ratio, decreased proportion of *Saccharomyces cerevisiae*, and increased proportion of *Candida albicans* (Sokol et al. 2017).

19.7 Mechanism of Gut Microbe/Biofilm in Pathogenesis of IBD

Gut microbes alter the milieu in host-mucosal surface–microbiome interface in several ways (Fig. 19.1). The exposure to an unusual antigen at the interface coupled with altered signaling steer the hosts' immune system to provoke inflammatory reaction. Though, the exact mechanisms of pathogenesis by a specific inciting agent differ from one to another pathogen (Sartor 2008) (Table 19.1).

Though the IBD is not caused by a single bacterial species, evidences favor the implications of a few bacterial species in its pathogenesis. Though the exact mechanism of bacteria-mediated disease development has not clearly been elucidated. The current knowledge about the bacteria-mediated mechanisms is summarized here:

(i) *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is known to produce a Crohn's disease like condition, named as granulomatous enterocolitis, in ruminants.

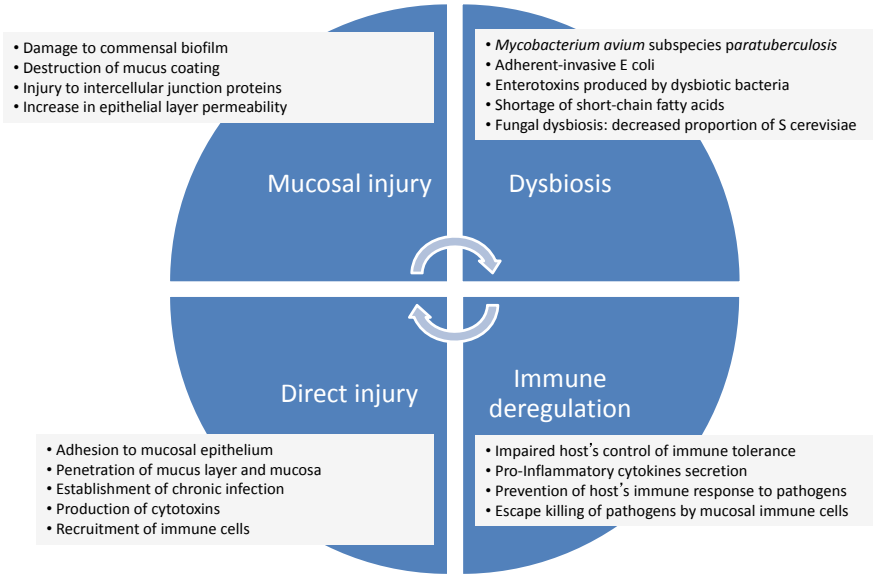

Fig. 19.1 Schematic diagram showing various mechanisms adopted by gut microbes and biofilms in pathogenesis of inflammatory bowel disease

(ii) Adherent-invasive *E. coli* (AIEC) are the strains of *E. coli* bacteria which are equipped with the capability to adhere and invade intestinal epithelial cells. Following epithelial invasion, they could replicate within macrophages without killing them. These strains have no virulence factor-encoding genes that are traditionally present in typical pathogenic species. These strains cause stimulation of the immune system in hosts with either abnormal mucosal immunity (particularly in Crohn's disease) or intestinal barrier dysfunction (particularly in Ulcerative colitis) (Palmela et al. 2018) and thus promote inflammatory response. The exact role played by AIEC in IBD initiation or aggravation of pre-existing inflammatory disease is not clear.

(iii) Enterotoxins, secreted by certain commensal or pathogenic bacteria in lumen, can induce intestinal inflammation. For example, *Clostridium difficile* toxin can reactivate quiescent IBD, enterotoxigenic *Bacteroides fragilis* can induce colitis in experimental models, *Staphylococcal* enterotoxin B induces inflammation in patients with Ulcerative colitis.

(iv) Dysbiosis or change in composition of gut microbiome is common in IBD and is characterized by reduced microbial diversity, reduced numbers of Firmicutes and Bacteroidetes, accompanied with relative increase in Proteobacteria and Actinobacteria. The Firmicutes are reduced primarily because of decreases in Clostridium XIVa and IV groups in Lachnospiraceae subgroups.

Table 19.1 Pathogens involved in inflammatory bowel diseases

Pathogen	Mechanism involved in pathogenesis
Protective role against IBD	
Intestinal parasites	• Modulation of innate and acquired hosts' immune response which keeps mucosal inflammation in check
Helicobacter pylori (Castano-Rodriguez et al. 2017; Yu et al. 2018)	• Increase in IL-18 production • Enhanced immune tolerance • Accumulation of suppressive regulatory T cells (Tregs) • Reduces gastric secretion of leptin which has a proinflammatory effects
Predisposes for IBD	
Giardia duodenalis (Allain et al. 2017; Beatty et al. 2017)	• Impairment of biofilm architecture • Destruction of mucus coating of the epithelium • Damage of epithelial cell barrier, physiology and survival • Induction of bacterial dysbiosis
Enterohepatic helicobacteria species (Hansen et al. 2011) (EHS)[a]	• Regulates the switching of a 'healthy' colonic microbiota to IBD predisposing dysbiosis • Chronic infection with these species
Campylobacter (Castano-Rodriguez et al. 2017)	• *Campylobacter spp*., in particular *C. Concisus* increase the risk for IBD • Production of inflammatory cytokines • *C. concisus* has potential to invade Caco2 cells and secrete cytolethal distending toxin (CDT)-like toxin • Upregulation of otherwise low level of TLR-4 expression in intestinal epithelium, which keeps the gut mucosal system in a state to tolerate commensal intestinal bacteria flora
Adherent–invasive *E. coli* (AIEC)	• AIEC genes promote motility, capsule and lipopolysaccharide (LPS) expression, serum resistance, iron uptake, adhesion to and invasion of epithelial cell, biofilm formation, degradation of mucins protease • AIEC properties empower them to escape oxidative reactive species, tumor necrosis factor α (TNF-α) and other proinflammatory cytokines which enhances the dysbiosis • Exploitation of host mechanisms of apoptosis in favor of their own intracellular replication and prevention of antimicrobial response

(continued)

Table 19.1 (continued)

Pathogen	Mechanism involved in pathogenesis
Pseudomonas aeruginosa	• Virulence-related attachment factor increases the paracellular permeability • Transform apical membrane of epithelial cells into basolateral membrane
Listeria monocytogenes	• Weakens the defensive mucosal barrier, leading to invasive infection with *L. monocytogenes*
Fungal dysbiosis	• Anti-inflammatory effects in colitis models • Prevention of antibiotic-associated diarrhea, acute diarrhea, Clostridium difficile infection and enteral feeding-related diarrhea

[a]EHS, non-pylori Helicobacter members in Helicobacteraceae family and colonizes the gastrointestinal tract

(v) *Clostridia and Bacteroides* species preferentially produce butyrate and other SCFA which serves as an energy source for colonic epithelial cells. The utilization of SCFA by colonocytes is reduced by hydrogen sulfide produced by reduction of luminal contents by sulfate-reducing bacterial species. In patients with IBD, decreased concentrations of SCFA producing bacterial groups coupled with overgrowth of sulfate-reducing bacteria result in depletion of epithelial nutrients.

(vi) Impaired host's immune response against luminal bacteria results in enhanced antigenic exposure, leading to pathogenic T-cell responses and chronic intestinal inflammation. The functional immune defects might be operating at one or several of the following levels such as secretion of antimicrobial peptides in lumen, enhanced permeability of mucosal barrier, inability to extrude xenotoxins, epithelial defect repair, innate and adaptive immune responses, secondary phagocytosis, and effective killing of bacteria which have translocated the epithelial barrier.

19.8 Probiotics and Probiotic Biofilm

Probiotics are the living microbial food ingredients that, when ingested in adequate amounts, alter the microflora and confer a health benefit to the host. Majority of probiotic preparations contain commensal bacteria which are present in gut of a healthy human. Probiotic activity is shown with *Lactobacilli*, *Bifidobacteria*, *Streptococcus*, *Enterococcus*, nonpathogenic *E. coli*, and yeast *S. boulardii*. In addition to being safe, the bacterial or yeast strains, used as probiotic, are armored with few additional capabilities such as to survive in acidic and alkaline conditions on the way to their site

of action, to adhere and colonize the intestinal and colonic epithelial cells, to compete with pathogenic microorganisms for nutrients and colonization sites, to secrete antibiotic-like substances called bacteriocins, and to provide nutritional support to the host by the synthesis of vitamins. The bacteria, administered as probiotics, may get incorporated into mucosal biofilm thus replacing the harmful bacteria from good commensal bacteria.

19.9 Potential Roles of Probiotics and Its Biofilm in IBD

The beneficial effects of microorganisms, administered in the form of either live bacteria or spores in probiotic preparations, in IBD are supposed to be mediated through following plausible mechanisms (Abraham and Quigley 2017):

(i) Boosting of or rebalancing the altered mucosal immune system by augmentation of various components of humoral and cellular immune response such as antibody production, natural killer cell activity, upregulation of anti-inflammatory cytokines (interleukin-10, transforming growth factor beta), and reduction of proinflammatory cytokines (tumor necrosis factor alpha, interferon gamma, and IL-8).
(ii) Repair of the leakiness of intestinal mucosal barrier by inhibiting the apoptosis of intestinal epithelial cells, enhanced synthesis of tight junction proteins, and augmentation of mucus layer.
(iii) Modulation of gut microbiota composition by bacteriocins which inhibit the growth of potentially pathogenic bacteria, creation of acidic milieu that inhibits the growth of proinflammatory bacteria but promotes the growth of anti-inflammatory bacteria such as lactobacilli and bifidobacteria.
(iv) Enhancing the bacterial diversity and reducing the fungal diversity.
(v) Increased production of fatty acids, in particular, short-chain fatty acids, which have anti-inflammatory and anti-carcinogenic properties.
(vi) The probiotic is likely to have an additional centrally mediated effect which subdues the perception of visceral sensations by probiotics which helps in reducing the symptoms and morbidities in IBD which are very frequent in these patients and thus improves the quality of life.
(vii) The above-mentioned effect of probiotics on central nervous system could also reduce the symptoms of other associated conditions such as neurosis and depression.
(viii) Enhanced production of SCFA which serves as nutrients for colonocytes and thus increases their repair and immune capabilities.
(ix) Probiotic mat directly suppresses the mucosal inflammation.
(x) Promotion of cell proliferation, tissue regeneration, and healing.
(xi) Increased transepithelial resistance and increased mucin expression.
(xii) Induction of cytoprotective heat shock proteins which increases the mucosal capability to fight against various pathogenic stresses or bacteria.

19.10 Probiotics in Management of IBD

The natural history of both forms of IBD, Ulcerative colitis and Crohn's disease, is characterized by episodes of active disease interspersed by disease-free intervals of varying durations. The IBD management strategies focus on controlling the inflammatory activity in patients to reduce the symptoms of active disease and maintain the remission in inactive disease. The pharmacological treatment, used in IBD, includes 5-aminosalicylates, glucocorticosteroids, immunomodulators, and biological agents. Some of these treatments are either costly or are associated with serious adverse events. Hence, probiotics, which are safe and are available in affordable cost, are widely explored as an alternative therapy.

Probiotics have been used as a therapeutic agent for the induction of remission or maintenance of remission in patients with Ulcerative colitis, Crohn's disease or pouchitis after resection surgery (Abraham and Quigley 2017; Derwa et al. 2017). Most of the studies, in these patients, have used species of *E. coli*, *Lactobacilli*, *Bifidobacter or Streptomyces* either alone or in various combinations. Majority of single species containing probiotic data are from *Lactobacillus rhamnosus* GG or *S. boulardii*.

19.11 Use of Probiotics for Induction of Remission in Active Disease

Data on role of probiotics in inducing the remission in Crohn's disease are very limited to draw a reliable conclusion and hence further data are warranted. Relatively larger studies have used probiotics for remission induction in Ulcerative colitis; though all of them have used probiotic as an add-on therapy on standard of care and included only the patients with mild to moderately severe disease. These studies collectively indicate that though the probiotic use, in combination with conventional therapy, fails to induce remission but may provide a modest benefit in terms of reducing disease activity. So, we have no data on benefit of using probiotic add-on therapy in patients with severe disease. At present, therefore, there are insufficient evidences to support or refute the use of probiotics, either alone or in combination with standard medical therapy, for remission induction in Ulcerative colitis.

19.12 Use of Probiotics for the Remission Maintenance in Inactive Disease

Several large, well-designed, randomized controlled trials have studied the role of probiotics in remission maintenance in patients with either Crohn's disease or Ulcerative colitis. Data suggests that there are no apparent benefits of regular use of

probiotics in the maintenance of remission in Crohn's disease as assessed on clinical and/or endoscopic criteria. Though, the studies of probiotics use as a prophylactic measure to maintain the remission in Ulcerative colitis suggest a limited clinical benefit.

19.13 Use of Probiotics in Patients with Pouchitis

Many times, an acute episode of Ulcerative colitis fails to resolve with drug therapy. Such patients are managed with proctocolectomy with ileal-pouch and anal anastomosis (IPAA). This artificially formed pouch frequently develops inflammation, secondary to several factors, which is called as pouchitis. Probiotics are frequently used in pouchitis.

One study compared Lactobacillus GG with placebo for remission induction in acute pouchitis. Though this study showed that the administration of the probiotic led to a change in bacterial flora in the pouch, this flora change was not associated with improved clinical or endoscopic outcomes. A critical review of probiotic studies in pouchitis reveals that these studies had used different types and doses of probiotics and also had limitations of small sample size and shorter duration of intervention.

In summary, the beneficial effects of probiotic in IBD are shown to be limited. The beneficial effect of probiotic, if any, is more in maintenance of remission than inducing remission, in particular, those with Ulcerative colitis than Crohn's disease.

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