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Gianfranco Donelli *Editor*

Biofilm-based Healthcare-associated Infections

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Gianfranco Donelli
Editor

Biofilm-based Healthcare-associated Infections

Volume I

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Editor
Gianfranco Donelli
Microbial Biofilm Laboratory
Fondazione Santa Lucia IRCCS
Rome, Italy

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Preface

The recently acquired knowledge on the pivotal role played by biofilm-growing multidrug-resistant microorganisms in healthcare-related infections has given a new dynamic to detection, prevention and treatment of these infections.

As a consequence, the investigation of biofilm-based infections is currently one of the "hottest" research areas in microbiology and infectious diseases. Particularly, an increased awareness of the possible causative role of bacterial and fungal biofilms in a number of healthcare-associated infections has emerged in the last two decades as a result of the progressive improvements in our knowledge on the structure and physiology of single- and multi-species biofilms. In fact, the milestone paper published in the *Journal of Bacteriology* back in 1991 by John Lawrence in collaboration with Bill Costerton reports horizontal and sagittal optical sections of *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* biofilms obtained by using a confocal laser scanning microscope and viable fluorescent probes. In this paper, bacteria were described as fully immersed in a highly hydrated matrix constituting 73–98% of extracellular substances, with the presence of large void channels allowing the circulation of nutrients, signalling molecules and microbial metabolites. The tridimensional structure of these microbial communities, the dynamics of their sessile growth and the cell–cell interactions as well as those with the surrounding environment strongly differentiated the sessile growth condition from the planktonic one. On the basis of scanning electron microscopy investigations performed in the mid-1990s at the Center for Biofilm Engineering in Bozeman (USA), Bill Costerton proposed the well-known mushroom model schematically drawn by Peg Dirckx in his widely used cartoon. This new outlook of the microbial world has led basic microbiologists and clinicians to the awareness of the predominance of biofilm-growing microorganisms, especially, but not only, in cases of foreign-body infections. In the last years, an impressive series of microbiological and clinical data have widely demonstrated the key role of biofilms as causative agents of severe, and often relapsing, infections in both immunocompetent and immunocompromised patients admitted to acute care hospitals and long-term care facilities.

The explosion of the interest of microbiologists, hygienists and infectious disease specialists in this field is also due to the recalcitrance of biofilm-

growing microorganisms to antimicrobial treatments. In fact, with respect to planktonic cells, it an up to 100–1,000 times higher tolerance to antibiotics and antiseptics has been reported for biofilm-growing bacteria. Even if a comprehensive model is still lacking for this multifactorial phenomenon, significant issues have been identified in the reduced antibiotic diffusion due to the exopolysaccharide matrix acting as a mechanical barrier and in the anaerobic conditions that are created in the inner part of the biofilm that, as it is well known, make ineffective a number of antibiotics including aminoglycosides, beta-lactams and fluorochinolones. On the other hand, "persister cells" developing in a small percentage within the biofilms are also known to be highly tolerant to antibiotics and have been typically involved in causing relapses of infections. Furthermore, the development of resistance to antibiotics is highly promoted by the horizontal gene transfer between biofilm-growing bacteria, and the classical mutational mechanisms play a major role in this process. In addition, recent studies have demonstrated that mutagenesis is intrinsically increased in biofilms, and hypermutations are able to play an important role in these phenomena.

Most of the currently available methods to investigate bacterial and fungal biofilms as well as their antibiotic resistance have been exhaustively illustrated and critically annotated by authoritative scientists, well known for their relevant expertise in the respective fields, in the 25 chapters of the recent book by Humana Press titled *Microbial Biofilms – Methods and Protocols* (G. Donelli Ed., Springer 2014).

In the present two volumes of the book *Biofilm-Based Healthcare-Associated Infections*, a collection of 20 chapters written by leading scientists covering well-investigated areas of biofilm-related infections is offered to the attention and desirable appreciation of all the interested "biofilmologists". The chapters deal with biofilm-based human infections affecting the oral cavity, the respiratory system, the gastrointestinal tract and the urogenital apparatus as well as other bacterial and fungal infections associated with orthopaedic surgery and breast implant, the use of gel fillers in cosmetic and reconstructive surgery, neonatal enteral nutrition and the insertion of various medical devices in the human body, including central venous catheters, endotracheal tubes and voice prostheses. A separate chapter is also dedicated to the persister cells in biofilm-associated infections, while other chapters focus on recently developed anti-biofilm strategies, including antimicrobial polymers, innovative drug delivery carriers and antimicrobial photodynamic therapy. On the whole, readers will have at their disposal a precious reference book that can be used as a working tool to recognize and treat biofilm-based infections also in the light of the most recent knowledge on the reduced antimicrobial susceptibility of causative agents.

I would like to express my gratitude to all the chapter authors for their excellent contribution to this book. Their efforts in writing comprehensive reviews on topics in such a fast-moving research field should be considered a generous gift to the scientific community. I am sure that readers will highly appreciate this book as it has happened to me.

Rome, Italy Gianfranco Donelli

Contents

Contributors

Kevin S. Akers Extremity Trauma and Regenerative Medicine Task Area, United States Army Institute of Surgical Research, Houston, TX, USA

Infectious Disease Service, Brooke Army Medical Center, Houston, TX, USA

Carla Renata Arciola Research Unit on Implant Infections, Rizzoli Orthopaedic Institute, Bologna, Italy

Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy

Georgios N. Belibasakis Section of Oral Microbiology and Immunology, Institute of Oral Biology, Center of Dental Medicine, University of Zürich, Zürich, Switzerland

Nagihan Bostanci Section of Oral Translational Research, Institute of Oral Biology, Center of Dental Medicine, University of Zürich, Zürich, Switzerland

Davide Campoccia Research Unit on Implant Infections, Rizzoli Orthopaedic Institute, Bologna, Italy

Anthony P. Cardile Infectious Disease Service, Brooke Army Medical Center, Houston, TX, USA

Georgios Charalampakis Department of Oral Microbiology and Immunology, Institute of Odontology, The Sahlgrenska Academy at University of Gothenburg, Gothenburg University, Gothenburg, Sweden

Tom Coenye Laboratory of Pharmaceutical Microbiology, Ghent University, Ghent, Belgium

Gianfranco Donelli Microbial Biofilm Laboratory, Fondazione Santa Lucia IRCCS, Rome, Italy

Charles E. Edmiston Jr. Department of Surgery, Medical College of Wisconsin, Milwaukee, WI, USA

Garth D. Ehrlich Center for Genomic Sciences, Institute for Molecular Medicine and Infections Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA, USA

Department of Otolaryngology, Drexel University College of Medicine, Philadelphia, PA, USA

Marcelo Faveri Department of Periodontology, Dental Research Division, Guarulhos University, Guarulhos, São Paulo, Brazil

Magda Feres Department of Periodontology, Dental Research Division, Guarulhos University, Guarulhos, São Paulo, Brazil

Luciene Cristina Figueiredo Department of Periodontology, Dental Research Division, Guarulhos University, Guarulhos, São Paulo, Brazil

Stephen J. Forsythe Pathogen Research Group, School of Science and Technology, Nottingham Trent University, Nottingham, UK

Mohamed A. Jamal Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Noha A. Juma Department of Medical Microbiology and Parasitology, Faculty of Medicine, King Abdulaziz University, Jeddah, KSA

David Leaper School of Applied Sciences, University of Huddersfield, Huddersfield, UK

Matthias Leonhard Department of Otorhinolaryngology and Head and Neck Surgery, Medical University Hospital Vienna, Vienna, Austria

Andrew J. McBain Manchester Pharmacy School, The University of Manchester, Manchester, UK

Lucio Montanaro Research Unit on Implant Infections, Rizzoli Orthopaedic Institute, Bologna, Italy

Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy

Clinton K. Murray Infectious Disease Service, Brooke Army Medical Center, Houston, TX, USA

Paula Juliana Pérez-Chaparro Department of Periodontology, Dental Research Division, Guarulhos University, Guarulhos, São Paulo, Brazil

Issam Raad Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Christopher Roberts Clinical Resolutions, Hessle, East Yorkshire, UK

Berit Schneider-Stickler Department of Otorhinolaryngology and Head and Neck Surgery, Medical University Hospital Vienna, Vienna, Austria

Jamil Awad Shibli Department of Periodontology, Dental Research Division, Guarulhos University, Guarulhos, São Paulo, Brazil

Bernd Stadlinger Clinic of Oral Surgery, Center of Dental Medicine, University of Zürich, Zürich, Switzerland

Ilse Vandecandelaere Laboratory of Pharmaceutical Microbiology, Ghent University, Ghent, Belgium

Claudia Vuotto Microbial Biofilm Laboratory, Fondazione Santa Lucia IRCCS, Rome, Italy

Joseph C. Wenke Extremity Trauma and Regenerative Medicine Task Area, United States Army Institute of Surgical Research, Houston, TX, USA

Ammar Yousif Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Biofilm Formation by Clinical Isolates and Its Relevance to Clinical Infections

1

Kevin S. Akers, Anthony P. Cardile, Joseph C. Wenke, and Clinton K. Murray

Abstract

Reports of biofilms have increased exponentially in the scientific literature over the past two decades, yet the vast majority of these are basic science investigations with limited clinical relevance. Biofilm studies involving clinical isolates are most often surveys of isolate collections, but suffer from lack of standardization in methodologies for producing and assessing biofilms. In contrast, more informative clinical studies correlating biofilm formation to patient data have infrequently been reported. In this chapter, biofilm surveys of clinical isolates of aerobic and anaerobic bacteria, mycobacteria, and *Candida* are reviewed, as well as those pertaining to the unique situation of cystic fibrosis. In addition, the influence of host components on in vitro biofilm formation, as well as published studies documenting the clinical impact of biofilms in human infections, are presented.

1.1 Introduction

Publications of biofilm-related studies in the medical and scientific literature have risen exponentially over the past few decades, beginning with microbiological reports in the 1970s. Only more recently have observations on the biofilm mode of microbial growth been extended into humans as a mechanism for recalcitrant clinical infection (Fig. 1.1), which may parallel the increasing use of implanted medical devices in humans with attendant rise in infectious complications. In the United States, cardiac device implantation rose by 54.7 % between 1997 and 2004, with a cumulative total of more than 2.2 million devices

K.S. Akers (✉)
Extremity Trauma and Regenerative Medicine Task Area, United States Army Institute of Surgical Research, Houston, TX, USA

Infectious Disease Service, Brooke Army Medical Center, Houston, TX, USA
e-mail: kevin.s.akers.mil@mail.mil

A.P. Cardile • C.K. Murray
Infectious Disease Service, Brooke Army Medical Center, Houston, TX, USA

J.C. Wenke
Extremity Trauma and Regenerative Medicine Task Area, United States Army Institute of Surgical Research, Houston, TX, USA

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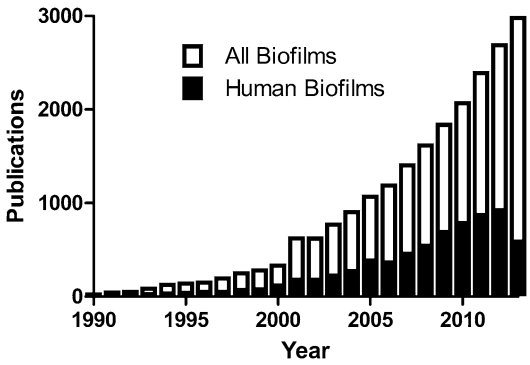

Fig. 1.1 PubMed-indexed literature citations for biofilms, 1990–2013. Annual study totals were obtained by searching the PubMed database using the term "biofilm" with and without application of the species record filter for "human"

implanted over this period (Zhan et al. 2008). Between the 1970s and mid-2000s, in Olmsted County, Minnesota (USA), rates of total knee and total hip arthroplasty rose from 31.2 and 50.2 to 220.9 and 145.5 per 100,000 persons (increases of 366 % and 340 %, respectively) (Singh et al. 2010). Similarly, increases of total knee and total hip arthroplasty increased by 36 % and 20 %, respectively, in Australia between 1994 and 1998 (Wells et al. 2002). These trends in the rising rates of implanted medical devices are likely to continue in developed countries with aging populations. Numerous microbiologically-focused in vitro studies have been performed using only commercially available reference strains, or single clinical isolates. Surveys of the biofilm-forming capability of wild-type bacterial isolates obtained from human populations began to be reported only in the mid 2000s, and population-based data detailing the clinical impact of biofilms in humans remain scarce. Thus much of the evidence supporting the theory of biofilms as a cause of recalcitrant infections is derived from in vitro studies using bacterial isolates which may not adequately reflect wild-type pathogens or in vivo conditions.

Among the available studies utilizing clinical isolates, there has been considerable variation in the experimental approach to the quantitation of biofilms produced by these organisms. Different investigations have used incubation periods from 12 to 48 h, varying concentrations of ambient oxygen and carbon dioxide during incubation, incubation temperatures, and supplemental substances in the biofilm cultures such as glucose, plasma, horse serum and sodium chloride (Sanchez et al. 2013). These can each potentially impact the degree of biofilm formation, which may be related to stimulation of gene transcription and/or metabolism by factors in the growth matrix. While most studies have used Crystal Violet staining followed by optical absorbance measurements of ethanol extracts to quantify biofilm biomass, there has been considerable variation in the duration of staining and destaining, the absorbance wavelength used for quantitation, and even the biofilm stain itself, as some studies have used safranin rather than Crystal Violet. Finally, converting the absorbance measurement (a continuous variable) to a binary interpretation of whether a single isolate is "positive" for biofilm formation requires comparison to a control specimen. These vary for each species but are generally an isolate reported to have either high or low biofilm formation such that they can be used as a point of reference in the assay. Given the heterogeneous approaches to the laboratory determination of biofilm-forming capacity in bacteria, and the significant impacts on biofilm formation which can occur from addition of supplemental factors to the biofilm culture, assay standardization (such as guidance provided by the Clinical and Laboratory Standards Institute in the United States for methods of planktonic MIC determination) would be beneficial to standardize data interpretation across studies.

1.2 Biofilms from Aerobic Gram-Positive and Gram-Negative Bacteria

Escherichia coli isolates causing genitourinary tract infection (device-unrelated) were examined for biofilm formation in several studies. In a large survey, 377 isolates were surveyed for 48-h biofilm production: 194 from cystitis, 76 related to pyelonephritis and 107 from prostatitis (Kanamaru et al. 2006). Prostatitis isolates were

associated with increased biofilm production and the curli fimbriae phenotype, suggesting a role for biofilm formation as a virulence factor in acute bacterial prostatitis. In a smaller study, 70 single-patient clinical isolates (43 from cystitis, 11 from pyelonephritis and 16 from urosepsis) from 34 children and 36 adults were examined for biofilm production following 72 h incubation in an anaerobic environment (Salo et al. 2009). In addition to Crystal Violet staining, 22 strains were examined by SEM and CLSM. Overall, 31 % of strains formed biofilms (26 % of cystitis, 55 % of pyelonephritis and 31 % of urosepsis strains), with more intense biofilm production noted in strains from pyelonephritis and antibiotic-susceptible versus – resistant strains.

Biofilm production has also been characterized in *E. coli* in diarrhea syndromes, wherein biofilm production is thought to mediate the enteroaggregative phenotype. In 1042 isolates from the same number of Japanese children with diarrhea, biofilm detection was used as a screening method for enteroaggregative *E. coli* (EAEC) strains (Wakimoto et al. 2004). Sixty-two strains (5.9 %) demonstrated high biofilm formation, of which 77 % possessed EAEC virulence genes *aatA* or *aggR*. In 75 proven EAEC isolates from 87 returning travelers (57 with diarrhea), 51 % of diarrhea isolates and 61 % of non-diarrhea isolates formed biofilms. Biofilm production was associated with the *aggR*, *set1A*, *aatA* and *irp2* virulence factor genes (Mohamed et al. 2007). Among 100 infants with acute diarrhea in India, 28 were confirmed to be EAEC by PCR demonstration of *aggR* and *east* virulence genes (Bangar and Mamatha 2008). Of these, 25 (89.3 %) formed biofilms. Collectively, these studies suggest that biofilm formation may play an important role in the pathogenicity of diarrhea caused by EAEC isolates. Biofilm production was also compared between 30 diarrheal isolates and 30 isolates of *E. coli* recovered from orthopedic implant infections (Cremet et al. 2012). High-biofilm isolates were observed in both the fecal and device-related isolates, with highly variable biofilm production overall. PCR surveillance for 19 different virulence genes found no clear association of virulence factors with device-related infecting isolates.

Given the central importance of biofilm formation to the theory of recalcitrance in device-related infections, isolates from these infections have been examined for biofilm formation in numerous studies involving various types of medical devices. Fifty-one catheter-related urinary isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) were characterized for biofilm formation in comparison to 58 isolates unrelated to catheters (Ando et al. 2004). The intensity of biofilm formation was significantly greater in catheter-associated isolates, and associated with hemolysin A and B (*hla*, *hlb*) and fibronectin binding protein A (*fnba*). Urinary *E. coli* isolates were similarly examined in patients with asymptomatic bacteriuria, with biofilm formation of 88 catheter-associated isolates compared to that of 88 catheter-unassociated urinary isolates (Watts et al. 2010). This study found no difference in biofilms between the two groups in microtiter plates, but noted enhancement of biofilm formation in selected isolates when they were grown on catheter segments in urine. This raises the question of what is the most clinically-relevant method by which to perform biofilm testing. Gad and colleagues examined biofilm formation by SEM in 53 *Staphylococcus* species (18 *S. aureus*, 35 *S. epidermidis*) from pre-insertion, post-extraction and device cultures among patients undergoing ureteral stent removal (Gad et al. 2009). The majority of patients had isolates recovered from direct stent culture, but not urinary culture. Fifteen of the *S. aureus* (83.3 %) and 35 of the *S. epidermidis* (88.6 %) were biofilm producers. All of the biofilm-producing strains possessed the intercellular adhesin genes *icaA* and *icaD*, whereas these genes, which encode intercellular adhesin proteins mediating cellular binding to surfaces and other cells, were absent from biofilm-negative strains. Wang and colleagues examined 96 catheter-related and 83 corresponding urinary Gram-positive and Gram-negative isolates from 45 patients (Wang et al. 2010). Catheters were colonized by multiple species, with *E. coli* present in high numbers. The extent of biofilm formation varied within and between species, but 95 % of the isolates exhibited at least one type of biofilm formation (adherence,

pellicle or clumping). Further investigation of the biofilm formation by *E. coli* found it to be associated with the *fluA* gene (which encodes cell surface protein Ag43), loss of the O-antigen and expression of type 1 fimbriae, and cyclic di-GMP was found to regulate adherence to the catheter surface. This implicates the intracellular second-messenger signaling pathway in biofilm formation, indicating that this process is likely a response to environmental stimuli.

Biofilm formation on medical devices are thought to play a major causative role in device infections, including orthopedic device-related infections. A survey of biofilm production among 26 staphylococcal isolates (13 *S. aureus*, 10 *S. epidermidis*, 1 *S. hominis*, 1 *S. warneri*, 1 *S. lugdunensis*) recovered from 23 orthopedic devices noted that all the strains formed biofilms and 19 (73.1 %) possessed the *ica* gene (Esteban et al. 2010). Biofilm production was also surveyed among 168 MRSA isolates (23 device-associated, 55 non-device associated and 90 nasal carriage isolates) from 87 infected and 95 colonized outpatients (Kawamura et al. 2011). This study found a greater intensity of biofilm staining among isolates from orthopedic devices. In addition, PCR demonstrated increased incidence of the accessory gene regulator (*agr*) locus, thought to be a virulence determinant in *S. aureus*, among these isolates. In a biofilm susceptibility study, MIC and MBEC values were determined for 21 isolates of methicillin-susceptible *S. aureus* (MSSA) recovered from peritoneal dialysis catheters, a situation in which catheter contamination leading to peritonitis is a feared clinical complication (Girard et al. 2010). As expected, biofilm MBEC values were significantly higher than planktonic MICs, with the surprising exception of gentamicin. In addition, rifampin (which penetrates biofilms more efficiently than any other antimicrobial) was found to improve the activity of vancomycin when given in combination. Revdiwala and colleagues undertook a broad examination of biofilm formation by isolates recovered from a variety of medical support devices, including endotracheal tubes, tracheostomy tubes, central venous catheters, Foley catheters, abdominal drains, nephrostomy tubes and suprapubic catheters (Revdiwala et al. 2012). Biofilms were compared by Crystal Violet and safranin stains. One hundred isolates were recovered, including 23 *Acinetobacter baumannii*, 23 *Pseudomonas aeruginosa*, 20 *Klebsiella pneumoniae*, 16 *E. coli*, 9 coagulase-negative staphylococci, 4 *E. cloacae*, 3 enterococci and 2 *S. aureus*. Crystal Violet staining identified 69 biofilm formers (11 Gram-positive, 35 Gram-negative) whereas safranin staining identified 88 isolates as biofilm formers (13 Gram-positive, 75 Gram-negative). This illustrates the influence of testing conditions and methods on the results, underscoring the need for standardization among biofilm assays and for awareness by investigators when contrasting the results of different studies.

A number of studies have also explored biofilm formation among invasive isolates recovered in the absence of medical devices. Forty viridans-group *Streptococcus* spp. were recovered from the bloodstream of 18 patients with endocarditis and 22 with neutropenic sepsis (Presterl et al. 2005). Forty-four percent of the endocarditis isolates and 27 % of those from neutropenic patients formed biofilms, which was decreased by co-incubation with teicoplanin or moxifloxacin, but not penicillin G. In Finland, 204 *Streptococcus pneumoniae* isolates (106 nasopharyngeal, 43 otitis media, 55 bloodstream) from children were examined (Tapiainen et al. 2010). No difference in biofilm formation was observed on the basis of isolate source or clinical syndrome. Some strain-specificity was demonstrated, however, with serotypes 14 and 33 producing greater amounts of biofilm than serotypes 3 and 38. In Japan, Moriyama and colleagues examined 109 isolates of non-typeable *Haemophilus influenzae* from 62 children with intractable otitis media, noting 84 % of isolates to be biofilm-formers (Moriyama et al. 2009). The prevalence of biofilm production was significantly higher among isolates recovered from patients whose condition was not improved by amoxicillin. Biofilm production by *Neisseria meningitidis*, a much-feared cause of transmissible bacterial meningitis featuring fulminant onset and high mortality, was examined in 16 invasive and 23 colonizing nasopharyngeal isolates (Yi et al. 2004). Only 12 % of invasive strains formed biofilms, versus 30 % of nasopharyngeal strains,

suggesting that biofilm formation may not play a major role in this disease.

Staphylococcal isolates causing infection have been examined for their biofilm production, including isolates from the potentially lethal syndrome of *S. aureus* bacteremia. Nine hundred and seventy-two isolates of *S. aureus* (763 MRSA, 209 MSSA; 31 % bloodstream isolates, 16 % nasal colonizing isolates, 13 % wound isolates) from Scotland were characterized to high, medium or non-quantifiable biofilm formation (Smith et al. 2008). 20.5 % of MRSA and 28.0 % of MSSA were high biofilm-formers, 53.8 % of MRSA and 43.5 % or MSSA were medium biofilm-formers, and 25.7 % of MRSA and 28.5 % of MSSA were non-quantifiable biofilm-formers. *S. aureus* isolates obtained from skin produced biofilms with a greater biomass than those from other sources, suggesting an important role for biofilm formation in *S. aureus* skin colonization. Isolates of *S. aureus* from South Korea (66 MRSA, 35 MSSA; 54 from surgical wounds, 20 from skin lesions, 12 from sputum, 10 bloodstream, 4 urine, 1 vaginal) were characterized for biofilm formation, genotyping by SCC*mec* gene, and the genetic insertion sequence element *IS256*, which has been associated with reduced glycopeptide susceptibility (Kwon et al. 2008). Thirty strains (30 %) formed biofilms, of which 25 were MRSA (SCC*mec* type IV). The *IS256* insertion sequence was associated with multidrug-resistant and high-biofilm forming phenotypes. Eight clinical isolates (four MRSA, four MSSA) from the Democratic Republic of the Congo were studied, noting that all formed biofilms within 4 h (Liesse Iyamba et al. 2011). Six of these were positive by PCR for the *icaA* intercellular adhesion gene. Both *S. aureus* (27 %) and coagulase-negative staphylococci (73 %) were examined among a collection of 104 isolates comprised of 74 invasive bloodstream and 30 non-invasive (peripheral IV contamination) isolates in Brazil (Reiter et al. 2011). Eighty-nine percent of invasive isolates and 64 % of colonizing isolates formed biofilms, and there was no correlation observed between biofilm intensity and SCC*mec* typing. The coagulase-negative staphylococci were further examined in a study of 30 isolates from Mexico in which 15 formed biofilms (Garza-Gonzalez et al. 2011), and 50 isolates from Uganda which included 30 ICU and 20 community isolates (Okee et al. 2012). In the latter study, 70 % of the ICU-obtained isolates formed biofilms, compared to 10 % of isolates obtained from non-hospitalized persons in the community.

Several studies have examined biofilm formation among isolates recovered from clinical specimens of chronic sinusitis. In a study of 19 patients from whom 31 isolates were recovered, 7 of 10 *S. aureus*, 7 of 11 coagulase-negative staphylococci and 8 of 10 *P. aeruginosa* formed biofilms (Bendouah et al. 2006). In a study including 139 isolates (including 53 *S. aureus*, 19 *P. aeruginosa* and 45 polymicrobial samples) from 157 patients, biofilm-forming bacteria were observed in 29 % of surgical specimens, with 15 % of *S. aureus* and 90 % of *P. aeruginosa* producing biofilms (Prince et al. 2008). Interestingly, biofilm formation was found to be correlated with the number of preceding surgical procedures. Several studies of chronic sinusitis isolates have characterized biofilm by SEM. Chen and colleagues used SEM only (no culture methods) to examine surgical specimens from 24 patients, observing biofilms on the mucosal surfaces of 13 specimens which were more commonly from revision surgeries (Chen et al. 2012a). Likewise, Tatar and colleagues performed a clinical trial of clarithromycin with or without mometasone treatment for 8 weeks in 32 chronic rhinosinusitis patients, obtaining surgical specimens before and after therapy in each group (Tatar et al. 2012). Biofilms were found in 75 % of specimens before therapy, and 44 % of specimens after therapy. No differences were observed in biofilm grading between treatment groups, however the study was noted to be underpowered. In a large cross-sectional study by Zhang and colleagues involving 518 patients, 108 (20.9 %) were noted to have biofilm-forming bacterial isolates recovered from surgical specimens (Zhang et al. 2011). Among the 145 isolates recovered were 145 *S. aureus*, 66 *P. aeruginosa*, 17 *H. influenzae*, 20 *S. pneumoniae*, and 14 *S. marcescens*. Biofilm formation was associated with positive bacterial cultures, prior sinus surgery and nasal steroid use within 1 month of the culture.

1.3 Biofilm Studies of Anaerobic Organisms

The clinical impact of biofilms formed by anaerobic organisms has considerably fewer reports in the literature, perhaps owing to the difficulties and technical expertise required to cultivate anaerobic organisms. An exception is the important field of dental anaerobic biofilms, which we will not address these here because it is beyond our scope of expertise. Suffice it to say that oral anaerobic organisms have been implicated in maladies such as dental caries and gingivitis, and a considerable amount of research effort has been applied to understanding these problems and developing innovative solutions.

In addition to the oral cavity, anaerobic organisms are abundant in the human gastrointestinal tracts. A situation somewhat analogous to that of orthopedic hardware contamination is that of biliary stents, which become colonized with gastrointestinal bacteria since the passages into which they are placed communicate openly with the gastrointestinal lumen. Noting that prophylactic ciprofloxacin prolonged stent patency in cats, 18 explanted patent biliary stents having a mean dwell time of 33 days were removed and cultured to identify colonizing bacterial species (Leung et al. 2000). Among 19 anaerobes recovered from 16 stents were *Clostridium perfringens*, *C. bifermentans* and *B. fragilis*. Twenty-eight obstructed biliary stents explanted from patients after a mean dwell time of 164 days were examined by conventional anaerobic culture, and the central areas of the obstructing sludge underwent DNA extraction and PCR-based identification ribosomal RNA amplification and sequencing (Guaglianone et al. 2010). Five species were identified on the basis of PCR analysis: *Atopobium rimae*, *Bifidobacterium breve*, *Bilophila wadsworthia*, *Mogibacterium diversum*, and *Peptostreptococcus stomatis*. Anaerobes recovered in culture (19 of the 106 recovered microbial strains, 17.9 %) were grown to biofilm over 8 or 18 h which were then stained and quantified by the Crystal Violet method. Of 12 g-negative anaerobes tested for biofilm, *Bacteroides fragilis*, *Fusobacterium necrophorum*, *Prevotella intermedia*, and *Veillonella* spp. were strong biofilm formers, while *Prevotella bivia* was a weak biofilm-former and *Bacteroides capillosus*, *B. distasonis* and *B. oralis* did not form biofilms. Five of six Gram-positive strains (*Clostridium baratii*, *C. perfringens*, *Finegoldia* (formerly *Peptostreptococcus*) *magnus*, *Veillonella* spp. and *F. necrophorum* were strong biofilm producers, whereas *C. bifermentans* was a weak biofilm producer. These isolates were subsequently examined in vitro, confirming strong 48-h biofilm production for *B. fragilis*, *F. necrophorum*, *P. intermedia*, and *Veillonella* spp., moderate for *B. oralis* and weak for *P. distasonis* (Donelli et al. 2012). Also notable in this study was the moderate biofilm production demonstrated by a strain of *Clostridium difficile*, and strong production by four additional species (*C. baratii*, *C. bifermentans*, *C. fallax*, *C. perfringens*) and *F. magna*. The clinical consequence of this intraluminal biofilm development, as is postulated to contribute to stent narrowing in vivo, is that bile flow is reduced, with the attendant potential for clinical consequences depending on the anatomic placement, and need for explantation.

Anaerobes also predominate in the human female genitourinary tract. The adherence of microcolonies of commensal anaerobic flora to vaginal epithelial cells has been suggested to represent normal human biology (Domingue et al. 1991). In contrast, a densely adherent overgrowth (i.e., biofilm) of *Gardnerella vaginalis* has been observed in the clinical syndrome of bacterial vaginosis (Swidsinski et al. 2005), which persist after therapy with moxifloxacin (Swidsinski et al. 2011), with 40 % recurrence rate 10–12 weeks after therapy. While relapse may reflect incomplete clearance of the biofilm, these were demonstrated 3 weeks following resolution of clinical symptoms following 5 days of oral metronidazole (Swidsinski et al. 2008), to which vaginal anaerobes are almost uniformly susceptible as planktonic organisms. More proximally in the GU tract, anaerobes have been recovered in culture from long-term intrauterine devices (IUD) with electron microscopy observation of biofilms (Pal et al. 2005). In a study of 127

explanted IUDs, anaerobes recovered from 51 devices indwelling for more than a decade included the following genera: *Prevotella*, *Porphyromonas*, *Bacteroides*, *Fusobacterium*, *Mobiluncus*, *Finegoldia*, *Propionibacterium*, *Bifidobacterium*, *Clostridium* and *Actinomyces*. Biofilms were not quantified in this study but were observed by electron microscopy of a single IUD.

Several studies have observed anaerobic biofilm growth on respiratory tract prostheses, including vocal prostheses, commonly placed to allow phonation following laryngectomy for malignancy, but which last on average only 3 months before biofilm-related deterioration. Vocal prostheses were explanted from 15 patients, most of whom had squamous cell carcinoma (Bertl et al. 2012). Following sonication, PCR-based detection identified 11 anaerobic and microaerophilic organisms: *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Prevotella intermedia*, *Peptostreptococcus micros*, *Fusobacterium nucleatum* (most common in this series), *Eubacterium nodatum*, *Aggregatibacter actinomycetemcomitans*, *Campylobacter rectus*, *Eikenella corrodens* and *Capnocytophaga* sp. As some of these organisms have been associated with periodontal disease, surveillance and treatment were recommended for laryngectomized patients in hopes of reducing the oral anaerobic burden and extending the life of the prosthesis. Interestingly, in a separate study, *P. gingivalis* (most commonly) and other oral anaerobic organisms were identified by PCR amplification of 16S rRNA in 50 % of coronary atheromatous plaques in patients with periodontitis, where *P. gingivalis* is known to form subgingival biofilms. This suggests the possibility that oral anaerobes may play a role in coronary artery disease (Marcelino et al. 2010). Biofilms were cultured in 23 explanted tracheal stents from children undergoing laryngotracheal reconstruction, 6 of which yielded anaerobic organisms of the genera *Fusobacterium*, *Bacteroides*, *Actinomyces*, *Prevotella*, *Propionibacterium*, *Eubacterium* and *Veillonella* (Simoni and Wiatrak 2004). The recovery of these organisms was suggested to be a potential cause of, or exacerbating factor for, granulation tissue forming in the stented trachea, a common problem in laryngotracheoplasty.

A diverse collection of other studies have identified anaerobic biofilms in a clinical context. The bacterial flora of cutaneous wounds >10 cm^2 associated with malignant breast cancer was studied with an aim to reduce unwelcome odors, which were associated with anaerobic growth in the wound but not with a single species or with the presence of biofilms (Fromantin et al. 2013). Strict anaerobes were detected in 70 % of wounds, with biofilms observed in 35 % by epifluorescence microscopy after applying stains to localize bacteria relative to their secreted extracellular matrix. In a study of bacteria adherent to surgical sutures removed from 158 patients (46 infected and 112 non-infected), biofilms were visualized by scanning electron microscopy (SEM) (Edmiston et al. 2013). Biofilms were present by SEM an all 15 sutures removed from infected sites, and also on 10 of 15 non-infected, culture-positive suture samples. Anaerobic organisms recovered in culture included *Finegoldia* spp., *B. fragilis*, *Clostridium* spp. and *Microsporum* spp. *Propionibacterium acnes*, a commensal skin anaerobe known to be associated with orthopedic device-related infections, was detected from sonicated biofilms of 120 retrieved prosthetic hip implants by immunofluorescence microscopy and 16S rRNA gene detection (Tunney et al. 1999). Finally, in a most unusual study, the potential for secondary bacterial infection caused by the ectoparasite *Tunga penetrans* (cause of tungiasis) was explored by aerobic and anaerobic swab culture of tungiasis lesions from 78 patients in northeastern Brazil following surgical parasite extraction (Feldmeier et al. 2002). An array of common human bacterial pathogens were demonstrated, including *Clostridium* spp. and *Finegoldia* sp. among the recovered anaerobes. In this malady, the *Tunga* parasite was proposed to facilitate infection by acting as a foreign body for biofilm formation, noting that bacterial infections persist until the parasite carcass is extruded or removed from the skin.

1.4 Biofilms in Clinical Isolates of *Mycobacterium* spp.

Mycobacteria are environmentally ubiquitous organisms, of which a minority of the species are infectious to humans. Little is known about the influence of mycobacterial biofilms in producing human infection. An early study documented biofilm formation by "aquatic mycobacteria" which included *M. kansasii*, a human pathogen (Schulze-Robbecke and Fischeder 1989). Similar to the "typical" bacteria, some non-tuberculous mycobacterial (NTM) species are able to colonize catheters and other medical devices. *M. chelonae* was recovered from nine patients with explanted orbital prosthetics (Samimi et al. 2013), *M. fortuitum* was recovered as a cause prosthetic valve endocarditis (Bosio et al. 2012), and NTMs have caused infections of peritoneal dialysis catheters (Nodaira et al. 2008), *Mycobacterium avium* complex has been reported from prosthetic joint infections in an immunocompromised patient (Gupta and Clauss 2009), and we reported a rare case of persistent *M. chelonae* bacteremia in a critically ill burn patient in whom no catheter or prosthetic device-associated infection could be proven (Boyer et al. 2010).

The prevalence of such infections, particularly among immunocompromised patients, may reflect the affinity of certain mycobacteria to exist within biofilms in human water supplies. In particular, *M. avium* complex has been recovered from potable household water (Wallace et al. 2013; Whiley et al. 2012), as well as hospital water supplies where NTM biofilms have been implicated in persistent bronchoscope contamination (Falkinham 2010) despite sterilization procedures. This may explain prior epidemiologic observations in the South Texas region of the United States correlating the presence of clonally identical NTM species in hospital and municipal water supplies (Conger et al. 2004). Furthermore, NTM biofilms have been reported from hospital room bathtub drain inlets (Nishiuchi et al. 2009) and appear to become concentrated in showerhead biofilms, yielding more than 100-fold increases in organism counts compared to water in the same pipes (Feazel et al. 2009).

Few biofilm surveys have been conducted among human clinical isolates of mycobacteria. Johansen et al. compared the biofilm-forming phenotypes of *M. avium* isolates from human, swine and bird origin, noting a higher frequency of biofilm formation in swine-origin than human-origin isolates (Johansen et al. 2009). In addition strain-dependent differences, they noted differences in biofilm formation depending on culture conditions of temperature and additives to the culture medium. Although biofilm formation was optimized by incubation for 28 °C for 3 weeks, practical concerns dictated that the set of 97 isolates was surveyed after incubation at 20 °C for 2 weeks. Under these conditions, 9 of 97 isolates (9.3 %) were considered biofilm-formers, all coming from swine. No *M. avium* isolates of bird or human origin formed biofilms under these conditions. Martin-de-Hijas and colleagues examined the biofilm formation of 167 strains of rapidly-growing mycobacteria recovered from patients in Spain over a 16-year period, 41 of which were deemed clinically significant after chart review on the basis of being associated with either device-related or device-unrelated infections of multiple and varied anatomic sites (Martin-de-Hijas et al. 2009). Notably, this collection included only five respiratory isolates, all obtained from patients with cystic fibrosis. The authors noted statistically significant differences, wherein clinically significant isolates more frequently produced biofilm (61.8 % vs 55.6 %) which was most prevalent among isolates of *M. abscessus* (87.5 %), *M. chelonae* (73.3 %) and *M. fortuitum* (61.1 %) and the only infection-related isolate of *M. peregrinum* (4.8 %). Logistic regression confirmed that the mycobacterial species, percent biofilm-covered surface over time, biofilm formation and sliding motility (lateral spread over time on agar) were all predictors of clinical significance among this group of isolates. Carter and colleagues examined 14-day biofilm formation in six bloodstream isolates of *M. avium* from AIDS patients, finding that biofilms were variable, enhanced by cationic supplementation, nutrient factors, supernatant from mature biofilm cultures (Carter et al. 2003). Interestingly, biofilm formation was prevented by sub-inhibitory concentrations of amikacin.

Mechanistic in vitro studies have attempted to illuminate the influence of biofilms on mycobacterial infections. Cultures of *M. avium* complex in with genetically-attenuated biofilm formation demonstrated reduced invasion of bronchial epithelial cells (Yamazaki et al. 2006), whereas wild-type *M. avium* complex biofilms resisted clearance by, and induced apoptosis in, human mononuclear phagocytes more rapidly than their planktonic cognate (Rose and Bermudez 2014). Curiously, *M. abscessus* biofilms were significantly enhanced in the presence of necrotic neutrophils, an environment mimicking the namesake clinical syndrome caused by *M. abscessus* (Malcolm et al. 2013). Perhaps this reflects both its success as a human pathogen as well as its particular tenacity in the face of treatment. Its treatment-refractory nature was demonstrated with antimicrobial testing against both biofilm-forming and non-biofilm-forming phenotypes (smooth- and rough- morphotypes, respectively) (Greendyke and Byrd 2008). In this study, neither amikacin nor clarithromycin (which has potent activity against many NTM) demonstrated significant activity against static-phase *M. abscessus* biofilms. Thus, biofilms may contribute to the difficulty in curing infections caused by this organism with medical therapy alone. In contrast, sub-inhibitory concentrations of clarithromycin were effective at preventing *de novo* biofilm formation (but could not eradicate established biofilms) among human bacteria isolates of *M. avium* (Carter et al. 2004).

1.5 Biofilms in Cystic Fibrosis

Cystic fibrosis (CF) is a heritable disease characterized by thickened, inspissated pulmonary secretions and chronic colonization and/or infection by a restricted group of organisms. Here we will review relevant biofilm studies in the CF literature, including those involving *P. aeruginosa*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia*.

Biofilm phenotypes of *P. aeruginosa* were examined in multiple reports. Small-colony variants from 12 CF patients in Germany were demonstrated to have increased capacity for biofilm formation in addition to increased fitness and twitching motility (Haussler et al. 2003). Among 96 isolates from 13 mono-infected children in Northern Ireland, a wide variation in biofilm production was observed (31 weak, 19 moderate, 46 strong) (Deligianni et al. 2010). Similar diversity in swimming, twitching and swarming motility characteristics was also found. This study suggested that motility was not required for biofilm formation, and that biofilm non-producing strains also can survive in the CF lung environment.

Whether the "mucoid" phenotype among CF isolates is can be interpreted as, or is contributory to, biofilm formation is unclear. At the genomic level, gene expression of quorum-sensing molecules and alginate production (a component of biofilm) were compared in paired mucoid and non-mucoid isolates obtained nearly a decade apart in each of same three CF patients (Lee et al. 2011). Transcriptional profiles and resulting phenotypes were significantly different, demonstrating loss of biofilm-forming capacity in the non-mucoid strains. However, a separate study documented similar increases in antimicrobial resistance in biofilms formed by both mucoid and non-mucoid isolates obtained from the same patients (Aaron et al. 2002).

Five clinical CF strains were cultured in a continuous-culture flow cell system, which produces shear forces in contrast to the static conditions of the microtiter plate method for producing biofilms (Kirov et al., 2007). The resulting biofilms were examined microscopically, revealing a proportion of dead bacteria distributed throughout the biofilm colony structure, seeding dispersal (organisms blebbing off of the colony), and hollow channels produced in the biofilm by motile bacteria. In addition, small-colony variants were recovered from the flow cell effluent for three of the five strains. In another study which evaluated biofilms formed in flow cells, significant diversity of biofilm formation and motility characteristics were found in 20 non-mucoid isolates from 8 CF patients (Lee et al. 2005). One patient in the series had six clonal isolates recovered over a 20-year period, one of which was collected after lung transplantation. The exuberance of biofilm formation among these isolates was generally below that of the reference

strain PAO1, and demonstrated progressively decreasing substrate adherence over the 20-year period of chronic infection. This provided further evidence that biofilm formation alone is not essential for the survival of *P. aeruginosa* in the CF lung.

Responding to uncertain relevance of conventional planktonic susceptibility testing in CF, several studies have examined antimicrobial susceptibility testing of *P. aeruginosa* isolates grown as biofilms (Hill et al. 2005). Sixteen multidrug-resistant CF isolates were tested against single agents and also using combinations of two or three antimicrobials. The isolates were grown aerobically, anaerobically (to reflect conditions in the CF lung environment) and as biofilms on pegs subjected to shear forces. Biofilm-grown and anaerobically-grown isolates were less susceptible than were aerobically-grown isolates, to both single and combination antimicrobials. The most potent regimen against biofilm-grown *P. aeruginosa* was the combination of meropenem, tobramycin 200 μg/mL and aztreonam. Similar increases in resistance associated with biofilms were observed in another study which examined both mucoid and non-mucoid isolates obtained from the same patients (Aaron et al. 2002). Mucoid and non-mucoid isolates exhibited similar susceptibilities, whereas isolates in biofilms or adherent monolayers were much less susceptible to two- and three-drug combinations compared to planktonic organisms. Clinical implementation of a laboratory biofilm test was proposed for *P. aeruginosa* following validation of an assay demonstrating only a 5.7 % serious error rate (Moskowitz et al. 2004). Using 94 isolates recovered from 41 CF patients, the anti-biofilm activities of 20 commonly used and 12 less-commonly used antibiotics in CF were tested in a standardized fashion for their ability to impede biofilm formation on plastic pegs, similar to the Calgary biofilm device (Ceri et al. 1999). Static biofilms formed over 20 h were challenged by constant exposure to antibiotics for 18–20 h and the biofilm-inhibitory concentration was determined spectrophotometrically. Results were reproducible in duplicate testing, with 5.7 % "serious" errors from discordant results (misclassification of resistant as susceptible, or vice-versa) and 9.2 % "minor" errors (misclassification of intermediate as susceptible or resistant). There was no predominant pattern of susceptibility among the isolate biofilms, suggesting that individualized testing might be necessary for this approach to be clinically useful. Interestingly, azithromycin appeared to have considerable anti-biofilm activity, ranking second in potency after ciprofloxacin. This is consistent with other reports wherein azithromycin at 8 μg/mL or less was observed to decrease biofilm inhibitory concentrations of primary anti-pseudomonal agents (Lutz et al. 2012). Several clinical trials have included azithromycin for CF therapy with salutary effects on pulmonary function (reviewed in (Schultz 2004)). Subsequently, the utility of biofilm susceptibility testing to guide antimicrobial therapy for chronic pulmonary infections by *P. aeruginosa* was examined in a multicenter randomized pilot clinical trial (Moskowitz et al. 2011). Twenty subjects were randomized to biofilm-directed therapy on the basis of testing results, versus 19 whose treatments were guided by conventional planktonic susceptibility testing. The type of susceptibility testing did not affect the antibiotic class selected for therapy in 19 of the 39 subjects enrolled. Both testing groups had comparable declines in sputum bacterial burden at the end of therapy, and there was no significant difference in pulmonary function as assess by the FEV_1. Garlic, an inhibitor of *P. aeruginosa* quorum sensing (a biochemical stimulus for biofilm formation), was tested in a randomized, placebo-controlled pilot clinical trial of 34 CF patients (Smyth et al. 2010). Garlic or olive oil (placebo) capsules were given at a dose of 656 mg daily for 8 weeks. Eight patients withdrew from the study. Although not statistically significant in this pilot study, improvements in FEV_1, clinical symptom scores and weight gain were suggested in the group receiving garlic oil. The quorum sensing molecule 3-oxo-C_{12}-homoserine lactone (produced by *P. aeruginosa*) was measured in plasma and sputum, but could not be compared between treatment groups due to insufficient measurable levels. Besides the therapeutic novelty of garlic oil, this study is laudable for attempting to identify

and utilize a clinically useful biomarker for in vivo biofilms, the lack of which represents a major obstacle for the clinical utility and application of the biofilm concept.

Other organisms pertinent to Cystic Fibrosis, such as *Burkholderia* spp. and *Stenotrophomonas maltophilia* have also been examined for biofilm-forming phenotypes among clinical isolates. *Burkholderia dolorosa*, a lesser-known species within the *Burkholderia cepacia* complex, were examined for in vitro biofilm formation in isolates from 4 CF patients and 1 environmental isolate, as well as 13 isolates belonging to 5 other *Burkholderia* species (Caraher et al. 2007). At 48 h, four of the *B. dolorosa* isolates formed strong biofilms, while *B. cepacia*, *B. stabilis, B. vietnamiensis* and one *B. dolorosa* clinical isolate of did not. From the sputum of a single CF patient in Portugal who died from the infection, 11 clonal variants of *B. cenocepacia* recovered over 3.5 years were examined for a variety of phenotypic parameters including biofilm formation (Coutinho et al. 2011). Genetic analysis by multilocus sequence typing identified the organisms as two clonal variants within the *B. cenocepacia* complex. Biofilm formation was variable among the isolates and did not clearly correlate with either MLST sequence type or chronologic recovery. With regard to *S. maltophilia*, 125 isolates from 85 CF patients underwent planktonic and biofilm susceptibility testing, revealing poor anti-biofilm activity of aminoglycosides and beta-lactams (Wu et al. 2013). Levofloxacin and colistin had the best anti-biofilm activity, tested at high concentrations to reflect aerosolized pulmonary delivery. Twelve *S. maltophilia* strains from individual CF patients were examined for the biofilm formation and ability to adhere to CF-derived bronchial epithelial cells (Pompilio et al. 2010). All the strains were variably adherent to the cells within 2 h and produced different amounts of biofilm (interpreted by total CFU); epithelial adherence was unrelated to the magnitude of biofilm. Lastly, a group of 98 *S. maltophilia* isolates (41 from CF patients) were evaluated in a comparative fashion for biofilm formation and other phenotypic properties (Pompilio et al. 2011). More biofilm formation was found more frequently among non-CF strains than those of CF origin (97.9 % vs 90.2 %), although clearly the rate of biofilm formation was very high in both groups. In addition, the magnitude of formed biofilm was lower overall among CF strains compared to non-CF strains. Flagellar and type IV pili motility mechanisms were linked to biofilm formation. Within this set of isolates, five isogenic strains recovered from a single patient over 3 years were found to produce variable amounts of biofilm. Finally, biofilm formation was compared between 20 CF and 22 non-CF bacteremia isolates of *S. pneumoniae*, finding that biofilm formation occurred significantly more frequently in the isolates of CF origin (80 % vs 50 %, respectively) and also demonstrated reduced susceptibilities to various antimicrobials, as measured by the MBIC (Garcia-Castillo et al. 2007).

1.6 Biofilms in Clinical Isolates of *Candida* spp.

Candida spp. are among the most studied organisms with respect to involvement of biofilms in human infection. Similar to other bacteria, these dimorphic fungi serve an important role as commensal organisms within the human host, but can be a potentially lethal cause of invasive bloodstream infection. Also like bacteria, growth conditions likely influence the development of *Candida* biofilms. In a study of 67 urinary isolates from 55 patients, biofilm formation was noted to vary depending on the growth medium (artificial urine versus RPMI medium), particularly for *C. albicans* (Jain et al. 2007). Optimal conditions for Candida biofilm development have been explored, recognizing the important shortcomings of in vitro test conditions to accurately represent the in vivo situation (Krom et al. 2009). Variables influencing biofilm development included not only the culture media used, but supplemental factors such as proteinaceous material, inoculum density, type and concentration of nutritional supplements such as glucose, and methods of biofilm evaluation. Whereas most bacterial biofilm studies have used Crystal Violet or safranin staining to measure biomass as a reflection of

biofilm magnitude, most studies of *Candida* biofilms have utilized a tetrazolium reduction assay (tetrazolium 2,3-bis (2-methoxy-4-nitro-5-sulfuphenyl)-5 [(phenylamino) carbonyl]-2H-tetrazolium hydroxide, abbreviated as XTT) which measures colorimetric change in proportion to the number of viable cells. Thus it is important to consider biofilm testing methodology in comparison of different study results. In a rigorous study involving five different techniques for biofilm quantitation, including Crystal Violet Staining, XTT reduction, counts of viable colony-forming units (CFU), automated cell-counting (ACC) and biofilm suspension turbidity (BST) were compared (Alnuaimi et al. 2013). While ACC and BST were equivalent to CFU or CV for determining biofilm biomass, they were better correlated with CFU counts than the CV method. This study also demonstrated that biofilm growth (measured by XTT) to a 72-h endpoint was optimal using an inoculum density of 10^7 CFU versus 10^3 or 10^4 CFU. The XTT growth signal was significantly higher among reference strains of *C. glabrata* and *C. albicans* at inoculum concentrations of 10^3 and 10^4 CFU, respectively, than at 10^7 CFU. CV/ACC/BST and XTT were used in conjunction in this study to characterize biofilms in a two-dimensional manner, segregating isolates into six combinations of mass (low, medium or high according to CV/ACC/BST signal) and growth (active or stationary, according to XTT signal). This study revealed that laboratory and reference strains can differ significantly with respect to growth rates and biomass, emphasizing the importance of both careful strain selection by investigators to ensure clinically-relevant results, and mindful interpretation by readers of their published reports as to the biological meaning of the results.

The biofilm formation of human candidemia isolates has been surveyed in a number of studies. Among 393 bloodstream isolates from Sweden, *C. albicans* was the most commonly recovered (61.8 %), but formed biofilm significantly less frequently (40.3 %) than the non-albicans Candida (88.7 %) (Pannanusorn et al. 2013). In Turkey, 8 of 68 (11.1 %) of *C. albicans* isolates were biofilm-positive, versus 13 of 31 (41.9 %) non-*C. albicans* isolates (17874282 (Gokce et al. 2007)). Among 58 Candida isolates recovered from immunocompromised patients in India (36 bloodstream and 22 oral isolates), 83.6 % of bloodstream isolates were found to form biofilm, versus 81.8 % from oral isolates. Biofilm formation was more common among non-albicans *Candida* (93.1 %) versus *C. albicans* (42.9 %) (Kumar and Menon 2006). Biofilm production was also investigated among 297 invasive isolates (276 from blood, 160 *C. albicans*) among ICU patients in Italy, finding 32.3 % of all isolates to be high biofilm producers (33.8 % for *C. albicans* versus 30.7 % for non-*C. albicans*). Clinical outcomes were compared between 77 and 53 patients infected by high and low biofilm-producing *Candida*, respectively, finding no differences in crude mortality according to biofilm formation (40 % versus 37.5 %) or species (59.3 % for *C. albicans* versus 59.4 % for non-*C. albicans*) (Prigitano et al. 2012). The biofilm formation of 107 sequential isolates from 32 candidemic patients was compared to single oropharyngeal isolates from 19 AIDS patients with oral lesions. The degree of biofilm formation was highly variable overall among all isolates, did not change significantly over time among serial candidemia isolates, and no association between biofilm formation and mortality could be demonstrated (Hasan et al. 2009). *Candida* isolates causing neonatal sepsis in India were compared with cervical swab isolates from their birth mothers with vulvovaginitis (Harakuni et al. 2012). Fourteen of the 16 bloodstream isolates (87.5 %, all non-albicans *Candida* spp.) in the infants were found to form biofilms, versus only 4 of 21 cervical isolates (19.1 %, all *C. albicans*).

Demonstrating the influence of species over site of collection, biofilm production among vulvovaginitis isolates of five different species from Brazil were compared, showing (in decreasing order of magnitude) biofilm production by *C. tropicalis*, *C. albicans*, *C. parapsilosis*, *C. guillermondii* and *C. glabrata* (Paiva et al. 2012). *C. tropicalis* was also found to be the highest biofilm producer among various species of 30 *Candida* isolates (Melo et al. 2011). Varying degrees of biofilm production were demonstrated

among 50 of 71 *Candida* vulvovaginitis strains from Northern India (48 % of *C. albicans*, 81 % of *C. parapsilosis*, and 81 % of *C. glabrata*) (Kumari et al. 2013). Among 17 isolates of *C. albicans* recovered from bronchial aspirates of intubated patients, all produced biofilm (Sacristan et al. 2011). Biofilm formation was investigated in a subset of 59 of 375 *C. albicans* bloodstream isolates collected from Italian patients, of which 23 (39 %) formed biofilms (Tortorano et al. 2005). Biofilms were noted to be significantly associated with serotype B versus serotype A.

A number of studies have examined the role of biofilm formation among clinical isolates of the *C. parapsilosis* complex. Among 49 bloodstream isolates recovered from children in Brazil (41 *C. parapsilosis* sensu strictu, 5 *C. orthopsilosis*, 3 *C. metapsilosis*), all were observed to form biofilms (Ruiz et al. 2013), as well as 9 *C. parapsilosis* sensu strictu and 2 *C. orthopsilosis* isolates in another study from Brazil (Abi-Chacra et al. 2013). Among a geographically diverse group of 62 isolates of *C. parapsilosis* sensu strictu representing superficial and deep-seated infections, more biofilm-forming isolates were observed in isolates from Argentina and Hungary than in isolates from New Zealand and Italy despite limited genetic variability (Tavanti et al. 2010). The majority of biofilm-forming strains represented bloodstream-infecting isolates. 42 isolates of *C. parapsilosis* complex from Turkey (38 *C. parapsilosis* sensu strictu, 1 *C. orthopsilosis*, 3 *C. metapsilosis*) were evaluated for biofilm formation, 11 (26.2 %) of the *C. parapsilosis* sensu strictu isolates demonstrating biofilm formation but not *C. orthopsilosis* or *C. metapsilosis* (Tosun et al. 2013). The dry weight of biofilms produced by five invasive strains of *C. parapsilosis* from a hospital outbreak in the state of Mississippi (USA) were compared to reference strains in Cleveland, Ohio (USA) (Kuhn et al. 2004). This demonstrated consistency of biofilm formation across multiple sites of recovery during the outbreak (catheter, blood, sputum, skin) and biofilm production was increased over sporadically-occurring isolates in the reference collection. This suggests that biofilm formation may have played a role in facilitating nosocomial transmission during the outbreak.

The clinical impact of biofilm formation among Candida has been investigated in several animal models and human studies. In the *Galleria mellonella* (wax moth) larvae model, which has a similar immune response to mammals, five biofilm-producing and four non-biofilm forming invasive human clinical isolates of *C. albicans* were used to assess virulence. Biofilm formation significantly decreased survival time of the infected larvae, with 80 % of larvae infected by biofilm-forming *C. albicans* dying by 72 h (Cirasola et al. 2013). An additional study utilized the *Galleria mellonella* model to examine the role of biofilm formation in virulence among 20 human clinical isolates 15 bloodstream and 5 catheter-related, 9 biofilm-forming) of *C. albicans* (Borghi et al. 2014). After optimizing the inoculum density at 10^5 CFU/larvae, significantly decreased survival was observed among larvae infected by biofilm-forming isolates compared to non-biofilm forming isolates (Hazard ratio 2.63, 95 % CI: 2.03–3.41), suggesting biofilm formation may be a virulence factor for poor outcomes. Additionally, infection by biofilm-forming strains resulted in a higher fungal burden at 48 h compared to non-biofilm forming strains and demonstrated more aggressive behavior, characterized by histopathologic evidence of increased hyphal invasion into the *Galleria* larval intestine. Six clinical isolates of *C. albicans* from one of the aforementioned studies (Hasan et al. 2009) were intravenously inoculated into mice, demonstrating significant differences in median survival time which correlated with the intensity of biofilm formation by the infecting isolate (high: 1–4 days; intermediate: 10–11 days; low: 20 to >40 days). These survival groups were correlated to fungal burden in kidney, liver and lung in post-mortem examination, supporting a role for biofilm formation in pathogenesis of *C. albicans* bloodstream infection.

A large biofilm survey was undertaken among 360 *Candida* spp. isolates collected from non-neutropenic patients in Korea over a 4-year period (Shin et al. 2002). These included bloodstream isolates from 101 patients and from other sites in 259 patients (97 from urine, 89 from the respiratory tract, 41 from pus or wounds, 12 from body fluids and 20 from other sites excluding

catheters). Bloodstream isolates exhibited biofilm production more frequently (79 %) than isolates recovered from other sites (52 %). Only 8 % of *C. albicans* isolates formed biofilms, versus 61 % of non-albicans *Candida* species. Notably, 86 % of *C. parapsilosis* isolates recovered from the bloodstream formed biofilms, compared to 47 % of *C. parapsilosis* from other sites. Among the non-albicans *Candida* species, 95 % of those which were related to central venous catheters formed biofilms, as did 94 % associated with total parenteral nutrition (TPN). There was no relationship observed in this study between biofilm positivity of infecting isolates and clearance of candidemia.

Human risk factors for candidemia and outcome predictors for the infection were determined at a single center in Italy in a comprehensive two-part study utilizing case-case-control (biofilm-forming isolates, non-biofilm isolates, non-infected hospitalized controls) and case-matched cohort (biofilm-forming versus non-forming) study design (Tumbarello et al. 2012). Two-hundred and seven case patients, 84 of whom were infected by biofilm-forming Candida isolates (32 *C. albicans*), were compared to 200 hospitalized controls. Multivariate logistic regression demonstrated that presence of a central venous catheter and receipt of total parenteral nutrition were statistically significant risk factors for candidemia associated with both biofilm and non-biofilm isolates. Additionally, antibiotic therapy or surgery in the past 30 days were found to be risk factors for candidemia involving a non-biofilm forming isolate. While these constitute established risk factors for candidemia, novel risk factors for bloodstream infection with a biofilm-forming isolate were diabetes mellitus (OR 4.47, 95 % CI 2.03–9.83) and presence of a urinary catheter (OR 2.40, 95 % CI 1.18–4.91). The cohort study of 84 patients with biofilm-forming isolates matched by age, sex, APACHE score and receipt of antifungal therapy, to 73 non-biofilm-forming isolates revealed a 23 % excess mortality among those infected by a biofilm-forming isolate, as well as increased antifungal therapy costs and increased hospital length of stay among survivors of candidemia. Perhaps most compelling is the observation that treatment with an echinocandin, previously shown to disrupt *Candida* biofilms in vitro (Fiori et al. 2011), was associated with significantly reduced mortality and hospital length of stay. Thus *Candida* biofilms appear to represent a potent virulence factor for potentially lethal human infection with patients placed at risk through the use of vascular access devices. Awareness of these risk factors and appropriate modification of antifungal therapy have the potential to favorably influence the outcome of the infection.

1.7 Influence of Host Factors on Biofilm Formation

There is increasing evidence that in vitro biofilm assays may not accurately represent in vivo biofilms (Bjarnsholt et al. 2013). For example, there are important differences between in vitro biofilm structures and in vivo biofilms associated with chronic infections in humans and animal models to include smaller physical dimensions, lack of mushroom-like structures, and embedding in host material (Bjarnsholt et al. 2013). Factors that may lead to discrepancies include experimental time spans, presence of host defenses, and the chemical microenvironment (Bjarnsholt et al. 2013). We believe that host factors in particular have many important implications on biofilm formation in the in vivo environment that are underappreciated in current in vitro models. In the in vivo microenvironment, as opposed to in vitro biofilm assay, there are numerous host factors present, to include blood constituents, blood cells (such as platelets), and other body secretions (saliva, urine, respiratory secretions, peritoneal fluid, etc.) that may impact biofilm formation (Fig. 1.2).

The most commonly utilized host factors in in vitro biofilm assays are blood constituents and most available data has been generated using *S. aureus*. Human plasma is typically the most common blood component utilized in in vitro biofilm assays. It is important to understand the difference between plasma and serum as they differ in composition and their effects on biofilms.

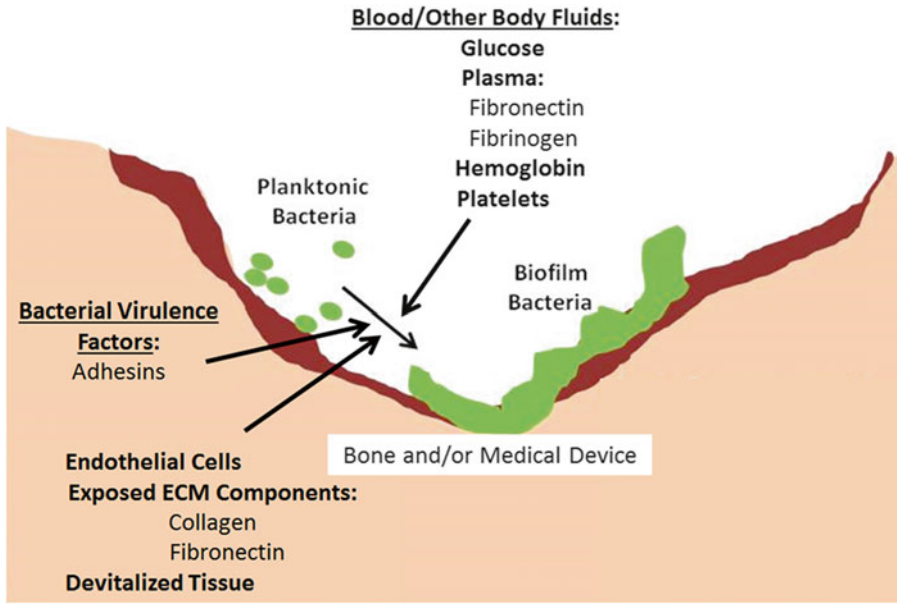

Fig. 1.2 Illustration of host factors which can contribute to biofilm formation in vivo

Plasma differs from serum in that it contains fibrin and other soluble clotting elements. Plasma is produced by the addition of anticoagulant (most commonly heparin) to whole blood to prevent clotting, followed by centrifugation to remove blood cells. Serum is obtained by separating whole blood into its solid and liquid components by centrifugation after it has been allowed to clot, thus removing cellular and subcellular components including coagulation factors which are consumed by clot formation. Plasma represents approximately 55 % volume/volume (v/v) of whole human blood (assuming a hematocrit of 45 %) and includes coagulation factors, albumin, globulins and other factors (Bridges et al. 1987; Espersen et al. 1990; Herrmann et al. 1988). Plasma is an attractive option for use in in vitro biofilm assays, as most human body fluids consist of plasma filtrates and plasma proteins are present in varying concentrations in human body fluids to include (percent, v/v): burn wound exudates (10–44 %), acute soft tissue wound exudates (23–36 %), interstitial fluid (10–27 %), nasal secretions (15–45 %), ascitic fluid (4–26 %), lymphatic fluid (10–50 %), and synovial fluid (1–73 %) (Chang et al. 1995; Henderson et al. 1980; Hourigan et al. 2010; Igarashi et al. 1993; Lehnhardt et al. 2005; Miller et al. 2000; Takeda 1966). In addition, implanted medical devices become coated with plasma proteins, and plasma coating of titanium surfaces has been shown to increase the adherence of *S. aureus* and *P. aeruginosa* (Francois et al. 2000; Sela et al. 2007; Vaudaux et al. 1989; Wagner et al. 2011).

Human plasma and its components such as fibrinogen and fibronectin have been demonstrated to enhance *S. aureus* biofilm formation, promote adherence to human cells, and mediate adhesion to medical devices (Aly and Levit 1987; Beenken et al. 2003; Chen et al. 2012b; Lower et al. 2011; O'Neill et al. 2009; Walker and Horswill 2012; Zautner et al. 2010). Typically, plasma is utilized to coat or condition a surface overnight, prior to biofilm formation, and it is has not been established if surface coating or inclusion as a part of the growth medium is optimal for biofilm production. The most commonly utilized plasma concentrations in in vitro assays that consistently enhance biofilm formation are 10 or 20 % (v/v) in both flow chambers under controlled shear flow and static well models (Chen et al. 2012b; Walker and Horswill 2012). It has also been observed that the response to plasma is

species-dependent; for example, biofilm formation by *Staphylococcus epidermidis* has been observed to be less responsive to plasma in vitro (Beenken et al. 2003). The mechanism by which plasma enhances *S. aureus* biofilm formation is unclear, and more studies are needed which include purified plasma components. However, for *S. aureus,* current hypotheses hold that surface-adherent plasma proteins interact with various receptors known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). A limitation to the use of human plasma is standardization, as plasma can come from a single donor or from a pool of donors, without knowledge of the concentrations of constituent proteins. In addition, it is likely that the plasma components may vary based on age, gender, and disease state. Thus, use of purified plasma components which simulate the in vivo environment may be the optimal approach for a standardized assay.

Of the plasma proteins which serve as ligands for MSCRAMM receptors, fibronectin and fibrinogen have been the most frequently studied, primarily using *S. aureus*. Fibronectin can be produced by fibroblasts, monocytes, endothelial cells in response to blood vessel injury, and/or be derived from blood plasma or serum (Clark et al. 1982; Martin et al. 1988). In vivo, fibronectin assists in providing a provisional substratum that is essential for cell migration and proliferation by coating tissue debris and non-viable cells, and is concentrated in areas of intense cellular activity such as wound repair (Clark et al. 1982; Martin et al. 1988). *S. aureus* has evolved to exploit the presence of fibronectin in wounds and areas of vascular injury. For example, polymorphisms in fibronectin binding protein A of *S. aureus* have been found to be associated with the infection of cardiovascular devices in 26 isolates from patients with infected devices when compared to 34 colonizing strains from asymptomatic subjects (Lower et al. 2011). Fibronectin has also been found to accumulate on medical device material and to promote adhesion of *S. aureus* and oral flora (Badihi Hauslich et al. 2013; Herrmann et al. 1988). Recently, expression of fibronectin binding proteins A and B have been demonstrated to be required for biofilm formation by the *S. aureus* USA300 strain LAC (McCourt et al. 2014). In group A streptococci, collagen-like protein-1, Scl1, has been found to mediate biofilm formation by targeting the extra domain A-containing variant of cellular fibronectin expressed in wounded tissue (Oliver-Kozup et al. 2013). In a study of 465 clinical isolates of *S. aureus*, the presence of fibronectin binding protein B was found to be a genetic risk predictor for strong biofilm producers (Lim et al. 2013). In particular, the N3 subdomain in a domain of fibronectin-binding protein B (isotype I) was found to be an independent risk factor which predicted biofilm formation of *S. aureus* clinical isolates (Kwon et al. 2013). There is also evidence that a novel *S. aureus* biofilm phenotype is promoted by fibronectin binding proteins (O'Neill et al. 2008, 2009; Shanks et al. 2008). Finally, reduced susceptibility to vancomycin has been reported compared to planktonic bacteria when *S. aureus* isolates were adhered to a fibronectin coated coverslip, but no comparison was made with biofilm formed on an uncoated surface (Chuard et al. 1993). The suggestion that host components may alter antimicrobial susceptibility calls into question the validity of in-vitro antimicrobial biofilm screening assays which omit host factors which are known to be involved in biofilm formation in vivo.

Fibrinogen has also been found to significantly impact biofilm formation. The primary role of fibrinogen is to provide scaffolding for the formation of intravascular thrombus. However, fibrinogen also participates in other biologic functions involving unique binding sites, some of which become exposed as a consequence of fibrin formation (Jaffe 1987). Fibrinogen has been found to promote adherence of *S. aureus*, and to a lesser extent *S. epidermidis*, and *E. coli* (Herrmann et al. 1988; Tedjo et al. 2007). In addition, it has been shown that transient fibrinogen depletion significantly reduces the bacterial burden and consequent morbidity and mortality during experimental infection with wild-type *S. aureus* (Rothfork et al. 2003). Fibrinogen has found to be the most consistently adherent component to shunt tubing used in chronic hemodialysis

(Francois et al. 2000). Biofilm formation by *S. aureus* cutaneous strains is enhanced by fibrinogen, but this is dependent on the conversion of fibrinogen to fibrin (Akiyama et al. 1997). Fibrinogen has also been found to promote *Streptococcus mutans* biofilm formation and its adherence to endothelial cells and to enhance biofilm formation by *Streptococcus suis* and enhances its antibiotic resistance (Bedran et al. 2013; Bonifait et al. 2008).

In contrast to fibronectin and fibrinogen, human albumin has been associated with inhibition of biofilm formation (Herrmann et al. 1988). In a study of seven *E. coli* isolates, pre-treatment of polystyrene plates with human albumin significantly reduced biofilm formation by all strains (Naves et al. 2010). Human serum albumin coating of polystyrene plates was found to significantly reduce bacterial adhesion and biofilm formation in *S. pneumoniae* (del Prado et al. 2010; Ruiz et al. 2011). There is also evidence that the anti-adherence effect is species-dependent as albumin coating of titanium surfaces decreased the adhesion of *S. mutans*, but neither *P. gingivalis* nor *F. nucleatum* (Badihi Hauslich et al. 2013). However, for *S. aureus*, the anti-adhesive effects of albumin can be overcome by the presence of fibrinogen in the growth medium (Jaffe 1987).

Like albumin, human serum has been shown to inhibit biofilm formation. While human serum has been found to support planktonic bacterial growth, it demonstrated potent inhibition of biofilm formation, with the inhibitory component (s) found to be protease-resistant and heat stable (Abraham and Jefferson 2010). *Candida albicans* biofilm formation was also inhibited by human serum, and this effect was similarly preserved after exposure to heat and protease treatments (Ding et al. 2014).

The impact of human hemoglobin on biofilm formation has been less well studied, but may be important given recent findings. Human hemoglobin promoted *S. aureus* surface colonization, and significantly decreased the inoculum necessary for nasal colonization (Pynnonen et al. 2011). When grown in human hemoglobin, compared to mouse hemoglobin, *S. aureus* preferentially recognized human hemoglobin, and harvested iron more efficiently (Pishchany et al. 2010). In addition, transgenic mice which expressed human hemoglobin were more susceptible to fatal *S. aureus* infection compared to mice expressing murine hemoglobin (Pishchany et al. 2010). Thus, considering that biofilms are implicated in diseases such as wound infections and endocarditis, where hemoglobin exposure is likely, the impact of human hemoglobin on biofilm formation merits further investigation. It may also be beneficial to examine the effects of various iron sources on biofilm formation, as it has been demonstrated in seven strains of *Actinobacillus actinomycetemcomitans*, that the incubation in the presence of FeCl (3) or hemin resulted in the formation of more aggregates and microcolonies compared to cells grown in the presence of the synthetic iron chelator dipyridyl (Rhodes et al. 2007).

In addition to blood constituents, whole blood has also been examined in relation to biofilm formation. Murga et al. investigated the effect of human blood on biofilm formation on the inner lumen of needleless central venous catheter connectors by *Enterobacter cloacae, P. aeruginosa*, and *Pantoea agglomerans*; and found that conditioning with whole blood resulted in significantly higher viable colony counts than non-conditioned controls (Murga et al. 2001). Another study examined gene expression of a single *S. aureus* USA300 isolate in response to human blood, and demonstrated that whole blood induced greater expression of fibronectin binding protein and extracellular fibrinogen binding protein, which could potentially impact biofilm formation (Malachowa et al. 2011). Franca et al. demonstrated that exposure of *S. epidermidis* to whole human blood resulted in increased transcription of genes involved in biosynthesis and metabolism of amino acids, small molecules, carboxylic and organic acids, cellular ketones, and most notably increased expression of iron utilization genes (Franca et al. 2014).

Biofilm formation may also be influenced by the presence of host cells, most notably platelets. This interaction has most commonly been noted in *S. epidermidis* contamination of stored platelets for clinical use, where the organism has been found to form biofilm on platelet aggregates and

on platelet bags (Greco et al. 2007). *S. aureus* adhesion to immobilized platelets is thought to play a role in the pathogenesis of invasive bloodstream infections or endocarditis (Herrmann et al. 1993). Although direct binding interactions are known to occur between staphylococci and platelets, adhesion to endothelial cells is significantly higher in the presence of plasma proteins under levels of shear stress found in the intravascular environment (George et al. 2009; Shenkman et al. 2001). Interestingly, platelets have been noted to be essential for in vitro biofilm formation by *Streptococcus mutans* or *Streptococcus gordonii* grown in human plasma (Jung et al. 2012). The biofilms were found to be composed of bacterial floes embedded with platelet aggregates in layers, and a similar architecture was also detected in vivo on the injured valves of a rat model of experimental endocarditis. The streptococci in biofilms were also able to induce platelet aggregation, which was found to facilitate multilayer biofilm formation. A most concerning finding was that directly entrapment of platelets enhanced the resistance of streptococcal biofilms to clindamycin (Jung et al. 2012).

There are a myriad of other host factors that may influence biofilm formation. For example, basement membrane proteins such as collagen and laminin may promote biofilm formation similarly to plasma proteins, and in some studies have also been found to alter antimicrobial susceptibility (Herrmann et al. 1988; Jagnow and Clegg 2003; Violante et al. 2013). Even glucose concentrations may impact biofilm formation, potentially influencing infections in patients with diabetes mellitus. For example, it has recently been demonstrated that biofilm biomass was increased at higher glucose concentrations for both *S. epidermidis* and *S. aureus* with a threshold response at 0–20 and 160–200 mg/dL for *S. epidermidis* and 200–240 mg/dL for *S. aureus* (Waldrop et al. 2014).

In summary, host factors can have the potential to influence biofilm formation, and in some studies exposure to host factors has been implicated in increased antimicrobial resistance. More studies are needed to elucidate the complex interactions at the host-biofilm interface. Standardization of in vitro biofilm assays, analogous to methods of planktonic MIC determination endorsed by the Clinical Laboratory and Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST), may aid in reducing variability in experimental results while better reflecting in vivo conditions and allowing for more direct comparisons between studies. Further exploration of the interactions between bacterial biofilms and host factors may lead to improved in vitro predictors of therapeutic outcomes for specific antimicrobials, and yield novel treatment approaches for biofilm-associated infections.

1.8 Clinical Studies of Patients with Bacterial Biofilms

While the theory of biofilm disease supports the inability to eradicate infection from solid substrates (artificial devices, bone, etc.) due to reduced antimicrobial susceptibility, clinical data linking biofilms to recalcitrant infection are sparse (Table 1.1). A prospective study of *E. coli* bacteremia recorded 185 episodes of bacteremia from 177 patients, examining microbiologic variables isolates, including biofilm production by the microtiter plate method with Crystal Violet method (incubation time not reported), correlating them to demographics, underlying conditions, fever, shock, white blood cell count, source of infection and mortality (Martinez et al. 2006). Associations were not demonstrated between biofilm production and demographic or clinical variables ($p = 0.8$ for mortality differences) and was less common in phylogroup A (16 %) versus other groups (48 %). Independent predictors of bacteremia included neutropenia, infected catheter, cirrhosis and pneumonia.

Several studies have examined the clinical outcomes of infected neonates in relation to biofilms, a population vulnerable to suffering poor outcomes from infection. Biofilm formation among coagulase-negative staphylococci (CoNS), which are a significant cause of neonatal sepsis, was examined in 164 septic episodes from 150 neonates (Klingenberg et al. 2005). Eighty-five

Table 1.1 Selected clinical studies relating biofilm formation to clinical outcomes

Organism/syndrome	Study design	Population	Summarized findings	Reference
Coagulase-negative *Staphylococcus* bacteremia	Retrospective cohort	150 neonates	No difference in biofilm formation between infecting and colonizing isolates	Klingenberg et al. (2005)
			Biofilm production more common in *S. epidermidis* and assoc. with 34 % reduction in C-reactive protein	
E. coli bacteremia	Prospective cohort	177 patients	Biofilm formation not associated with outcomes	Martinez et al. (2006)
			Predictors of *E. coli* bacteremia: neutropenia, catheter-assoc. infection, cirrhosis and pneumonia	
Cystic fibrosis	Pilot randomized, controlled trial of garlic capsules to inhibit quorum sensing	34 patients with cystic fibrosis	Treatment group had non-significant increases in FEV_1, clinical symptom scores and weight gain	Smyth et al. (2010)
Chronic rhinosinusitis (CRS)	Retrospective cohort	24 patients with medically refractory CRS	Patients with polymicrobial biofilms had worse symptoms and radiologic severity	Foreman and Wormald (2010)
			H. influenzae was associated with milder symptoms and faster resolution, whereas 10 patients with persistent disease had *S. aureus* biofilms requiring more clinic visits	
Chronic rhinosinusitis	Prospective cohort	90 patients with CRS, 20 without CRS	Biofilms observed by scanning electron microscopy in 71 % of CRS group versus 0 % of control group	You et al. (2011)
			6-/12-month symptom and endoscopy scores worse among those demonstrating biofilms	
Candida spp. fungemia	Retrospective case-control	207 cases, 200 controls	Risk factors: central venous catheter, total parenteral nutrition, recent surgery or antibiotic therapy, diabetes mellitus, presence of urinary catheter	Tumbarello et al. (2012)
			23 % excess mortality associated with biofilm-forming isolates	
			Echinocandin therapy reduced mortality	
Ureaplasma spp./ bronchopulmonary dysplasia (BPD)	Retrospective cohort	43 neonates	95 % of isolates formed biofilms	Pandelidis et al. (2013)
			MBIC for macrolides was below planktonic MIC	
			67 % developed BPD including 4 who died	

(continued)

Table 1.1 (continued)

Organism/syndrome	Study design	Population	Summarized findings	Reference
Various bacterial species, invasive and non-invasive syndromes	Retrospective observational	150 patients	61 % of organisms formed biofilms, assoc. with multidrug-resistant (MDR) phenotype	Sanchez et al. (2013)
			Trend toward increased biofilm formation among isolates from non-fluid sites	
			Isolates from relapsing infections displayed strong biofilm formation	
Persistent wound infections	Retrospective case-control	25 patients (35 wounds) with persistent infection and 60 patients (69 wounds) with non-persistent infection	Risk factors for persistent infection included biofilm formation, MDR phenotype, and polymicrobial infection	Akers et al. (2014)
			Biofilm formation by *A. baumannii* associated with persistence	
			Multivariate model prevented by small sample size	

recovered isolates were regarded as true bacteremia, whereas 79 were considered blood culture contaminants. Biofilm formation was graded as none, weak or strong. While there were no differences in biofilm production between invasive and contaminating isolates, biofilm production (weak or strong) was more common in *S. epidermidis* than in other species of CoNS, and associated with 34 % lower levels of C-reactive protein. Biofilm formation among CoNS isolates was thus postulated to represent a mechanism for evasion by the pathogen of the host immune system response. In another study, biofilm formation by *Ureaplasma* spp., an atypical bacterial pathogen associated with bronchopulmonary dysplasia (BPD), was examined for its contribution to macrolide resistance (erythromycin and azithromycin) and BPD outcomes were recorded (Pandelidis et al. 2013). However, 95 % of the isolates formed biofilm, preventing this parameter from being used as a comparator. Interestingly, the minimum biofilm inhibitory concentration for 50 % ($MBIC_{50}$) for the isolates was lower than the planktonic MIC, which is in contrast to the usual situation for "typical" aerobic bacteria. In terms of clinical outcomes, 4 neonates died (9.1 %), 15 had moderate or severe BPD (34.1 %), 10 had mild BPD (22.7 %) and 14 escaped the infection with no BPD (31.8 %).

The clinical consequences of biofilm formation have also been examined in chronic rhinosinusitis (CRS). A study from China found bacterial biofilm formation (judged by scanning electron microscopy of sinus mucosal specimens) occurred 64 of 90 CRS patients (71.1 %) (You et al. 2011). These were compared to a control group of 20 patients without CRS who also underwent sinus mucosal biopsy. Patients were followed forward in time for changes in symptom scores as well as objectively evaluated by endoscopy. No biofilms were observed by SEM among control group samples, and follow-up measures indicated less symptom improvement and worse endoscopic appearance among those who exhibited biofilms on the mucosa. A separate retrospective study utilized fluorescence in-situ hybridization (FISH) to query for biofilms with presence of *S. aureus*, *H. influenzae* or *P. aeruginosa* (alone or in combination) in ethmoid sinus mucosal samples from 24 patients with medically-refractory CRS (Foreman and Wormald 2010). Symptom and radiology-based scores were evaluated and patients had a median follow-up of 11 months. Eleven patients had complete disease resolution at the end of follow-up, with ten of those with persistent disease having evidence of *S. aureus* biofilms. Patients with polymicrobial biofilms had significantly higher symptom and

radiology scores compared to single-species biofilms. *H. influenzae* was noted to be associated with milder symptoms and more rapid resolution of disease. Thus, the post-operative clinical course in CRS may be influenced not only by biofilm formation, but also by the composition of the involved species.

We evaluated the biofilm-forming capacity of 205 clinical isolates from 150 patients with relapsing infections where the clonal identity of relapse isolates was identical to the initially recovered strain (Sanchez et al. 2013). Isolates were recovered from multiple anatomic sites, including wounds, bone, respiratory tract, urinary tract and blood. Clonality was assessed using pulsed-field gel electrophoresis (PFGE), and biofilm formation was determined in triplicate by Crystal Violet staining of biofilms grown for 48 h in appropriate media for gram-positive or gram-negative organisms. Overall, 61 % of the organisms formed biofilms. Biofilm formation was very heterogeneous among the organisms, but trends were evident supporting increased biofilm formation among isolates recovered from non-fluid sites (superficial/deep tissue, bone or respiratory samples) compared to fluid sites (blood or urine). In addition, biofilm formation was statistically related to organisms having a multiple drug resistant (MDR) phenotype. This is potentially concerning in light of the ongoing global epidemic of MDR pathogens, given that antimicrobial effectiveness would then be limited not only by the acquired (e.g. plasmid-mediated) resistance mechanisms but also by innate resistance attributable to the biofilm.

Subsequently, we utilized a case-control study design to determine risk factors, including biofilm formation, in the persistence of clinical wound infections (meeting CDC NHSN criteria for skin/soft tissue infection (Horan and Gaynes 2004)), which has been a major source of morbidity among U.S. military casualties injured in Iraq and Afghanistan (Akers et al. 2014). Data from the Trauma Infectious Disease Outcomes Study (TIDOS), a prospective database of U.S. military combat casualties, was used (Tribble et al. 2011). A wound was considered to be persistently infected if isolates were recovered at least 14 days apart. Reflecting our clinical experience with the most frequent infecting pathogens in these patients, the study was restricted to *S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa* and *E. coli*. Comparing 35 wounds with persistent infection from 25 patients were compared to 69 wounds from 60 patients having a single episode of infection, biofilm formation was found to be a risk factor for infection persistence with an odds ratio (OR) of 29.49 (95 % CI, 6.24-∞) . We could not demonstrate an association with implanted medical devices (primarily orthopedic hardware, in this population) but this was limited by the poor granularity of these data. Additional risk factors identified in univariate analysis included MDR phenotype (OR 5.62, 95 % CI 1.02–56.92), packed red blood cell transfusion within the first 24 h after injury (OR 1.02, 95 % CI 1.01–1.04), number of operating room visits prior to and on the date of infection diagnosis (OR 2.05, 95 % CI 1.09–4.28), anatomic location of infection (OR 5.47, 95 % CI 1.65–23.39) and polymicrobial infection (OR 69.71, 95 % CI 15.39-∞). A multivariate model could not be successfully executed due to limited sample size. *A. baumannii* was the predominating isolate, all of which displayed an MDR phenotype, and the only species for which higher biofilm formation was statistically associated with persistence of infection. No differences were seen with *P. aeruginosa* or *E. coli*, and data from *K. pneumoniae* and *S. aureus* were too few to be analyzed. This study provides an important link between the biofilm formation of infecting isolates and persistence of soft tissue wound infection, further supporting the biofilm theory of disease with human clinical data.

1.9 Conclusion

Several decades after being implicated as a pathogenetic mechanism affecting human health, the concept that biofilms can enable the survival of microorganisms in various compartments of the human body, including on the artificial surfaces of prostheses and therapeutic devices, is firmly supported by numerous in vitro studies. In contrast, far fewer studies have demonstrated the manner in which biofilm phenotypes influence

the clinical outcomes of infected human patients. Our understanding of the natural history of this important state of bacterial growth thus remains limited. While biofilms have been demonstrated in vitro in the studies reviewed in this chapter, it is important to note that with rare exception, biofilms were not perfectly correlated with disease. This indicates that although biofilms may be important as a virulence factor in some clinical scenarios, microorganisms can still cause infection in the absence of a biofilm-forming phenotype. Clinical studies which associate biofilm phenotypes of microorganisms as risk factors for adverse clinical outcomes are likely to advance our understanding of the role of biofilms in human disease, while serving to identify targets upon which to focus therapeutic efforts. Standardization of laboratory methods of biofilm characterization would help to ensure meaningful comparison of data across studies and species.

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Biofilm-Based Implant Infections in Orthopaedics

2

Carla Renata Arciola, Davide Campoccia, Garth D. Ehrlich, and Lucio Montanaro

Abstract

The demand for joint replacement surgery is continuously increasing with rising costs for hospitals and healthcare systems. Staphylococci are the most prevalent etiological agents of orthopedic infections. After an initial adhesin-mediated implant colonization, *Staphylococcus aureus* and *Staphylococcus epidermidis* produce biofilm. Biofilm formation proceeds as a four-step process: (1) initial attachment of bacterial cells; (2) cell aggregation and accumulation in multiple cell layers; (3) biofilm maturation and (4) detachment of cells from the biofilm into a planktonic state to initiate a new cycle of biofilm formation elsewhere. The encasing of bacteria in biofilms gives rise to insuperable difficulties not only in the treatment of the infection, but also in assessing the state and the nature of the infection using traditional cultural methods. Therefore, DNA-based molecular methods have been developed to provide rapid identification of all microbial pathogens. To combat biofilm-centered implant infections, new strategies are being developed, among which anti-infective or infective-resistant materials are at the forefront. Infection-resistant materials can be based on different approaches: (i) modifying the biomaterial surface to give anti-adhesive properties, (ii) doping the material with antimicrobial substances, (iii) combining anti-adhesive and antimicrobial effects in the same coating, (iv) designing materials able to oppose biofilm formation and support bone repair.

C.R. Arciola (✉) • L. Montanaro (✉)
Research Unit on Implant Infections, Rizzoli Orthopaedic Institute, Bologna, Italy

Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy
e-mail: carlarenata.arciola@ior.it; lucio.montanaro@unibo.it

D. Campoccia
Research Unit on Implant Infections, Rizzoli Orthopaedic Institute, Bologna, Italy

G.D. Ehrlich
Center for Genomic Sciences, Institute for Molecular Medicine and Infections Disease, Drexel University College of Medicine, Philadelphia, PA, USA

Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA, USA

Department of Otolaryngology, Drexel University College of Medicine, Philadelphia, PA, USA

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2.1 Projections of Increase in Arthroplasty Numbers

The demand for joint replacement surgery is continuously increasing resulting in higher and higher costs for patients, hospitals and healthcare systems.

The expansion of arthroplasty surgery and the need to follow its outcome and control booming costs have promoted the institution of many registries at the regional, national and international levels.

Historical trends in total hip replacement drawn from the Swedish Hip Arthroplasty Register over the 45 year period, from 1968 to 2013, can interestingly provide some of the most accurate data in the world and can be used to provide projections of the future demand for arthroplasties (www.shpr.se). Similarly the National Joint Registry (NJR) of England, Wales and Northern Ireland, which has now published the 9th Annual Report 2012 (www.njrcentre.org.uk) provides data on all hip, knee, ankle, elbow and shoulder joint replacements across the National Health System and independent healthcare sector, thus providing trending data and providing for future projections.

Accurate statistical analyses of the data in the Swedish Hip Arthroplasty Register and projections to 2030 of total hip replacement in Sweden have been recently published (Nemes et al. 2014). The authors of this report have utilized two types of regression analysis, in order to forecast the incidence of THR operations per 10^5 Swedish residents aged 40 years or older in the decades after 2012 and to estimate the maximum incidence per 10^5 Swedish residents aged 40 years. If a Poisson regression analysis is used, which estimates the expected number of THRs per year and assumes a continuous growth, the incidence can reach, at least theoretically, 10^5 of 10^5 persons and, if the results are used for projections, unreasonably high numbers are reached. Secondly, a regression framework that assumes the existence of an upper threshold, i.e. an asymptote that depicts the forecasted maximum incidence, was adapted. Poisson regression should forecast that the incidence of THRs would increase exponentially in the next years, with a predicted incidence of 784 total hip replacements per 10^5 Swedish residents in 2030 and 1,133 in 2040. With an expected Swedish population in 2030 of 10,660,344 persons, about 83,600 total hip replacements can be forecasted, in respect to the 16,021 THRs performed in 2013, with a fivefold increase of incidence. The projections based on asymptotic modeling, gives instead a THRs expected number of 20,152, only 1.25-fold increase of incidence in respect to 2013.

Sometimes the registries announce good news. The National Joint Registry for England and Wales, 9th annual report 2012, reports that, among the 409,096 patients operated on for primary hip replacement during 8 years, 1,743 patients died within 90 days of surgery, with a substantial decrease in mortality, from 0.56 % in 2003 to 0.29 % in 2011, even after adjustment for age, sex, and comorbidity (Hunt et al. 2013). Based on the registered clinical data, the authors ascribe the decreased mortality to several clinical factors: posterior surgical approach, mechanical thromboprophylaxis, chemical thromboprophylaxis with heparin (with or without aspirin), spinal versus general anesthetic, while the type of prosthesis was unrelated to mortality. The authors do not report data about infections, except the observation that only 19 patients had AIDS/HIV infection and none died.

A symposium report developed from the 9th Report of the National Joint Registry for England and Wales, found that a total of 8,639 hip revisions were reported in 2011, among which 87 % were single-stage revisions, 12 % were two-stage revision and less than 1 % removal of the prosthesis. The infection was the indication for revision in 12 % of cases but in 11 % of cases an adverse soft tissue reaction was recorded, confirming that the failure of metal-on-metal replacement is a rising cause of revision, while the lowest rates of revision were associated with cemented metal or ceramic on polyethylene combinations. With regard to knee replacement, a total of 5,135 revision operations were performed in 2011, the main indications being aseptic loosening (35 %) and infection (23 %).

2.2 Infection Burden for Arthroplasties

As Sculco wrote more than 30 years ago "Infection in total joint replacement is a devastating and life-threatening complication for the patient. It can also be an economic disaster for hospitals that treat large numbers of these patients" (Sculco 1993).

The infection burden, as a proportion of the total number of primary and revision total hip arthroplasties (THA), has been reported to have increased from 0.66 % in 1990 to 2.18 % in 2009 (Kurtz et al. 2008, 2012).

The incidence of post-operative infections has also been calculated for total knee arthroplasties, which are often managed with two-stage revisions, and ranges from 0.7 to 2.4 % (Kurtz et al. 2012; Whitehouse et al. 2002).

The rates of periprosthetic joint infection (PJI) after primary procedures range from 1 to 9 %, depending on the types of arthroplasty, being less than 1 % in hip and shoulder prostheses, about 2 % in knee prosthesis, and about 9 % in elbow prosthesis. The rates of PJI reach the significantly higher level of about 40 % after revision procedures (Corvec et al. 2012).

The current cumulative annual cost of revisions for periprosthetic joint infections has been estimated to exceed $566 million in the United States and is expected to exceed $1.6 billion by the year 2020 (Kurtz et al. 2012). According to the projections that the number of total knee arthroplasty procedures are yearly increasing, the projected cost of managing these surgical site infections is expected to become a huge problem for patients, physicians, and healthcare institutions (Kapadia et al. 2014).

The authors of population-based studies hypothesize stability in the incidence of infections over the nearly 40-year time span. This hypothesized stability is tentatively ascribed to the increased patient morbidity and risk factors for infection counterbalanced by improvements in aseptic techniques, surgical skills, and infection prevention and control measures (Tsaras et al. 2012).

Whether the infection incidence per person-joint-years is increasing or not, the total number of periprosthesis infections will certainly increase in the ensuing decades, owing to the increasing number of primary implants being performed and the cumulative number of arthroplasties that remain in place (Tande and Patel 2014) for longer periods of time.

2.3 Current Classification of Prosthetic Infections

The American Association of Orthopedic Surgeons divides prosthetic infections into the following four types (Leone and Hanssen 2006):

Type 1 (positive intraoperative culture): two intraoperative cultures turning out positive;

Type 2 (early postoperative infection): infection occurring within the first month after surgery;

Type 3 (acute hematogenous infection): hematogenous seeding of site of previously well-functioning prosthesis;

Type 4 (late chronic infection): chronic indolent clinical course; infection present for more than 1 month.

A slightly different classification of prosthetic joints infections has been proposed by Zimmerli et al. (2004):

Early (those that develop less than 3 months after surgery);

Delayed (3–24 months after surgery), or;

Late (more than 24 months after surgery).

For the Total Hip Arthroprosthesis three main types of postoperative surgical site infection are considered, with a classification almost superimposable to the Zimmerly-Trampuz's classification: (1) acute postoperative (early onset), appearing within 3 months postoperatively; (2) delayed deep, appearing 3–12 months postoperatively; and (3) late hematogenous, appearing more than 1 year postoperatively (Fitzgerald 1995; Lindeque et al. 2014).

The etiopathogenic significance implied by these classifications resides in that the early and delayed infections are acquired by contamination at the time of surgery, while the delayed being caused by less virulent microorganisms so that

the onset of infection occurs not before the first 3 months. Late infections, occurring between 12 and 24 months after surgery, are often due to a haematogenous infection or, less frequently, to an indolent infection acquired at surgery time.

2.4 Epidemiology of Periprosthesis Infections in Our Experience

Approximately a decade ago, we studied a collection of 1,027 clinical isolates from 699 orthopedic patients with surgical infections (Arciola et al. 2005). We compared the etiology of infections associated with medical devices (MDs) to those developed in the absence of implant materials (no MDs). MDs included infections associated to knee and hip prostheses, external and internal fractured bone fixation systems, materials for tendon and ligament reconstructions and other orthopedic implant materials.

The isolates from infections associated with medical devices accounted for over 70 % of all the bacteria consecutively isolated from orthopedic infections of patients referred to the specialized hospital Rizzoli Orthopaedic Institute. Among these microorganisms 775 (75.5 %) were identified as belonging to the *Staphylococcus* genus, 82 (8 %) to the *Enterobacteriaceae* family, 75 (7.3 %) to the *Pseudomonas* genus, 54 (5.3 %) to the *Enterococcus* genus and 20 (1.9 %) to the *Streptococcus* genus.

Staphylococcal species were found to be the most prevalent etiological agents of orthopedic infections, representing 75.5 % of all strains, ranging from 68.3 % in infections without MDs to 78.1 % of the isolates with MDs. Among the species belonging to the *Staphylococcus* genus, *S. aureus* generally exhibited the highest prevalence (35.5 % overall prevalence, 33.8 % in MDs and 40.3 % in the no MD group). The overall prevalence of *S. epidermidis* was 27.5 %, ranging from 16.5 % in infections without MDs to 31.5 % of the isolates with MDs, as in infections associated with fracture fixation devices and in pelvis surgery. No single bacterial species, except for *S. aureus* and *S. epidermidis*, exceeded a frequency of 7 %, giving emphasis to the critical importance of these two species in the epidemiology of orthopedic infections. However, it should be noted that cultural methods often miss anaerobes, fastidious pathogens, and organisms with long interval doubling times (Costerton et al. 2011).

We have analyzed an up-dated collection of isolates recovered from 242 orthopedic patients covering the period between 2007 and 2011. The results of the new survey confirmed those of the previous epidemiological investigation. Again, the prevalence of staphylococci in the entire collection was approximately 75 %, slightly higher in the case of MDs (82.3 %) and lower for no MDs (65.4 %). *S. aureus* again represented approximately 35 % of all the isolates and *S. epidermidis* 29.9 % of all the isolates. When these data were analyzed for the presence of MDs, unlike the previous collection, a prevalence of *S. epidermidis* (39.0 %) over that of *S. aureus* (31.7 %) was found in infections associated with MDs (Montanaro et al. 2011a).

In a very recent look at epidemiologic data on orthopedic infections treated between February 2011 and May 2014 at the Rizzoli Orthopaedic Institute, the *Staphylococcus* genus is always the leading etiologic agent, but the slight prevalence of *S. epidermidis* over *S. aureus* does not seem to be confirmed. Figure 2.1 shows up-to-date findings analyzed as a function of the absence or presence of MD and the type of surgery. Infections without MD represent 31.1 % and polymicrobial infections 9.4 %.

2.5 The Steps of Infection: From First Adhesion on Implant Materials to Biofilm Production

Surface adhesion of bacteria to implant surfaces is the initial step in the pathogenesis of implant-related biofilm infections, initiating the colonization of biomaterial surfaces. During the first step, the initial interactions between bacteria and a biomaterial are nonspecific in nature and driven by different forces, as hydrophobic, electrostatic

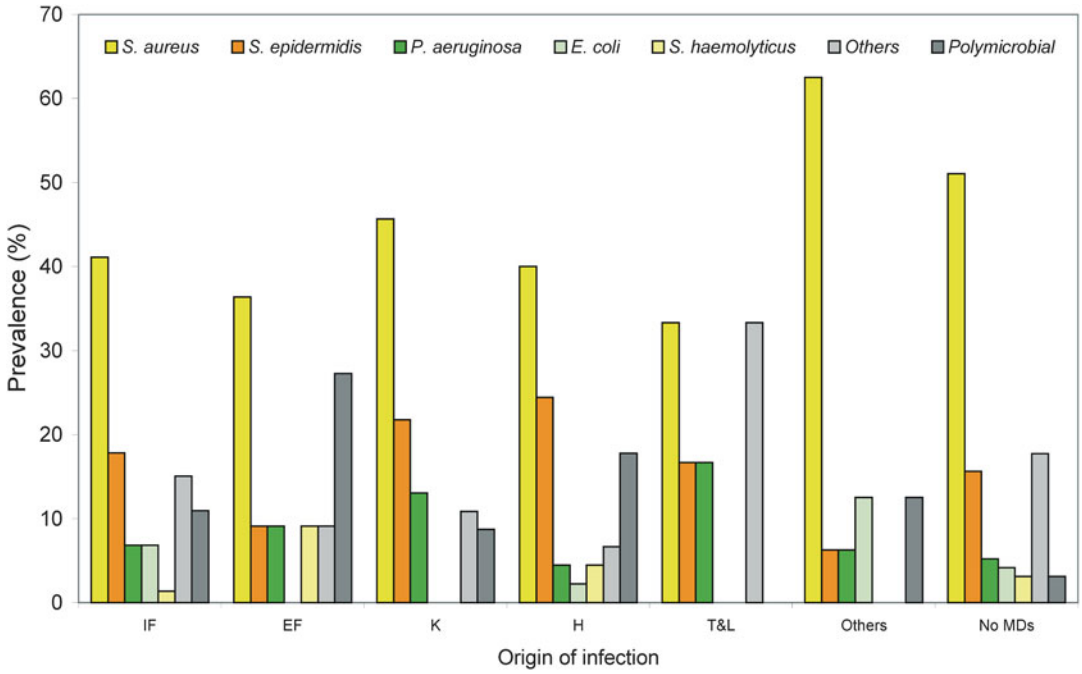

Fig. 2.1 Prevalence of the most frequent pathogens as a function of the origin of the orthopedic infection in a collection of 338 clinical isolates obtained from 309 patients in the period February 2011–May 2014 (29 polymicrobial infections) (*IF* Internal fixation, *EF* External fixation, *K* Knee, *H* Hip, *T&L* tendons and ligaments, *Others* other medical devices, *No MDs* no explicitly reported presence of medical devices at the site of infection (Arciola CR, Campoccia D, Cangini I, Montanaro L, unpublished results))

and Van der Waals forces. In this phase, bacteria are therefore passively adsorbed onto the material surfaces. In addition to this passive adhesion, specific proteins have been identified that mediate the binding to the abiotic surfaces, the autolysins, first described by Heilmann et al. (1997). Autolysins possess a double function: enzymatic (being peptidoglycan hydrolases) and adhesive. In *S. epidermidis*, the major autolysin/adhesin is AtlE, a 148 kDa protein, which mediates attachment to polystyrene. In *S. aureus*, the autolysin/adhesin is AtlA, a 137 kDa protein, highly homologous to AtlE (Foster 1995). Both AtlA and AtlE, in particular their glycine-tryptophane dipeptide repeats, are involved not only in surface association and biofilm production but also in a novel mechanism of staphylococcal internalization by host cells (Hirschhausen et al. 2010).

Passive bacterial adsorption spontaneously occurs on material surfaces, but active stable anchorage of the bacterial cells is established by adhesins, which bind to host proteins adsorbed on the implant surface following exposure to physiologic fluids. Therefore, in the early phases of infection, adhesins play a primary role, acting even as invasins and, furthermore, intervene in the process of bacterial internalization into host cells.

S. aureus harbors approximately 50 accessory genes, encoding for factors either secreted or expressed on the bacterial surface, all having a function in pathogenesis (Sittka and Vogel 2008). Among them, adhesins are an important group of virulence factors responsible for interactions between microbial cells and host cells and extracellular matrix (ECM).

S. aureus adhesins comprise the cell wall-anchored microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Patti et al. 1994a; Speziale et al. 2009), as well as the secretable expanded repertoire adhesive molecules (SERAMs), ionically rather than covalently

associated to the bacterial cell wall (Chavakis et al. 2005). MSCRAMMs are receptorial proteins anchored to the bacterial cell wall through a typical cell wall signal LPXTG motif. The *S. aureus* enzyme sortase A, located on the extracellular side of the membrane, cleaves the LPXTG anchor motif, covalently anchoring the adhesin to the cell wall peptidoglycan. Bound to the peptidoglycan and exposed on the bacterial surfaces, MSCRAMMs recognize specific host extracellular matrix (ECM) proteins. Through the interaction with ECM proteins, certain MSCRAMMs, acting as invasins, can facilitate the process of internalization into host cells. Other MSCRAMMs mediate bacterial cell accumulation, contributing to biofilm formation by means of accretion as opposed to the classical formation via elaboration of exopolysaccharides typical of most *S. aureus* strains and associated with the expression of an *ica* locus encoding for the polysaccharide intercellular adhesin (PIA).

2.6 Prevalence of Adhesin Genes in Collections of Clinical Isolates from Periprosthesis Infections

S. aureus MSCRAMMs play an important role in various processes of infection pathogenesis such as tropism, invasion, intracellular penetration; and, in the peri-implant tissues, bacterial adhesion on biomaterials coated by host extracellular matrix (ECM) proteins. Many adhesins involved in adhesion on indwelling devices appear multifunctional, as fibronectin-binding proteins A and B (FnBPA and FnBPB) and clumping factors A and B (ClfA and ClfB) (Greene et al. 1995; Herrmann et al. 1993), which bind more than one specific ligand. FnBPA in addition to fibronectin can bind fibrinogen and elastin, and ClfA binds fibrin besides the fibrinogen γ-chain. Moreover, binding of fibronectin by FnBPs was found to be crucial in the invasion of eukaryotic cells, where the ECM protein serves as a bridging molecule between the adhesin and the integrin $\alpha_5\beta_1$ (Sinha et al. 1999; Hauck and Ohlsen 2006), enabling the internalization of the bacteria within the cells.

The FnBP-fibronectin internalization mechanism into osteoblast cells is thought to trigger apoptosis, osteolysis and, ultimately, destructive osteomyelitis (Arciola et al. 2012a; Montanaro et al. 2011b).

An interesting subgroup of MSCRAMMs characterized by the Serine-Aspartate repeat (Sdr) proteins, among which SdrE, and the bone sialoprotein-binding protein (Bbp). Bone sialoprotein, the binding target of Bbp, is an ECM highly glycosylated and sulphated phosphoprotein that is found almost exclusively in mineralized connective tissues (Ganss et al. 1999), where it represents 10 % of the non-collagenous proteins of the matrix, being mostly synthesized in osseous tissue. Bone sialoprotein-binding capacity, together with collagen-binding capacity, was found in all staphylococci associated with septic arthritis (Patti et al. 1994b; Ryde'n et al. 1997), thus suggesting that Bbp and Cna could represent important virulence factors.

In our collection of 200 *S. aureus* isolates from orthopedic implant-associated infections, categorized by genotyping by a RiboPrinter® and dendrogram analysis, an epidemic cluster has been identified. In this predominant ribogroup, consisting of 27 isolates, the *bbp* gene encoding bone sialoprotein-binding protein appeared to be an important virulence trait, found in 93 % of the isolates. The *bbp* gene was instead found in just 10 % of the remaining isolates of the collection. In this epidemic cluster, co-presence of *bbp* with the *cna* gene, encoding collagen adhesion, was a pattern consistently observed (Campoccia et al. 2009).

The same collection of 200 *S. aureus* isolates from orthopedic implant infections was also typed for their *agr* groups, and screened for the presence of adhesin and leukotoxin genes. Interestingly, specific virulence gene patterns emerged in association with *agr* groups. The *agr* groups I and II, were associated with the presence of *sdrE*, *fib* (*agr* II more than *agr* I), *fnbB* (*agr* I more than *agr* II), and *lukE/lukD* (*agr* II more than *agr* I). The third most frequent *agr* group, *agr* III, differed clearly from *agr* I and II, exhibiting high prevalence of *bbp*, generally not harbored by *agr* I and II, and copresence of *bbp* with *cna* (Montanaro et al. 2010). These studies

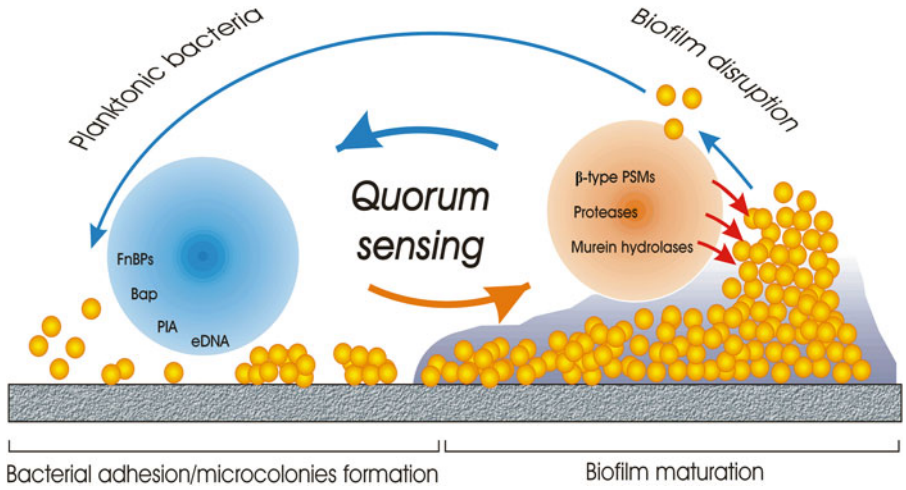

Fig. 2.2 Simplified view of the four-step process of biofilm formation in *Staphylococcus aureus*. Planktonic cells adhere and anchor to the material substrate through adhesins such as the fibronectin-binding proteins (FnBPs), also implicated in the following steps of biofilm formation. Factors playing a fundamental role in biofilm formation include the biofilm-associated protein (Bap), the intercellular polysaccharide adhesin (PIA) and a series of other polymeric extracellular substances among which extracellular-DNA (eDNA). Once a mature biofilm has formed, under the fine control of the *Quorum sensing* system, enzymes such as proteases and murein hydrolases, and phenol-soluble modulins (β-type PSMs) with surfactant-like properties determine the disruption and detachment of the biofilm leading to the release of bacteria in the planktonic phase

indicate that specific adhesins may synergistically act in the onset of implant-related infections and that anti-adhesin strategies should be usefully targeted to adhesins conjointly present.

2.7 Biofilm: Role of Biofilm in Implant Infections

After the initial adhesin-mediated implant colonization, *S. aureus* and *S. epidermidis* produce biofilm. Biofilm is a structured consortium of bacteria, which encase themselves in an extracellular matrix and firmly stick them to the implant surface (Fig. 2.2).

Biofilm formation is classically viewed as a four-step process: (1) initial attachment of bacterial cells; (2) cell aggregation and accumulation in multiple cell layers; (3) matrix elaboration and biofilm maturation and (4) detachment of cells or rafts from the biofilm into a planktonic state or flock to initiate a new cycle of biofilm formation elsewhere (Costerton et al. 2005; Mack et al. 2004).

During the second step, the biofilm is progressively established on the colonized surface. Then, in the subsequent step, the maturation of biofilm takes place and characteristic structural features of the biofilm, specific for the bacterial species, are developed. During the final stage, the bacteria previously encased and protected in the biofilm structure return to their initial planktonic form of life, ready for a new invasive phase. Bacterial detachment and dispersion therefore characterize this final step of the bacterial life cycle (Arciola et al. 2012b).

2.8 Biofilm Structural Components: The Extracellular Polymeric Substance

Composition, structure, formation and regulation of the *Staphylococcus* biofilms have been illustrated and discussed by Arciola et al. (2012b) and by Speziale et al. (2008) in dedicated reviews to which the readers could be refer for an in-depth treatise.

In staphylococcal orthopedic infections, the extracellular polymeric substance of the biofilm is composed of polysaccharides, proteins, and extracellular DNA.

2.8.1 PIA and *ica* Locus

The principal polysaccharide of the staphylococcal biofilm matrix is a linear homoglycan composed of at least 130 residues of β-1,6-linked *N*-acetylglucosamine, partially deacetylated (15–20 % of the residues) and therefore positively charged. This polysaccharide was initially discovered and characterized in *S. epidermidis* (Mack et al. 1996) where its biosynthesis is encoded by the intercellular adhesion (*icaADBC*) locus (Heilmann et al. 1996). For a long time the *ica* locus was considered a virulence determinant peculiar to *S. epidermidis* strains responsible for catheter- or indwelling device-related infections. Later the presence of the *ica* locus was documented in the *S. aureus* species (Cramton et al. 1999), and recognized also in clinical isolates of *S. aureus* from catheter-associates infections (Arciola et al. 2001).

The product of the *icaA* gene is a transmembrane protein with a *N*-acetylglucosaminyltransferase activity that synthetizes short PIA oligomers from UDP-*N*-acetylglucosamine as substrate. The product of the *icaD* gene is required for the optimal efficiency of IcaA. The product of *icaC* is involved in externalization of the nascent polysaccharide. The product of *icaB* is an *N*-deacetylase, responsible for the partial deacetylation of the *N*-acetylglucosamine polymer.

2.8.2 *ica*-Independent Biofilm Production

Besides the demonstration of the important role of the *icaADBC* operon and of the PIA components in the biofilm extracellular polymeric substance, new evidence highlights the existence of *ica*-independent mechanisms involved in biofilm formation both in *S. aureus* and in coagulase-negative staphylococci, in particular *S. epidermidis* and *S. lugdunensis* (O'Gara 2007).

This alternative mechanism of biofilm synthesis relies on the ability of *S. aureus* to express a variety of adhesion proteins that favor the attachment of bacterial cells to many different surfaces. These proteins, which are anchored to the cell-wall of *S. aureus*, maintain a cell-to-cell interaction inside the biofilm. Among the adhesive proteins that are implicated in biofilm formation, an important role is played by a biofilm-associated protein termed Bap, which was demonstrated to be essential both for both initial adherence and for intercellular accumulation during biofilm development of *S. aureus* strains isolated from bovine chronic mastitis infections (Cucarella et al. 2001).

The *bap* gene is present in other *Staphylococcus* species, including *S. epidermidis*, *Staphylococcus chromogenes*, *Staphylococcus xylosus*, *Staphylococcus simulans*, and *Staphylococcus hyicus* (Tormo et al. 2005).

While the *S. aureus bap* gene has been detected only in strains isolated from bovine mastitis and never in strains isolated from human infections, in coagulase negative staphylococci (CoNS) the presence of the *bap* gene has been found in clinical isolates from human nosocomial infections in Brazilian hospitals (Potter et al. 2009). Thus, the role of Bap in human infections, at present, seems to be limited to CoNS species and its presence in *S. aureus* strains isolated from human infections has not been confirmed.

With regard to orthopedic infections, Rohde et al. have investigated the presence and expression of biofilm-associated genes in clinical isolates of *S. aureus* and *S. epidermidis* from total hip and total knee infected arthroplasties. All *S. aureus* strains and nearly 70 % of *S. epidermidis* strains produced biofilm. Among the *S. epidermidis* biofilm-producing strains, 27 % were PIA-independent and at least in part involved the expression of the accumulation associated protein (Aap) (Rohde et al. 2007).

In *S. aureus* other surface proteins are involved in the formation of biofilm. Among these SasG has been shown to promote formation of biofilm. This protein exerts its action during the biofilm accumulation phase when, in the presence of physiological concentrations of Zn^{2+}, it supports cell-to-cell interactions (Geoghegan et al. 2010).

Moreover, the fibronectin-binding proteins (FnBPs) were demonstrated to be part of the proteinaceous component of biofilm formed in the presence of glucose, while a PIA/PNAG-dependent biofilm was shown to be produced under osmotic stress conditions (Vergara-Irigaray et al. 2009; Houston et al. 2011). There is therefore evidence that *S. aureus* can modulate its metabolism switching from the production of a proteinaceous to an exopolysaccharidic biofilm matrix, as an adaptation to the external conditions.

2.8.3 Extracellular DNA in Biofilm

Another biofilm matrix component, recently attracting attention, is the extracellular DNA (eDNA), which has been shown to be important for biofilm structural stability. Starting from the observations of Arciola et al. on strong biofilm production by epidemic clones of *Enterococcus faecalis* (Arciola et al. 2008), Thomas et al. have described the relationship between DNA release, role of proteases and biofilm production in *E. faecalis* (Thomas et al. 2008).

After having given evidence that the mechanisms underlying eDNA production is autolysis, they advanced the concept of two modes of autolysis: an altruistic suicide and a fratricide killing of different sub-populations of bacterial cells. In *S. aureus* altuistic suicide predominates, in which *altruist* cells commit suicide by programmed cell death (a process similar to apoptosis in eukaryotic cells), for the common sake of the larger community with salvage of *survivor* cells. In *E. faecalis*, *Bacillus subtilis* and *Streptococcus pneumoniae* the fratricide mechanism prevails: *attacker* cells release killing factors (a process similar to necrosis in eukaryotic cells) that destroy *target* cells. The *attackers* themselves are protected from self-destruction by specific immunity proteins they express (Thomas and Hancock 2009).

The mechanisms of eDNA production have been thoroughly investigated in *Pseudomonas aeruginosa*, in which eDNA originates by lysis of a bacterial subpopulation. Lysis is controlled by *quorum sensing* systems, based on acyl homoserine lactone (AHL) and on *Pseudomonas* quinolone signaling (PQS) (Allesen-Holm et al. 2006). In *S. epidermidis*, eDNA is a major component required for initial bacterial attachment to surfaces, as well as for the subsequent early phase of biofilm development. In this case too, eDNA originates from lysis of a small subpopulation of the *S. epidermidis* bacteria. DNA release from *S. epidermidis* appears to be mainly mediated by the autolysin protein AtlE, since inactivation of *atlE* drastically reduced DNA release (Qin et al. 2007).

The presence of eDNA in biofilms accomplishes three important roles, which are treated in (Montanaro et al. 2011c):

(i) Stabilization of the biofilm matrix, as demonstrated by the effect of DNase I in preventing the formation of a stable biofilm and in impairing the attachment of bacterial cells to culture flow-chambers.

(ii) Part of gene-transfer mechanisms. Extracellular DNA present in bacterial biofilm communities constitutes a dynamic gene pool from which bacteria competent for natural transformation can derive genetic information by horizontal gene transfer (Ehrlich et al. 2005, 2010). The impact of horizontal gene transfer is exemplified by bacterial acquisition of virulence traits and antimicrobial drug resistance.

(iii) Conditioning of the innate immune response, prevention of phagocytosis, and attenuation of inflammation. The components of biofilm matrix (eDNA, proteins and exopolysaccharides) are microbial structural motifs recognized by the innate immune system via the TLR family of pattern recognition receptors (PRRs). Upon phagocytosis and digestion of *S. aureus* in the phagosome, bacterial DNA is liberated and engages TLR9. TLR9-dependent activation can be triggered not only by phagocytosis of whole *S. aureus* cells but also by that of extracellular DNA, extensively contained in the biofilm matrix. After TLRs engagement, the behavior of immune response appears different between biofilm-encased and planktonic bacteria. Turlow et al. have demonstrated that *S.*

aureus biofilms actively attenuate classical antibacterial immune responses, inducing a significant reduction in cytokine/chemokine production in biofilm infected tissues (Thurlow et al. 2011).

2.9 The Problem of Etiological Diagnosis in Biofilm Infections

The encasing of bacteria in biofilms gives rise to insuperable difficulties not only in the treatment of the infection owing to the high antibiotic resistance of bacteria embedded in biofilm, but even in assessing the state and the nature of the infection (Costerton et al. 2003). The traditional culture methods turn out often to be inefficacious in reaching a proper diagnosis of the microbial species responsible for the infection (Ehrlich et al. 2012). The only laboratory techniques approved by the U.S. Food and Drug Administration to detect and identify bacteria responsible for human infections are cultures, which necessarily depend on the ability of bacteria to grow and produce visible colonies when seeded on the surfaces of appropriate agar plates. However, this 100-year-old technology is able to detect, under ideal circumstances, only one or two out of dozens of bacterial species that may be present in a wound. The agar plate culture technique may fail completely in the detection of bacteria present in very large numbers in orthopedic infections (Wolcott and Dowd 2011; Costerton and DeMeo 2011).

In implant infections, particularly in orthopedics, a rapid and sensitive identification of the etiological agents is mandatory for undertaking efficacious therapeutic measures. The accurate assessment of the infecting pathogen and its identification at species- and strain-level are needed (Ehrlich and Post 2013) to establish the virulence potential, the antibiotic resistance profiles, and to predict the biofilm-forming capacity in order to optimize appropriate therapeutic approaches. Therapeutic measures can go from local and systemic antibiotic therapy, to surgical debridement, and lastly to the removal and replacement of the implant.

Classically, methods used to diagnose prosthesis-related infections start with the in vitro culture of bioptic samples taken from periprosthetic tissues, to ascertain any bacterial growth. The definite characterization of an infection as a biofilm infection should be based on the microscopic demonstration of matrix-embedded bacterial colonies in affected tissues, but, for routine clinical use, this diagnostic procedure is invasive, costly and time intensive.

In the field of biofilm-centered implant infections, these classical culture methods, developed for acute infective diseases caused by planktonic bacteria, have encountered rising skepticism. Etiological diagnosis is seriously limited by the frequent failures in detaching and collecting biofilm cells from infected tissues and in culturing them on agar, since planktonic bacteria produce colonies on agar, whereas biofilm-forming bacteria do not.

The difficulties or even the impossibility to isolate the bacterium responsible for an implant infection often leads to the greatly abused diagnosis of "aseptic loosening", even in cases in which clinical signs of infection clearly exist, with the serious consequence to fail rational basis for the therapy (Jacovides et al. 2012).

Thus, DNA-based molecular methods not relying on cultural methods have been developed to provide rapid identification of all microbial pathogens.

Benefits and limits of molecular methods for etiological diagnosis and for identification of virulent strains have been discussed by Arciola et al. (2011).

The new advanced technologies for rapid bacteriological identification demonstrate a shift from the traditional biochemical and molecular testing methods towards those using mass spectrometry (MS) for the analysis of microbial proteins and genetic elements (Ehrlich et al. 2014)

Costerton and his colleagues have in-depth reviewed the plethora of molecular techniques that could replace cultures in the diagnosis of bacterial diseases and have identified the new IBIS technique that is based on base ratios (not base sequences), as the molecular system most likely to fulfill the requirements of routine diagnosis in orthopedic surgery (Costerton et al. 2011).

Another mass spectroscopy-based technology, MALDI-TOF, has earned some diagnostic interest, but this technology, while rapid and useful for species identification (Harris et al. 2010), nevertheless requires, in the first step of analysis, a colony plating and, thus, suffers from all of the disadvantages of the microbial culture approach (Arciola et al. 2011).

2.10 Clinical Diagnosis of Periprosthetic Infections

Together with diagnostic molecular methods, highly sensitive and specific biochemical and hematological markers are searched, which can be applied to both serum and joint fluid aspirate for early diagnosis.

Recent reviews have scrutinized current research efforts in the field of these markers, to evaluate their features and their positive or negative predictive values in diagnosing implant infections (Rak et al. 2013; Hansen et al. 2012). C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), white blood cells (WBC), and leukocyte esterase (LE) are the markers studied for their sensitivity, specificity, and positive and negative predictive values in diagnosing periprosthetic joint infections (PJI) (Parvizi et al. 2011).

According to the majority of Authors, a clinical diagnosis of PJI can be suspected when at least of one of the following criteria is present: (i) cutaneous sinus tract communicating with the prosthesis, (ii) visible purulence around the prosthesis, (iii) histopathological characteristics of acute inflammation, (iv) increased leukocyte count and differential in the synovial fluid, or (v) positive culture of the synovial fluid, periprosthetic tissue or sonication fluid cultures (Osmon et al. 2013).

None of the routine blood tests, including WBC, ESR, CRP and procalcitonin is sufficiently sensitive or specific enough to diagnose or exclude a PJI with high accuracy. In particular, normal values of ESR or CRP do not exclude PJI, especially in cases of low-grade infection. Moreover, ESR and CRP usually increase after surgery and reflect post-interventional inflammation. Therefore, rather than a single value, serial post-operative measurements are needed for accurate interpretation (Zimmerli et al. 2004).

The investigation of synovial fluid is more helpful than blood. The synovial-fluid leukocyte count is highly sensitive and specific for infection. Additional tests in synovial fluid, such as glucose, lactate or CRP were not shown to bring additional information regarding the diagnosis of infection (Schinsky et al. 2008).

2.11 Sonication of Removed Implants

Sonication of the removed prosthesis, followed by culture of the sonication fluid is a method of diagnosing implant-associated infections reported few years ago. Sonication of explanted prosthetic components in bags for diagnosis of PJI was first associated with risk of contamination due to bag leakage and subsequent risk of microbial contamination, especially due to non-fermentative, Gram-negative bacilli (Trampuz et al. 2006).

Nevertheless, the culture sensitivity of sonication fluid is superior to that of standard periprosthetic tissue (75 % versus 54 %, respectively (Corvec et al. 2012).

A cutoff of 50 colony-forming units (CFU)/ml of sonication fluid yields a sensitivity of 79 % and a specificity of 99 % for the diagnosis of PJI based on a study involving 331 patients with total knee prostheses or hip prostheses (Puig-Verdié et al. 2013).

Sonication is mainly recommended when an implant failure does not have clear signs of infection and in patients with delayed implant failure. In early failure, culture of fluid obtained by sonication is not superior to culture of peri-implant tissues for the diagnosis of infection and, therefore, is not recommended as a routine diagnostic test in these patients (Esteban et al. 2014).

In conclusion, sonication of the implant increases the sensitivity of the culture of periprosthetic tissues and is being increasingly adopted by many centers. Molecular diagnostic

methods compared with intraoperative tissue culture, especially if combined with sonication, have a higher sensitivity, a faster turnaround time and are not influenced by previous antimicrobial therapy. However, molecular methods still lack a system for detection of antimicrobial susceptibility, which is crucial for an optimized and less toxic therapy of periprosthetic joint infections (Esteban et al. 2014).

Recently, Parvizi et al. have discussed the problem of negative results of culture methods in the diagnosis of periprosthetic joint infections, when many clinical signs indicate an infection. According these Authors, the most important reason is the administration of antibiotics prior to obtaining culture samples. In the presence of a suspect of infection, antibiotics should not be given until the diagnosis is confirmed. Alternatively, aspiration of the joint should be delayed for at least 2 weeks after the last dose of antibiotics. Different and appropriate technical suggestions are given in the cited Parvizi's article in order to enhance the likelihood of obtaining a positive result, including biomarkers and molecular techniques (Parvizi et al. 2014).

How the embedding of causative microorganisms in a biofilm is responsible for the negative culture diagnosis comes from the clear lesson of Bill Costerton, and his suggestion to recourse to advanced molecular methods instead of culture procedures has been highlighted by Ehrlich and Arciola (2012).

2.12 Future Perspectives: Infection-Resistant Materials

Among the new strategies to combat biofilm-centered implant infections, antibiofilm agents, able to inhibit biofilm formation or disrupt formed biofilm, are subjects of extensive researches, and this item is treated in other chapters of the book.

Especially in orthopedics, the recourse to anti-infective or infective-resistant materials is at the forefront in the biomaterial science.

Achievement of infection-resistant materials can be based on different approaches: (i) modification of the biomaterial surface to give anti-adhesive properties, (ii) doping the material with antimicrobial substances, (iii) combining anti-adhesive and antimicrobial effects in the same coating, (iv) realization of materials able to oppose biofilm formation and, at the same time, to support bone repair (Fig. 2.3).

Two recent reviews have surveyed the different approaches for obtaining efficacious infection-resistant materials and the reader could be referred to them for an extensive treatise of this subject (Arciola et al. 2012b; Campoccia et al. 2013).

The first approach is based on adsorption of molecules conferring hydrophilic properties to the material surface and competing with the interaction between bacteria and host matrix proteins that film the implant. Heparin, with its strong hydrophilic properties, has been proposed long ago to be able to hamper adhesion of bacterial cells. Besides acting by increasing hydrophylicity, forming a highly hydrated layer between the bacteria and the surface (Arciola et al. 1993, 1994, 1995, 1998; Legeay et al. 2006), heparin has been proved that can interfere with *S. epidermidis* adhesion by specifically inhibiting the binding of bacterial adhesins FnBPs to fibronectin that film the biomaterial surfaces (Arciola et al. 2003; Bustanji et al. 2003). Bacterial adhesion on implant surfaces can be inhibited by hydrophilic polymeric brushes based on poly(ethylene glycol) (PEG) or poly(ethylene oxide) (PEO). A coating of these highly hydrated polymer chains on a surface inhibits protein and bacterial adhesion (Neoh and Kang 2011).

The second approach is mainly based on the local delivery of antimicrobial agents, in particular antibiotics, through carrier biomaterials. The risk of inducing antibiotic resistance is an intrinsic drawback of the antibiotic-loaded materials (Campoccia et al. 2010). Another antibacterial substance that avoid the limits of antibiotic-loading is the natural cationic polysaccharide chitosan, which besides having an antibacterial action, is a promising biopolymers for tissue engineering.

Recent results by Zhao et al. indicate that chitosan-lauric acid may be successfully

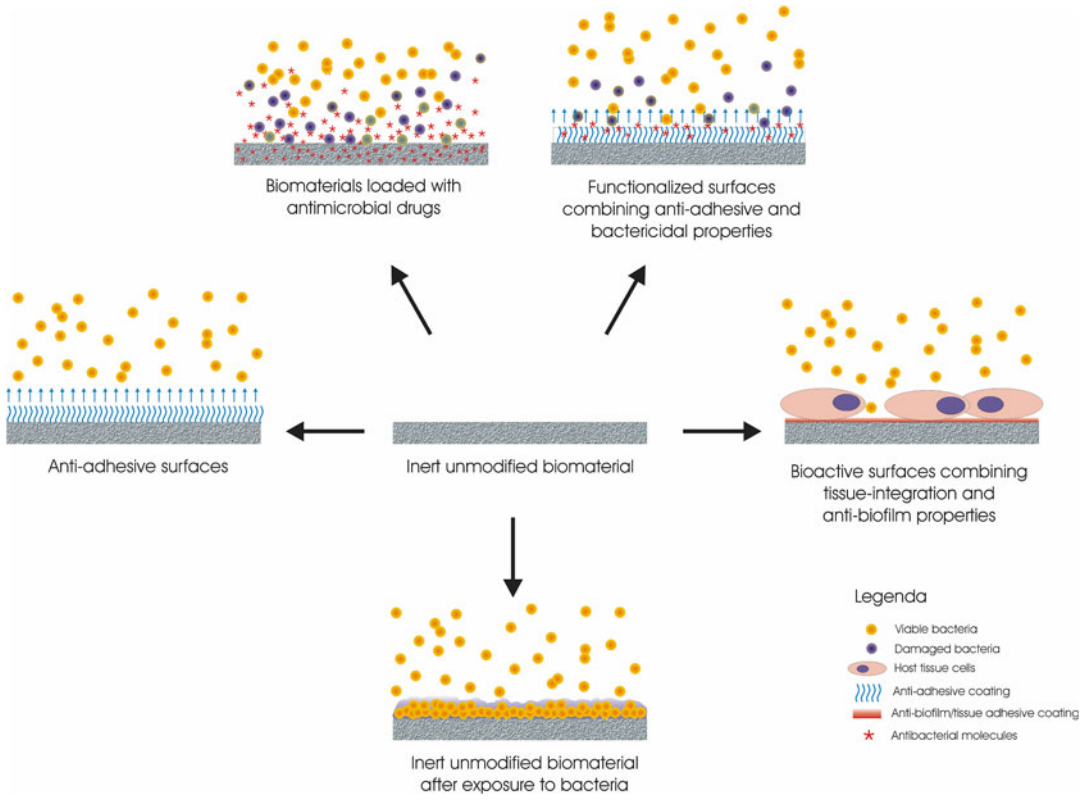

Fig. 2.3 Different strategies to achieve infection-resistant biomaterial surfaces

immobilized onto the surfaces of Ti substrates. The chitosan-functionalized titanium promotes osteoblast cell adhesion, cell viability, intracellular alkaline phosphatase activity and mineralization capacity of osteoblasts. Antibacterial assays against *S. aureus* and *P. aeruginosa* showed that titanium functionalized with chitosan-lauric acid conjugate efficiently inhibited the adhesion and growth of bacteria. The Zhao's study represents a promising approach to fabricate functional Ti-based orthopedic implants, since this surface-modified material enhances the biological functions of osteoblasts and concurrently reduces bacteria adhesion (Zhao et al. 2014).

To the specific aim designed to search for new biomaterials having intrinsic antibacterial properties, able to hamper the formation of a biofilm, new quaternised chitosan derivatives appear promising. PMMA loaded with quaternised chitosan-loaded inhibits surface biofilm formation by antibiotic-resistant staphylococci, more strongly than PMMA alone, gentamicin-loaded PMMA and chitosan-loaded PMMA. Moreover the quaternised chitosan-loaded PMMA markedly down-regulates expression of *ica locus* genes, encoding essential enzymes for biofilm biosynthesis, and also down-regulates the expression of *mecA*, which is responsible for methicillin resistance (Tan et al. 2012).

The third approach is illustrated, as an example, by the multilayer film constructed by assembling layer-by-layer heparin and chitosan, obtaining an antiadhesive and antibacterial biomaterial. This new multilayer material not only reduced the bacterial adhesion but also killed the bacteria adhered onto the surface, proving to be a powerful anti-infective coating (Fu et al. 2005).

The possibility to combine anti-adhesive and antimicrobial effects in the same coating, without recurring to antibiotic-loading is offered by the new evidence of the antimicrobial activity of cationic antimicrobial peptides. These peptides

are an important component of innate immune defenses and have been shown to kill a broad variety of Gram-negative and Gram-positive bacteria and are promising tools to treat multidrug-resistant bacteria (Kang et al. 2012). Different biomaterials can be employed as surface supports for immobilizing cationic antimicrobial peptides, such as resin beads, gold surfaces, polymer brushes, cellulose membranes and block copolymers. The antimicrobial peptides immobilized onto a hydrophilic polymer has been proved to give a robust coating with antiadhesive and antimicrobial properties, highly effective in combating biofilm formation. Since polymer brushes with immobilized antimicrobial peptides can be synthesized on most of the current implant material surfaces, the coating will be widely applicable for combating implant-associated infections (Hancock and Sahl 2006; Bagheri et al. 2012).

The fourth approach, the achievement of materials able to oppose biofilm formation and, at the same time, to support bone repair, is of outstanding interest in orthopedics. Hydroxyapatite, besides its properties as infection-resistant material (Arciola et al. 1999), have been proposed as a coating surface undergoing slow in vivo degradation and as a stable interface for osseointegration and bone fixation (Campoccia et al. 2003).

An innovative osteointegrative and antibacterial biomimetic coating on titanium has been obtained by Anodic Spark Deposition (ASD) treatment. The anodization treatment creates a chemically and morphologically modified titanium oxide layer, characterized by a microporous morphology enriched by calcium, silicon, phosphorous, and silver. A biological characterization of this coating has shown an optimal adhesion of osteogenic SAOS-2 and proliferation as well as a strong antibacterial effect (Della Valle et al. 2012).

Bioglasses are of wide interest since they spontaneously bond and integrate with living bone in the body. By varying the glass chemistry and/or by adding some dopants, it is possible to improve their clinical applications. A bioglass doped with gold nanoparticles has been developed, which showed efficient antibacterial properties against *S. aureus*, in addition to its bone reconstruction property (Grandi et al. 2011).

2.13 Conclusion

The significant worldwide impact of periprosthetic joint infections and the loss of efficacy of antibiotic-based conventional therapies urgently demand new preventive strategies able to effectively limit the infection burden that parallels the increasing total number of primary and revision arthroplasties.

Many categories of anti-infective biomaterials are currently available and new ones are rapidly advancing. The use of materials coated with immobilized antibacterial substances, particularly cationic antimicrobial peptides, appears very innovative and promising. Nanotechnologies and nanomaterials in medical research have created new therapeutic horizons and are rapidly growing.

The biomaterial science offers powerful and valuable tools. Their potential is often well proved in vitro and in preclinical models. However, clinical trials, appropriately designed at multicenter scale, together with well-implemented international registries are necessary to obtain evidence-based data on the benefits of the scientific advancements in the field. In this way, we may reach the aim at identifying the most effective anti-infective strategies.

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Clinical and Microbiological Aspects of Biofilm-Associated Surgical Site Infections

3

Charles E. Edmiston Jr., Andrew J. McBain, Christopher Roberts, and David Leaper

Abstract

While microbial biofilms have been recognized as being ubiquitous in nature for the past 40 years, it has only been within the past 20 years that clinical practitioners have realized that biofilm play a significant role in both device-related and tissue-based infections. The global impact of surgical site infections (SSIs) is monumental and as many as 80 % of these infections may involve a microbial biofilm. Recent studies suggest that biofilm- producing organisms play a significant role in persistent skin and soft tissue wound infections in the postoperative surgical patient population. Biofilm, on an organizational level, allows bacteria to survive intrinsic and extrinsic defenses that would inactivate the dispersed (planktonic) bacteria. SSIs associated with biomedical implants are notoriously difficult to eradicate using antibiotic regimens that would typically be effective against the same bacteria growing under planktonic conditions. This biofilm-mediated phenomenon is characterized as antimicrobial recalcitrance, which is associated with the survival of a subset of cells including "persister" cells. The ideal method to manage a biofilm-mediated surgical site wound infection is to prevent it from occurring through rational use of antibiotic prophylaxis, adequate skin antisepsis prior to surgery and use of innovative in-situ irrigation procedures; together with antimicrobial suture technology in an effort to promote wound hygiene at the time of closure; once established, biofilm removal remains a significant clinical problem.

C.E. Edmiston Jr. (✉)
Department of Surgery, Medical College of Wisconsin, Milwaukee, WI, USA
e-mail: edmiston@mcw.edu

A.J. McBain
Manchester Pharmacy School, The University of Manchester, Manchester, UK

C. Roberts
Clinical Resolutions, Hessle, East Yorkshire, UK

D. Leaper
School of Applied Sciences, University of Huddersfield, Huddersfield, UK

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3.1 Introduction

In 2010, the Centers for Disease Control and Prevention (CDC) reported that 51.4 million in-patient surgical procedures were performed in the United States (CDC 2010). It has been estimated that approximately 400,000 surgical site infections (SSI) occur in the United States each year with an associated mortality approaching 25 % (100,000) (Reed and Kemmerly 2009; Shepard et al. 2013; De Lissovoy et al. 2009; Herwaldt et al. 2006). While these numbers have historically been extrapolated from in-patient procedures alone, the actual number of SSIs is likely to be much higher since recent CDC data suggests that more than 34 million additional surgical procedures are performed in outpatient ambulatory surgical centers (ASC) in the United States (CDC 2009). The global impact of SSIs is therefore monumental and as many as 80 % of these infections may involve a microbial biofilm (Edward and Harding 2004; Hall-Stoodley et al. 2004; Percival 2004; Romling and Balsalobre 2012; NIH 2002). Many of the microbial populations associated SSIs have been observed to exist primarily within a biofilm matrix, often as a polymicrobial (heterogeneous) community in selective disease processes (Dowd et al. 2008; Edmiston et al. 2013a). The presence of a microbial biofilm within host tissue or on the surface of a biomedical device poses a significant challenge when attempting to eradicate these infections in-situ. In addition, biofilm-mediated infections exhibits resistance to host defense, reportedly contributing to a chronic inflammatory response, leading to complement activation and formation of immune complexes which in turn leads to tissue injury through an excessive inflammatory response (Hoiby et al. 2011; Jenson et al. 2011). A recent study suggests that biofilm producing organisms play a significant ($p = 0.024$) role in persistent skin and soft tissue wound infections in post-surgical military personnel with deployment-related injuries compared with a cohort control group (Akers et al. 2014). The authors suggest that this presence of a polymicrobial biofilm (71.4 %) was a significant risk factor for relapsing infection in skin and soft tissue infections due to increased bioburden, severity of disease, increased antimicrobial resistance and enhanced inflammatory response within the affected-tissues. *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were by far the predominant isolates recovered in both monomicrobial and polymicrobial infections, 24.0 % and 23.7 %, respectively. While microbial biofilms have been recognized as being ubiquitous in nature for the over 40 years; it has only been within the past 20 years that clinical practitioners have realized that biofilm-mediated disease plays a significant role in both device-related and tissue-based infections. The present discussion will focus on the microbial etiology, pathogenesis and treatment of selective biofilm-mediated acute and chronic surgical site infections.

3.2 Biofilm-Mediate Acute and Late Onset Infection in the Surgical Patient

What indications exist to suggest the presence of a biofilm mediated infection? Several biofilm investigators have suggested a diagnostic guideline that may serve to suggest the presence of a biofilm-based infection within the host tissue (Hall et al. 2014; Hall-Stoodley et al. 2012):

(a) Microbiological evidence of a localized or foreign body-associated infection post-surgery,
(b) Microscopic (light or electron optic) evidence of microbial aggregation,
(c) Medical history, documenting a biofilm predisposing condition such as implanted biomedical device, infective endocarditis, previous device-related infection,
(d) Recurrent infection (site specific) with organisms that are clonally identical,
(e) Documented history of antimicrobial failure or therapeutic recalcitrance (persistent infection) despite selection of appropriate antimicrobial agent (both dose and duration), and
(f) Presence of local or systemic signs and symptoms of infection that resolved primarily with appropriate antimicrobial therapy, only to recur following termination of therapy.

Under selective scenarios it may be assumed that a biofilm-based infection is present. However in the case of a chronic inflammatory process involving fascia (deep) or organ-space involvement, the true nature of the infection may not be evident until the wound is actually viewed at surgical revision. While selective diagnostic criteria may be helpful in elevating one's index of suspicion that a biofilm-associated infection is present, the "heavy-lifting" involves selecting an appropriate course of therapy in the presence of an often recalcitrant disease process.

The incidence of sternotomy SSI ranges from 1 to 8 % with an associated mortality approaching 40 %, which is significant from both a patient outcome and resource utilization perspective (Mauermann et al. 2008). In the United States alone, the number of open cardiac surgery procedures exceeds 600,000 a year (Owen et al. 2010). The time period between cardiac surgery and presentation of a sterna complication can range from 2-weeks to 3-months postoperatively. The staphylococci are the most commonly reported clinical isolates associated with these postoperative infections and both *Staphylococcus aureus* and *Staphylococcus epidermidis* are recognized as organisms that possess the ability to produce biofilm (Otto 2008). A study published in 2013 examined sternal tissues and stainless steel wires extracted from infected and non-infected sternal wounds which were analyzed by traditional culture methodology, immunofluorescence and electron optics. The infections in all subjects (6) were characterized as deep sternal wound infections. Positive wound cultures were obtained from two of the patients-Methicillin-resistant *S. aureus* (MRSA) and Methicillin-sensitive *S. aureus* (MSSA)-while four were culture negative. All blood cultures collected prior to debridement were negative. Identification of staphylococci in the debrided tissues was confirmed by immunofluorescence. No evidence of staphylococci was observed in debrided tissue remote to the infected wound. Confocal laser scanning electron microscopy (CLSM) confirmed that the staphylococci were organized in three-dimensional clumps and that these clumps represented a thick biomass, occupying 70 % of the infected tissue segments. The extracted stainless steel wires from the infected cases were examined using scanning electron microscopy (SEM), and revealed a metal surface that was coated with a thick extracellular matrix which included cluster of staphylococci. It is interesting to note that all attempts to recover staphylococci from the infected sternal material and sutures using conventional culture techniques were unsuccessful (Elgharaby et al. 2013). Three observations are worthy of consideration; first sternal (deep) tissues and stainless steel wire sutures revealed a staphylococcal biofilm suggesting that there was intraoperative contamination of the surgical field prior to closure. Secondly, the presence of biofilm necessitated the complete removal of all foreign such as the stainless steel wire sutures and a wide (deep) debridement of sternal tissues to facilitate adequate control of the infected tissues. The recalcitrant nature of these infections requires that all infected tissue be excised since residual biofilm will not respond to traditional antimicrobial therapeutic measures even though the planktonic (free-floating) form of the responsible organisms will often be susceptible to traditional antimicrobial agents (Edmiston 1993). Finally, unlike other biofilm-mediated device-related infections (to be discussed later) which are traditionally viewed as device-centric infections and not tissue-based, these deep sternal wound infections often involve both tissue and prosthetic material, contributing to the significant morbidity and mortality associated with these serious infections.

Bacterial biofilms are now recognized as a causal etiology of dental peri-implantitis, leading to chronic infection in selective oral surgery patients (Subramani et al. 2009). The process of post-implant infections requires the formation of a plaque which is similar to that seen on "native" teeth. The biofilms associated with these infections are highly heterogeneous, and may involve the following bacteria; streptococci, *Actinomyces*, *Porphyromonas*, *Prevotella*, *Capnocytophaga* and *Fusobacterium* species. The surface characteristics of a prosthetic implant serves to foster microbial adherence and colonization by biofilm-forming oral microbiota. The rough surface of

titanium implants enhances microbial adherence and plaque formation, leading to subsequent inflammation of the gums (Ray and Triplett 2011). Biofilm-mediated osteomyelitis can also be associated with dental peri-implantitis and the primary causative pathogens are *S. aureus* and *S. epidermidis*. However, biofilm-mediated osteomyelitis of the jaw can also be associated with oral streptococci, *Bacillus* species and *Actinomyces* species. Similar to other biofilm-mediated infections, antibiotic therapy has limited effectiveness and surgical debridement is still the treatment of choice.

Biofilm formation often plays a significant role in the etiology of periorbital implant infections with *Micobacterium chelonae*, *S. aureus* and *P. aeruginosa* being the three most common organisms recovered from explanted periorbital devices (Samini et al. 2013). The median time from implant to disease presentation is approximately 13 weeks. Implanted devices which can be affected include orbital spheres, enucleated spheres, lacrimal stents, Jones Tubes and sclera buckles. Electron microscopy optic studies have documented that the biofilm-associated with infected periorbital implants involve a heterogeneous collected of microorganisms encased in a thick exopolysaccharide matrix and the definitive therapy for treatment of these infection involves device removal (Sugita et al. 2001; Parsa et al. 2010; Holland et al. 1991).

According to the American Society of Plastic Surgeons, breast implants represent the leading type of cosmetic plastic surgery with over 300,000 breast implant surgeries performed annually in the United States (American Society of Plastic Surgeons 2011). The reported incidence of infection in breast reconstruction is between 2.8 and 28 % which suggests a high probably risk of infection for women undergoing these procedures (Neto et al. 2002; Tran et al. 2002; Vandeweyer et al. 2003; Nahabedian et al. 2003; Olsen et al. 2008). Bacterial contamination of the implant at the time of insertion can result in persistent low-grade inflammation of the surrounding tissues, leading to capsular fibrosis and capsular contraction (Rieger et al. 2013). A recent meta-analysis has documented a significant risk for infection associated with expander/implant reconstruction compared with reconstruction using autologous abdominal tissue (Tsoi et al. 2014). While most traditional (swab) cultures of explanted devices typically yield few organisms, sonication of explanted devices and tissue biopsies have yielded microbial recovery in 38.5 and 89.5 % of respective samples (Netscher 2004). *S. epidermidis* is hypothesized as the causative organism associated with capsular contraction, contaminating the device at the time of insertion, persisting on the device surface and resulting in subclinical infection. A recent study has documented that staphylococcal contamination of the surface of the expander/implant devices leads to biofilm-formation in 80 % of culture positive devices (Jacombs et al. 2014). The authors' of this study have suggested that textured breast implants significantly potentiates biofilm formation compared with implants having a smooth surface. While the biologic advantages of a textured surface leads to better tissue incorporation and therefore less potential contracture, surgeons should be aware that this advantage can be negated if the device is contaminated at the time of insertion. The current treatment for an infected tissue expander or implant is device removal which is often catastrophic for the patient, potentially delaying additional therapy such as chemo or radiotherapy in patients who have undergone breast reconstruction follow mastectomy.

The practice of neurosurgery has witnessed an explosion in the number of devices that have been developed to treat patients. These include complex spinal instrumentation/hardware, pulse generator, indwelling Silastic catheters and shunts and synthetic bone flaps after delayed cranioplasty (Braxton et al. 2005). The estimated rate of infection associated with implantation of spinal hardware ranges from 2 % to approximate 9 % (Braxton et al. 2005; Massie et al. 1992). Implant infections are characterized by increased utilization of healthcare resources including prolonged length of stay, increased cost of antibiotic therapy, additional surgical revisions and extended rehabilitation post discharge. *S. aureus* and *S. epidermidis* are the two most common

isolates recovered from documented spinal hardware infections. Early infections occur within approximately 2 weeks of implantation and are associated with intraoperative infection or wound contamination in the immediate postoperative period. Infections which present years postoperatively have been thought to occur following an unrelated hematogenous event. But they could also be associated with phenotypic changes of sessile bacteria to their planktonic forms in device-related biofilms. Resolution requires reoperation and removal of the infected device. The incidence of infection associated with deep brain stimulators is reported to be approximately 3.7 %, and similar rates have been suggested for dorsal column stimulators (3.4 %) (Umemura et al. 2003; Cameron 2004). Approximately 20,000 ventriculoperitoneal (VP) shunts are placed annually in the United States and the incidence of infection can exceed 15 % with virtually all infected devices harboring a biofilm (Bondurant and Jimenez 1995; Davis et al. 2002; Wood et al. 2001). The same analogy can be made for ventriculostomy catheters of which nearly 140,000 are placed yearly for many indications, including acute hydrocephalus to ICP monitoring and management of neurotrauma (Lozier et al. 2002). The reported infection rate following catheter insertion in this patient population is approximately 10 % and ventriculitis is a potentially life-threatening biofilm-mediated complication. The morbid nature of ventriculoperitoneal and ventriculostomy infections has led to the development of antimicrobial impregnated technologies that resist bacterial adherence, thereby limiting biofilm development (Braxton et al. 2005). Unfortunately infection remains a serious outcome in this patient population. Infection is a major complication of delayed bone flap cranioplasty and most cryopreserved bone grafts when cultured by conventional methodology are negative, which is suggestive of culture-resistant biofilm contamination (Braxton et al. 2005). Because of the difficulty in treating these serious life-threatening infections current emphasis focuses on fastidious technique and appropriate skin-antisepsis that can also include innovative irrigation techniques using effective biocidal agents (Barnes et al. 2014).

Over 900,000 abdominal wall hernia repairs are performed yearly in the United States (Engelsman et al. 2007). A recent meta-analysis has suggested that use of synthetic mesh prosthesis for abdominal wall closure significantly increases the risk infection (Scott et al. 2002). Contamination of the implanted mesh usually occurs at the time of implantation or exogenously in the early postoperative period. Several clinical studies suggest that the infection rate is highly variable and dependent upon the type of mesh used for abdominal repair ranging from 2.5 to >6 % with polypropylene; less than 1 to >9 % with expanded polytetrafluoroethylene (ePTFE); while polyester mesh demonstrates an infection rate similar to polypropylene (Luijendiik et al. 2000; Leber et al. 1998; Bauer et al. 1999; Hamy et al. 2003; Machairas et al. 2004). The level of microbial-contamination (bioburden) that develops upon the surface of a synthetic mesh is dependent upon the type of material and the structural surface characteristics of the device. For example, meshes made of multifilament Dacron support a luxurious and dense biofilm (Engelsman et al. 2008). Meshes which exhibit a hydrophobic surface such a ePTFE initially inhibit bacteria adherence. However with prolonged exposure to bacterial contamination both Gram-positive and Gram-negative organisms will form a dense biofilm. So it would appear that surface hydrophobicity as a deterrent against bacterial adherence has only a short-term benefit in vivo (Davidson and Lowe 2004). Biofilm-associated mesh infections adversely impact the wound healing process by interfering with the ingrowth of host tissues through the mesh. *S. aureus* colonization of the mesh surface induces fibroblast death (apoptosis), thereby inhibiting the proliferation of these cells during the maturational period of wound healing (Bellon et al. 2004; Edds et al. 2000). Biofilm formation on the surface of a synthetic mesh can result in chronic infection, draining sinuses, mesh extrusion and enteric fistula formation. Most chronic biofilm infections has been associated with *S. aureus* but mesh infections following abdominal surgery may also involve selective Gram-negative bacteria. A recent analysis has suggested that relapsing

infections of the type observed with chronic mesh infections is almost always associated with biofilm-forming microorganism and many of these isolates also express multi-drug resistance (Sanchez et al. 2013). Resolution of these infections requires complete mesh removal, along with removal of other foreign bodies such as residual suture material, followed by simultaneous reconstruction often using a monofilament polypropylene mesh. The affected patients will often require a prolonged period of follow-up to monitor the possibility of occult infection following mesh replacement (Birolini et al. 2014).

The pathobiology of a vascular graft infection is best understood as a biofilm-mediated infection. An excellent example of a biofilm-mediated vascular graft infection is the development of a groin sinus tract following insertion of an aorto-femoral prosthetic bypass graft. These infections are characterized as late-onset, occurring weeks to months post-implantation and the presentation may be occult with no systemic signs of infection. Traditional culture methodology often fails to recover any isolates, However when the graft segment is sonicated, *S. epidermidis* is often recovered in numbers which exceed 6-logs (Hasanadka et al. 2007). The establishment of a biofilm-mediated vascular graft infection requires a series of sequential events (Bandyk and Black 2005; Edmiston et al. 2005). First, the device is contaminated at the time of insertion by a biofilm-forming organism; a process that is facilitated by surface conditioning by blood and tissue fluid proteins. Once the organism adheres to the surface of the graft a microcolony aggregation may to form followed by the elaboration of an extracellular matrix which can eventually progress to development of a mature biofilm. The organization and maturation of the bacterial biofilm is a dynamic process that ironically requires very few contaminating organism to initiate the process. A low metabolic activity, due to limited substrate availability and production of a luxurious extracellular matrix, contributes to the physiological conditions that foster resistance to both host immune defenses and antimicrobial therapy (recalcitrance). Typically, the biofilm spreads slowly over the exterior surface of the graft,

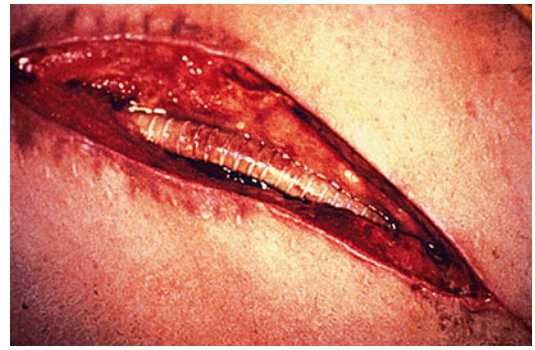

Fig. 3.1 Acute-onset vascular graft infection, MRSA infection and wound dehiscence at 10-days post-implantation of fem-popliteal vascular graft

eventually involving the graft-to-artery anastomosis, reducing anastomotic tensile strength, leading to development of a pseudoaneurysm and eventual graft failure that may be heralded by catastrophic hemorrhage. During this process there is little or no spread to the perigraft tissues nor does one observe the development of fulminant sepsis unlike early-onset vascular graft infections involving *S. aureus* or Gram-negative pathogens (usually *Escherichia coli*). The incidence of SSI following vascular surgery is reported to be in the range of 5–15 %; higher in diabetic patients, patients colonized with MRSA and after procedures requiring a groin incision (Armstrong and Bandyk 2006; Frei et al. 2011). Early-onset infections are characterized by wound dehiscence and purulent drainage often within days of surgery (Fig. 3.1). Late onset infections are more indolent, clinical recognition can be delayed for months or even years. Signs of SSI may include failure of graft healing (incorporation) within the surrounding tissues, sinus tract formation, pseudoaneurysm formation or late erosion into adjacent bowel mediated by a chronic inflammatory process (with attendant enteric hemorrhage), due to the presence of a mature bacterial biofilm (Fig. 3.2) on the surface of the graft (Frei et al. 2011). While historically, treatment of a late-onset vascular graft infection involved an extra-anatomic revascularization, this approach has been replaced by in-situ graft replacement, which is associated with significantly less morbidity and mortality (Hart et al. 2005). Future developments

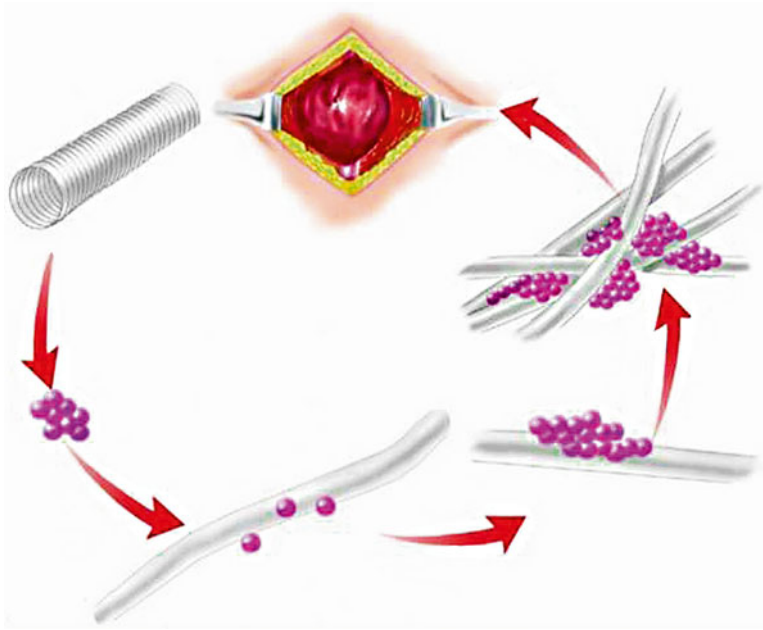

Fig. 3.2 Developmental presentation of late-onset vascular graft infection involving *S. epidermidis*; intraoperative contamination leads to causal contamination of external surface of vascular graft, organism down-regulates its metabolism growing slower over week to month, biofilm finally reached critical density resulting in chronic inflammatory leading to pseudoaneurysm and possible graft failure

in graft technology to reduce microbial adherence, along with effective intraoperative risk reduction technologies are warranted.

Over the past 20 years the number of annual total hip replacements in the United States has increased twofold to >250,000 while the number of total knee replacement has increased almost fivefold to >500,000 (Del Pozo and Patel 2009). The risk of infection following total joint replacement is 0.5 % to approximately 2 % for hips and knees and 2 % to approximately 9 % following ankle replacement (Laffer et al. 2006; Kessler et al. 2012). Biofilm-mediated infection of orthopedic implants can occur either as an exogenous process, with contamination of the device occurring during surgery (or early in the postoperative period) or hematogenously via the bloodstream at any time after surgery. The vast majority of exogenous infections occur in the acute postoperative phase, especially in patients experiencing poor wound healing. Periprosthetic joint infections (PJI) can be classified as "early", "delayed" or "late." Early infections occur within the first 2 months; delayed infections occur between the third and 24th month postoperatively and late infections are diagnosed >2 years post-implantation. The most common organisms associated with PJI are the coagulase-negative staphylococci (30–43 %) and *S. aureus* (12–23 %). These are followed by streptococci (9–10 %), enterococci (3–7 %), Gram-negative bacilli (3–65 %) and miscellaneous anaerobes, such as *Propionibacterium* and *Peptostreptococcus* spp. (2–4 %) (Pandey et al. 2000; Steckelberg and Osmom 2000). Polymicrobial infection is observed in approximately 10 % of cases and 10–30 % of clinical cases present as culture-negative. As with other biofilm-mediated infections, traditional antibiotic therapy has limited utility in the treatment of in situ PJI. Therapeutic efficacy dictates that a sufficient concentration of antibiotic greater than the MIC^{90} for most likely pathogens must migrate from the blood into the tissue space (second compartment) then into the biofilm, which is essentially a third compartment. Previous studies have documented that the MIC required to inhibit

or kill most microorganism within a mature biofilm is often 100–1,000 times the traditional MIC^{90} for selective device-related microbial pathogens (Edmiston 1993; Costerton et al. 1995). The use of antibiotics in the treatment of PJI is adjunctive to surgical management, which often involves a two-stage debridement and revision, requiring the removal of the infected prosthesis with implantation of a new device at a later operation. A temporary articulated antimicrobial-impregnated spacer is constructed for use until the second operation. The success rate of this procedure approaches 90 % but failures require additional interventions. In some scenarios debridement and retention of the original implant may be successful if: (a) the infection is detected early (<3-weeks), (b) there is absence of a sinus tract, (c) the organism is susceptible to traditional antimicrobial therapy (suggesting little if any biofilm involvement), and (d) the implant is stable (not loose). Another option involves a one-stage procedure; removal of the infected implant with immediate re-implantation of a new device, but the patient must have intact or slightly compromised soft tissue to qualify for this approach. However, the involvement of multi-drug resistant pathogens will often preempt this approach (Zimmerli and Moser 2012). A recent report has suggested that biofilm-forming organisms recovered from culture-positive cases are commonly associated with a polymicrobial infection (37.5 %), posing an additional therapeutic challenge (Fernandes and Dias 2013). These infections are among the most catastrophic for both the patient and practitioner, management may involve years of additional medical and surgical care without a guarantee of successful resolution. A proportion of device-adherent (biofilm) organisms are metabolically locked in a stationary growth phase and therefore the usual achievable antibiotic serum and tissue concentrations are inadequate to resolve the in-situ infection, leading to therapeutic failure. This most often leads to therapeutic failure. The optimal strategy is prevention which requires putting into place an appropriate interventional bundle that significantly reduces the risk of postoperative infection, therefore minimizing the opportunity for wound/device contamination (Kim et al. 2010).

Traditional studies of device-related infection have focused primarily on the device itself and little if any time is spent considering the role that sutures may play in initiating or potentiating the risk of postoperative infection. While wound closure technologies such as surgical sutures, have not always been viewed in the same light as other implantable biomedical devices, surface characteristics of these devices make them a susceptible substrate for bacterial adherence and/or contamination. The classical studies conducted by Varma, Elek and Raju documented the microbial burden required to produce an infection in a clean surgical wound (Varma et al. 1974; Elek and Cohen 1957; Raju et al. 1977). These studies further characterized the role of suture material as a foreign body, functioning as a nidus for infection in the presence of wound contamination. Recent reports by Kathju and colleagues would suggest that contamination of surgical sutures at the time of implantation by biofilm-forming organisms leads to recalcitrant infection, necessitating eventual removal of the infected material (Kathju et al. 2009, 2010). In a recent study, suture segments were explanted from 158 surgical patients, 46 (29.1 %) were recovered from documented infected cases. A bacterial biofilm was observed by scanning electron microscopy (SEM) on 100 % of infected sutures. Biofilms associated with infected explanted sutures were observed in both deep incisional and organ-site infections. In the majority of these infected cases the microbial burden exceeded $>10^5$ cfu/cm suture surface (Edmiston et al. 2013a). In three separate cases involving infected mesh segments, the primary device had been removed but recurrent infection required exploration and removal of retained suture segments. All three of these suture segments (polypropylene) exhibited a polymicrobial microbial flora comprised of Gram-positive (2 MRSA, 1 MSSA), Gram-negative aerobic (*E. coli*) and anaerobic bacteria (*Peptostreptococcus* and *Bacteroides* spp.) enmeshed in a luxurious biofilm (Fig. 3.3). These findings are complimentary to previous in-vitro studies, which suggest that bacterial adherence to surgical sutures is associated with the formation of a luxurious bacterial biofilm (Henry-Stanley et al. 2010; Williams and

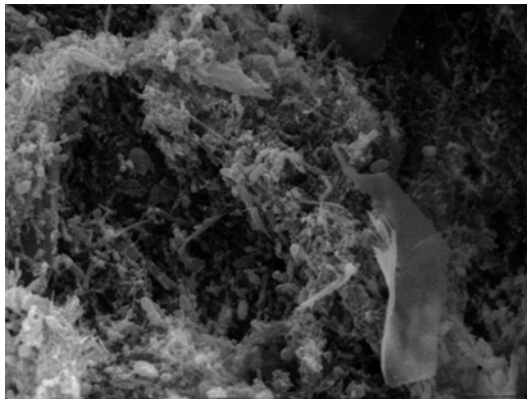

Fig. 3.3 Suture sample recovered from chronic polypropylene mesh infection where device eroded into the peritoneal cavity, polymicrobial recovery from suture segments includes aerobic and anaerobic bacteria, magnification ×5,875

Costerton 2012). Preventing microbial adherence and biofilm formation on the surface of a multifilament or monofilament suturing devices would appear to be a beneficial risk reduction strategy. Several investigators have documented a reduction in bacterial adherence (Gram-positive and Gram-negative) to the surface of multifilament and/or monofilament sutures which are coated with the biocide, triclosan (Rothenburger et al. 2002; Ming et al. 2007; Edmiston et al. 2006a).

3.3 Mechanistic Aspects of Biofilm Formation in Host Tissues and Implantable Devices

A biofilm is an organized community of bacteria attached to a surface and enveloped within a self-produced matrix (Costerton et al. 1999). The formation of a differentiated multicellular community gives a biofilm defense against UV light, bacteriophages, biocides, antibiotics, immune system responses, and many environmental stresses. The biofilm, on an organizational level allows the bacteria to survive many intrinsic and extrinsic defenses that would inactivate the single cell (planktonic form) bacteria. The first step in establishment of a biofilm-mediate infection involves adherence of the organism to a conditioned surface such as host-tissues or implanted device. Biofilms can functions as a partial physical barrier against penetration of antibiotics, antibodies and granulocytic cell populations (Akiyama et al. 1997; Hoyle and Corsterton 1991). In the presence of host tissue protein (plasma), *S. aureus* forms a biofilm that has a unique composition, composed of sheaths of fibrin and glycocalyx (Nemoto et al. 2000). These substances serve to anchor the matrix to the infected cell or inert biomedical device surface.

According to a much cited model, in the process of microbial biofilm formation the microbial cells attach irreversibly to surfaces (i.e., those not removed by gentle rinsing) and will begin cell division, forming microcolonies, and produce the extracellular polymers that define structural components of the biofilm (Fig. 3.4). These extracellular polymeric substances (EPS) consist of polysaccharides, proteins, DNA and other materials of microbial and host cell origin, which and can be detected microscopically and by chemical analysis. EPS provides the matrix or structure for the biofilm. The biofilm matrices are highly hydrated (98 % water) and tenaciously bound to the underlying surface. The structure of the biofilm is not a mere homogeneous monolayer of slime but is heterogeneous, both in space and over time. The presence of "water channels" allow transport of essential nutrients and oxygen to the cells growing within the biofilm (Evans and Lewandowski 2000). In certain situations, biofilms have a propensity to act almost as filters to entrap particles of various kinds, including minerals and host components such as fibrin, RBCs, and platelets.

Phenotypic heterogeneity of biofilm-associated bacteria is an important biofilm characteristic and biofilm-associated organisms can grow more slowly than planktonic organisms occupying the same niche due to localized nutrient and/or oxygen depletion (Donlan 2001b). Bacterial cells may detach from the biofilm as a result of physical disturbance, cell growth and division, or the spontaneous release or biofilm cell aggregates (Donlan 2001a). These detached cells can potentially metastasize to distant sites in the host causing a systemic infection. Formation of a mature biofilm requires a complex series of events, involving different organisms (motile

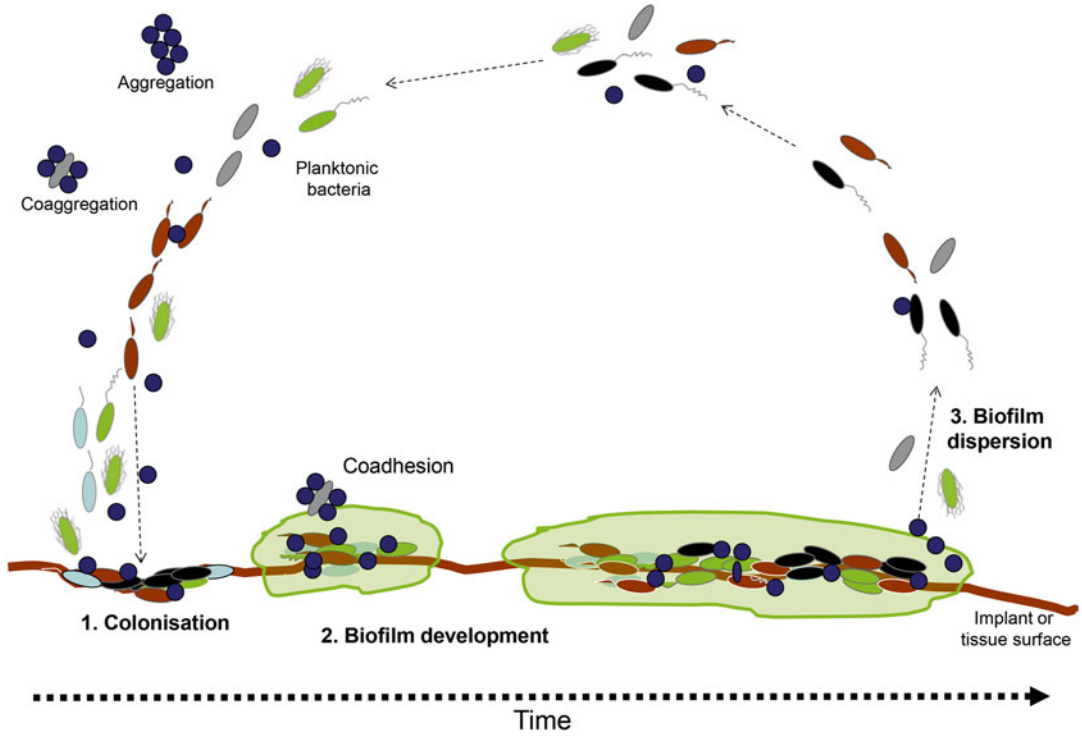

Fig. 3.4 A simplified general model showing the sequence of events through which biofilms develop. In this scenario, a mixture of bacterial species in planktonic form adheres to the implant or tissue surface (*1*) and develops into a biofilm (*2*) with the associated deposition of enveloping matrix material. Dispersion (*3*) where it occurs can enable cells to colonize elsewhere. The diagram also shows coaggregation, where taxonomically distinct bacteria form aggregates through specific surface receptor interactions and aggregation (where cell clusters form through a range of mechanisms)

and non-motile), the material on to which the biofilm forms and the external environment including fluid flow, oxygen levels and availability of nutrients. It can be viewed as a multistage process beginning with adherence (adherent stage) followed by proliferation and differentiation of the attached cells (maturation stage). From a molecular perspective, in some bacteria, these two stages are controlled by surface adhesins and cell-to-cell signaling pathways, respectively. To understand attachment, it is important to closely examine the properties of the material or tissues upon which the biofilm will form. Implanted medical devices can range from various hydrophilic materials such as plastic or PTFE, which is often used for vascular prosthesis or hydrophilic materials such as various metals used in orthopedic implants (Meier-Davis 2006). Other materials that may serve as a substrate for bacterial adherence and biofilm formation can include xenografts such as implantable porcine heart valves.

Planktonic bacteria with special appendages such as fimbria, cilia, and flagella, allow for motility but also give the bacteria a sense of "touch". Under some conditions when a bacterium encounters a surface that has been conditioned with small organic molecules, it explores the surface (Singh et al. 2002; Stoodley et al. 2002). This process is called "twitching." According to this model, the bacterium attaches irreversibly to the surface, a radical change in the phenotype of the bacterium occurs (Sauer et al. 2002). Over 800 new proteins can be expressed within the first hour during which the bacterium attaches (Sauer et al. 2002). The microorganism undergoes division to form a young community of cells, and after only several hours, the beginning of an immature biofilm is visible (Harrison-Balestra et al. 2003). Once irreversible attachment has occurred, the bacterium is

committed to a biofilm phenotype. Rapidly, the bacterium changes its phenotype and begins to grow and differentiate. The bacteria begin to excrete extracellular polymeric substances (EPS), which can contribute to the communal defense of the nascent bacterial biofilm. These cells can share a similar genotype but eventually differentiate into a variety of phenotypes. This emerging microbial population can be viewed as a of a "microcolony." The microcolony continues to develop and mature. It takes cue from cell-to-cell signaling molecules such as quorum sensing molecules which work to guide its maturation (Parsek and Greenberg 2005). Neutrophil accumulation within the biofilm can initiate self-injury through the released of oxidants which in turn compromises host defense mechanisms. Debris from the necrotic neutrophils serves as a biological matrix that facilitates additional biofilm formation (Walker et al. 2005).

The mature biofilm releases planktonic 'seeds' cultures which will stimulate the host immune response to mount an intense inflammatory response. The biofilm will derive nutrients from the host exudate that accompanies this inflammatory response. In this way, the sacrifice of a few bacteria promotes the survival of the community through continual nutrient acquisition.

3.4 Why Are Biofilms Difficult to Eradicate Using Antimicrobials?

As described earlier from the clinical perspective, surgical site infections (SSIs) associated with implants are difficult to eradicate using antibiotics regimens that would typically exhibit effectiveness against the same bacteria growing under planktonic conditions (Gilbert and McBain 2001). In addition, clinical laboratory results based upon in-vitro susceptibility of planktonic cells provide little clinical therapeutic guidance (Edmiston 1993). An understanding of the mechanisms which underlie biofilm recalcitrance is useful because it contributes to the development of more effective strategies for biofilm control and aids clinicians in utilizing current therapeutic option more effectively. It is known multiple mechanisms are responsible, most of which relate to phenotypic changes and multi-cellularity, rather than the genetic adaptation responsible for antibiotic resistance exemplified by MRSA. This is evidenced by the fact that cells dispersed from a biofilm, if tested before significant cell division occurs will exhibit comparable susceptibility to planktonic cells (Gilbert et al. 2002). The following discussion outlines the main mechanism which is believed to underlie biofilm resistance and is based on the schematic presented in Fig. 3.5.

3.4.1 Penetration Failure

This is probably the most intuitive reason for the tenacity of biofilms under antimicrobial stress but paradoxically it is least likely to be the main reason for antimicrobial recalcitrance. An antimicrobial agent must penetrate a biofilms effectively in order to achieve a high level of bacterial inactivation. However, diffusivity of biofilms is normally sufficient to allow significant penetration and furthermore, water channels that are a feature of some biofilms have been likened to a primitive circulatory system that will further enhance drug penetration. Cationic antimicrobials or biocides such as gentamicin and chlorhexidine can bind to anionic sites within the biofilm matrix in a process termed reaction-diffusion limitation, which perturbs penetration but probably only temporarily. Extracellular products of bacterial growth, including β-lactamases and other drug inactivation enzymes can concentrate with the biofilm matrix and thus enhance penetration failure of some antimicrobials, but in all of these situations, this protective mechanism can be overcome if the antimicrobials are delivered to the site for a sufficient length of time and concentration (Gilbert et al. 2002).

3.4.2 Phenotypic Heterogeneity and Biofilm-Specific Phenotypes

Growth-rate is a key mediator of bacterial susceptibility to many antimicrobial agents even in planktonic cells. Bacteria which are not actively

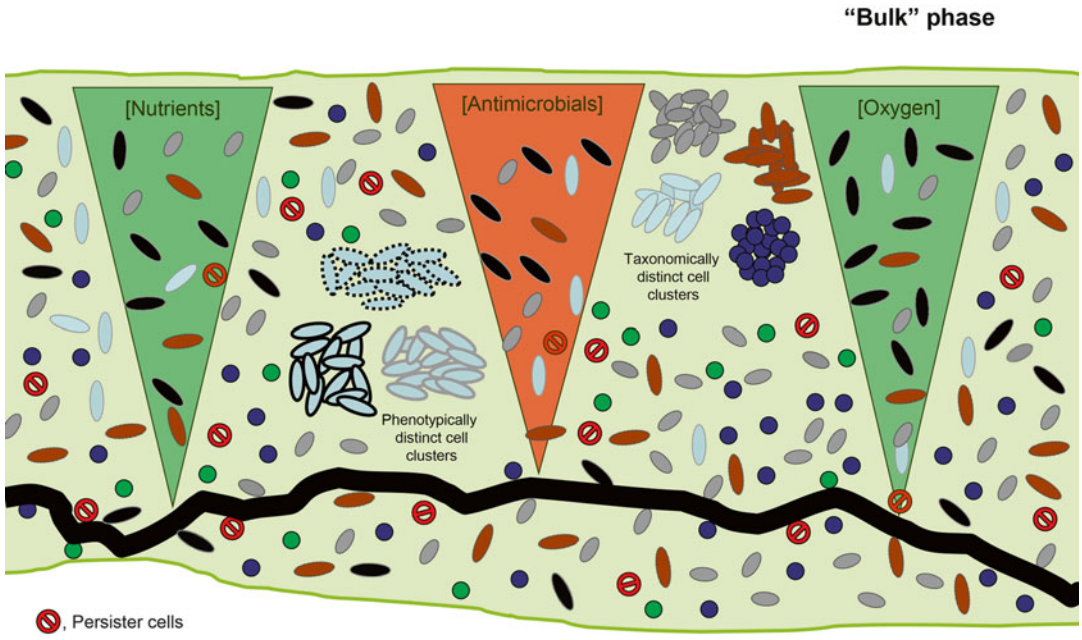

Fig. 3.5 Biofilm recalcitrance is a complex and incompletely understood phenomenon. This diagram presents some of the main mechanisms believed to be responsible all of which depend on the heterogeneity afforded by the biofilm matrix. (1) gradients of nutrient and gases may be establish resulting in local variations in bacterial phenotype including areas of dormancy; (2) the penetration of antimicrobials may be perturbed by the matrix and by enzymes within the matrix including β-lactamases. This can reduce the effective concentration of active agent delivered to the deeper layers. (3) persister cells, which are phenotypically specialized recalcitrant variants can be protected from immune cells by the matrix and thus may survive sub-inhibitory antimicrobial exposure allowing regrow following treatment

dividing may be refractory to some antimicrobials through a process that has been termed "drug-indifference" (Jayaraman 2008). This is where the role of the biofilm matrix is particularly important since immobilization can confer marked phenotypic variation in cellular growth-rate within the biofilm due to localized depletion of nutrients and oxygen (for aerobic cells). This has been depicted in Fig. 3.5 as "phenotypically distinct cell clusters" but in mixed species biofilms, taxonomically distinct clusters of bacteria (also illustrated) may occur and represent pockets of bacterial survival. The reason phenotypic heterogeneity alone is unlikely to account for the totality of biofilm recalcitrance is that during antimicrobial treatment, biofilms normally undergo a considerable level of cellular death; survival being a pyrrhic victory in which small pockets of viability facilitate re-growth. In such circumstances the initial bacterial death which occurs at the biofilm periphery on exposure to antimicrobial exposure will expose deeper lying cells to nutrients and oxygen reducing heterogeneity hence lowering localized recalcitrance.

3.4.3 Persister Cells

The above explanation probably does not fully account for the extent of biofilm recalcitrance. The persister hypothesis which was originally proposed by Bigger in 1944 answers many of the questions one has when contemplating the process of biofilm recalcitrance (Bigger 1944). It was noted that strains of staphylococci could not be sterilized in situ with penicillin; an antibiotic to which it was highly susceptible. Surviving cells which were called "persisters" occur at a

frequency of approximately one per million of the bacterial cell present in the original culture, for which penicillin is bacteriostatic and only very slowly, if at all, bactericidal. Another important early paper (Gunnison et al. 1964) made similar observations for *S. aureus*, demonstrating that persister cells were phenotypic variants that did not result from stable genetic alteration. It was noted that that the proportion of survivors was not changed even by a 1,000-fold increase in the dose of penicillin, or following the addition of streptomycin. As with the earlier study, these persister organisms were essentially drug indifferent. More recently, it has been shown that biofilm recalcitrance in *P. aeruginosa* is largely attributable to slow growth rate and the presence of persister cells (Spoering and Lewis 2001). Therefore persisters, which many species of bacteria can form, represents an important facet in the current explanation of why there is biofilm recalcitrance, with survival drug treatment and proliferation afterwards while being protected from phagocytosis by the biofilm matrix.

3.5 Biofilms, Infection and Wound Healing in Chronic Surgical Wounds

Biofilms can occur in practically any hydrated non-sterile environment. Their dispersed or "planktonic" counterparts, upon which microbiologists have based much of their understanding of microbial behavior, differ markedly in pathogenicity and in their responses to antimicrobials and the immune system. Biofilms are may be present in up to 70 % of open wounds, healing by secondary intention and this figure probably increases in chronic wounds particularly when there are multiple, underlying and unmet co-morbidities. There is less evidence of their presence in acute wounds but, since biofilms take time to form, it is highly likely that their incidence may be lower in acute wounds (James et al. 2008; Percival et al. 2012).

Increasing knowledge and understanding of the formation and inhibitory influence of biofilms on wound healing processes has changed many aspects of wound management. The early stages of biofilm formation may occur rapidly even in acute wounds such as burns and sutured surgical wounds healing by primary intention, and may therefore resulting in delayed healing or a greater risk of developing overt infection (Percival et al. 2012; Costerton et al. 1999). The microorganisms which can cause wound infection are diverse and in the case of chronic wound infect they are often polymicrobial. It is commonly observed that the presence of bacteria (or the bioburden) in chronic, open wounds presents as a continuum from contamination through colonization to local and systemic infection. Although the precise definition has not been agreed on, a pre-local, or covert, infection phase has been referred to as "critical colonization" (Kingsley 2001). In critically colonized wounds, there may be no clear signs of acute infection in (the Celsian calor, rubor, dolor et tumor), but instead there may be unexplained stalled or delayed healing, usually associated with increasing pain, exudate or smell, and abnormal or excessive granulation tissue, and maceration of surrounding skin (Cutting and Harding 1994; Gardner et al. 2001). This may be worsened by underlying pathological processes such as venous or arterial insufficiency, diabetes, pressure damage or an occult malignant process. The difficulty of assessing infection, particularly in these chronic wounds, is not made easier by conventional processing of microbiological swab analysis. Even when the Levine technique (based on the rotation of the swab, under pressure, in the wound swab over a 1 cm^2 area) is used, to harvest organisms deep in the wound bed, or sequential biopsies are taken to assess the quantity of colony forming units/gram of tissue and progress of treatment (which has been accurate only in burn management and is unpopular with patients and Ethics Committees!) only planktonic bacteria are identified. If this report is to the caregiver and if the sensitivities are included, there is a high likelihood that an inappropriate antibiotic will be prescribed with attendant risk of developing antibiotic resistance.

Biofilms may be involved in this continuum of micro-organism proliferation (Davis et al. 2008). The establishment of bacterial biofilms in wounds

may hypothetically equate to uncontrolled critical colonization and failure to prevent or manage this process, risks invasive tissue infection. The sequence of biofilm persister-cell phenotype to biofilm planktonic-cell phenotype would seem to turn the Koch postulates on infection upside down (Percival et al. 2010; Wolcott et al. 2010a). As a result conventional swabbing and microbiological testing in this situation is unlikely to reveal the "culprit" planktonic organisms, since no single bacterial species is responsible, or aid with a treatment strategy. The presence of bacterial biofilms on a wound surface can encourage and excite an underlying, inappropriate and excessive host inflammatory response (through stimulation of neutrophils and macrophages) to cause a prolonged release of nitric oxide, inflammatory cytokines and free radicals and delayed healing (Wolcott et al. 2008). This delay is not an inert biological process; quite the reverse, with persistence, the early inflammatory processes and healing cascades may be out-of-phase. This concept, which helps to explain why chronic wounds fail to heal, is not new (James et al. 2008; Percival et al. 2012; Costerton et al. 1999; Kingsley 2001; Bjarnsholt et al. 2008). The same concept likely applies to surgical wounds and explains why some surgical wounds fail to heal, often with superficial skin dehiscence, without clinical signs of acute infection (cellulitis, pus formation and pain) and a failure to identify/harvest micro-organisms from the dehisced wound. Although there is likely to be biofilm-related structures in or on most wounds these cannot be recognized without sophisticated laboratory testing. However, if there is a lack of clinical progress in the healing of an open surgical wound, or of any hard-to-heal, chronic wound, it is reasonable to assume that there is critical colonization together with reformation of biofilm. It is misguided to believe biofilms are visible to the naked eye although they may be present when the wound bed is heavily exuding or covered with fibrinous material or necrotic tissue that needs debridement.

The ideal way to manage a biofilm-mediated surgical wound would be to prevent it from occurring through the rational use of antibiotic prophylaxis in addition to adequate skin antisepsis prior to surgery or the use of antimicrobial-coated sutures. Once there is bacterial attachment to the wound bed and biofilm formation it is probable that only maintenance debridement can control it. Prevention of biofilm reformation in open wounds involves adequate wound irrigation or cleansing using antiseptics, the use of negative pressure wound therapy (NPWT), or the use of antimicrobial dressings. The diversity of biofilm phenotypes directly relates to successful attachment and infection in the wound, together with resistance to host response and antimicrobial therapy (whether it is a topical antiseptic or systemically administered antibiotic), particularly in the non-planktonic state (Sauer et al. 2002; Cho and Caparon 2005; Leid et al. 2005; Stewart and Costerton 2001; Costerton and Stewart 2001; Mah and O'Toole 2001). The role that biofilms may play in delaying healing in sutured surgical or traumatic wounds is less clear, although it may account for early dehiscence of wounds after sutures or staples have been removed. Separation of the sutured skin is often accompanied with little evidence of acute inflammation or pus formation and cultures obtained from the margins of the wound are often negative, failing to yield responsible organism. Critical colonization and biofilm formation may also be the cause of failed split thickness skin grafts. Several unanswered questions remain, particularly for acute surgical wounds: does biofilm formation turn an acute wound into a chronic one (this may be relevant in early diabetic foot ulcers); does biofilm formation precede donor or recipient site infection, or burns colonization prior to infection, particularly with Pseudomonas? Microbial attachment and biofilm formation may also occur within deep wounds and these biofilms would be clinically relevance in orthopaedic and vascular surgical site infections. For example, in a persister-cell state organisms sequestered within a biofilm attached to a prosthetic graft, may when conditions are optimal, begin to re-grow, thus perpetrating the infection. This may occur many months post-surgery and in the case of late-onset vascular graft infection occur as an occult process. The use of an antimicrobial suture technology, with a prolonged antiseptic release, may be a valid

strategy for preventing initial microbial adherence in an environment of wound contamination (Edmiston et al. 2013b).

3.6 Methods for Biofilm Removal: Maintenance Debridement

Table 3.1 identifies the major strategies for wound debridement. Biofilms in wounds often defy eradication and in selective cases can only be suppressed, rather like the suppression of MRSA colonization using mupirocin plus chlorhexidine to reduce the risk of SSI; however biofilm decolonization is unlikely (Edmiston et al. 2013b). Debridement should therefore be undertaken regularly, at least weekly has been suggested in critical ischemia (Wolcott and Rhoads 2008). Biofilm suppression can be effective in surgical wounds using similar methods used for macro-debridement of slough and necrotic material in preparation of the wound bed; both should be undertaken simultaneously at dressing changes (Wolcott et al. 2009). The concept of "preparation of the wound bed" is taken from plastic surgical practice in which a recipient wound site is made as clean and receptive as possible for a split thickness skin graft. In open wounds, where the infection and biofilm is controlled by these same techniques, healing can progress successfully by secondary intention with adequate wound and dressing care alone; coupled of course with attention to holistic and correction of underling disease processes (Leaper et al. 2012a). The use of regular maintenance debridement has been shown to enhance therapeutic interventions using topical antimicrobials and dressings to reverse delayed or stalled healing, presumably by delaying biofilm reformation (Wolcott et al. 2010b; Phillips et al. 2010a).

Table 3.1 Current debridement methodologies

Surgical (use of anaesthesia with scalpel and scissors)
Mechanical (including "wet-to dry") and sharp (loop curette)
Wound irrigation (at all dressing changes, including antimicrobials)
Hydrosurgery (pulsed lavage)
Autolytic (hydrogel and hydrocolloid dressings)
Enzymatic (collagenases)
Larval (maggot) therapy
Chemical (antiseptics/hypochlorite's)
Antimicrobial dressings (silver, PHMB, povidone-iodine)
Negative pressure wound therapy (including antimicrobials)
Others (ultrasound, laser, electrical, hyperbaric oxygen)

All the methods of "macro"-debridement shown in Table 3.1 can equally be used for maintenance debridement of biofilm from critically colonized chronic, infected or dehisced surgical or burn wounds. It is probably universally accepted that open wounds heal optimally with debridement of any type; the more complete the debridement is (with removal of biofilm), the more the frequency of intervention can be lessened (Wolcott et al. 2009; Leaper et al. 2012b; Cardinal et al. 2009; Falanga et al. 2008). Methods of surgical, mechanical or sharp debridement are the most widely used at dressing changes at which the wound can also be cleansed or irrigated (with or without antiseptics). An even more effective removal of biofilm, and slowing its reformation, may be expected from the use of hydrolavage and negative pressure wound therapy (Vanwijck et al. 2010; Caputo et al. 2008; Mosti et al. 2005; Allan et al. 2010). The use of antiseptics at dressing changes to complement wound cleaning, irrigation and debridement reduces bacterial colonization, biofilm formation and reformation. This principle has also been used successfully in NPWT and instillation techniques. Polyhexamethylene biguanide (PHMB) has been shown to be effective for NPWT-instillation but other antiseptics such as cadexomer-iodine and silver as irrigants or in dressings are also effective in control of bacterial colonization and biofilm formation (Allan et al. 2010; Andriesson and Eberlein 2006; Kaehn and Eberlein 2009; Phillips et al. 2010b; Sibbald et al. 2011; Leaper et al. 2011, 2012, 2013; Back et al. 2013; Dowsett 2013; NIHCE 2013). The use of topical antimicrobials such is more effective after the biofilm has been disrupted by debridement (Dowsett 2013).

The practicality of who is responsible for maintenance debridement, or who is qualified to undertake it, may be abrogated by the use of effective but slower techniques such as enzymatic, larva-therapy or autolytic debridement. A simple technique that can be used by a wide range of general practitioners involves the use of a soft, monofilament fiber pads which can be used to regularly "brush" the wound, just as a biofilm can brushed off teeth and gingiva (White 2011). This technique however is somewhat superficial and does not compare with sharp, surgical or loop curette debridement or even the use of a surgical brush. The requirement for a qualified wound nurse to oversee this process has been questioned but regardless there are plenty of opportunities to learn and develop confidence with a myriad of debridement strategies, offered widely through wound healing societies and workshops (White 2011).

3.7 Future Strategies for Identify and Managing Chronic Surgical Wound

What's next in biofilm research that will benefit the future of wound care? The use of a diagnostic criteria that would hasten the recognition of the presence of a biofilm at the patient bedside and give proof of adequate suppression after treatment will clearly be welcome (Alavi et al. 2012; Percival 2011). This could be based on PCR technology, which would help to "fingerprint" which micro-organisms were present in wound and in what numbers, despite a negative conventional swab and microbiological analysis. Other diagnostic strategies might include detection of signaling molecules, bacterial products or the use of host cell lines. Alternative innovative diagnostics could also aid in deciding on the best method of general and maintenance debridement, including new technologies, together with monitoring and how often debridement would be needed, and specific targeting with antimicrobials (Attinger and Wolcott 2012; Dissemond et al. 2011). The eradication of wound biofilm, once there are accurate diagnostics, may come with the development of quorum-sensing inhibitors or other anti-biofilm modalities (Rhoads et al. 2008; Sun et al. 2008; Wolcott and Cox 2013; Zhao et al. 2013). In acute wounds, which are at risk, perhaps the targeted prophylactic use of antimicrobial agents may become an appropriate element of an effective wound care bundle.

3.8 Treatment and Management of Biofilm-Mediated Infections

At present there are no evidence-based studies focusing on the therapeutic efficacy of selective strategies for managing biofilm-associated surgical site infections. However, in general the current therapeutic options can be characterized succinctly as follows:

(a) Tissue-Based Infection: Surgical debridement to remove devitalized tissue, followed by copious irrigation preferable with a biocide agent followed by parenteral antibiotics (Barnes et al. 2014; Cardinal et al. 2009; Edmiston et al. 2013c; Leaper et al. 2011).
(b) Device-Related Infection: Removal of an infected device followed by insertion of antimicrobial adjunctive technology such as antimicrobial spacer, beads or suture technology plus parenteral antibiotics (Del Pozo and Patel 2009; Edmiston et al. 2013d; Griffin et al. 2012).
(c) Antimicrobial Agents: Selection of therapeutic or agents that appear to penetrate microbial biofilms include linezolid, daptomycin, rifampin and possibly ceftaroline (Edmiston et al. 2006b; Seaton et al. 2013; Barber et al. 2014).

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Peri-Implant Infections of Oral Biofilm Etiology

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Georgios N. Belibasakis, Georgios Charalampakis, Nagihan Bostanci, and Bernd Stadlinger

Abstract

Biofilms are complex microbial communities that grow on various surfaces in nature. The oral micobiota tend to form polymicrobial biofilms, particularly on the hard mineralized surfaces of teeth, which may impact on oral health and disease. They can cause inflammation of the adjacent tooth-supporting (periodontal) tissues, leading to destructive periodontal disease and tooth loss. The emergence of osseointegrated dental implants as a restorative treatment option for replacing missing teeth has also brought along new artificial surfaces within the oral cavity, on which oral bacteria can form biofilms. As in the case of natural teeth, biofilms on implant surfaces may also trigger infection and cause inflammatory destruction of the peri-implant tissue (i.e. peri-implantitis). While there are strong similarities in the composition of the mixed microbial flora between periodontal and peri-implant infections, there are also a few distinctive differences. The immunological events underlying the pathogenesis of peri-implant infections are qualitatively similar, yet more extensive, compared to periodontal infections, resulting in a faster progression of tissue destruction. This chapter summarizes the current knowledge on the microbiology and immunology

G.N. Belibasakis (✉)
Section of Oral Microbiology and Immunology, Institute of Oral Biology, Center of Dental Medicine, University of Zürich, Plattenstrasse 11, 8032 Zürich, Switzerland
e-mail: george.belibasakis@zzm.uzh.ch

G. Charalampakis
Department of Oral Microbiology and Immunology, Institute of Odontology, The Sahlgrenska Academy at University of Gothenburg, Gothenburg University, Box 450, Gothenburg 405 30, Sweden

N. Bostanci
Section of Oral Translational Research, Institute of Oral Biology, Center of Dental Medicine, University of Zürich, Plattenstrasse 11, 8032 Zürich, Switzerland

B. Stadlinger
Clinic of Oral Surgery, Center of Dental Medicine, University of Zürich, Plattenstrasse 15, 8032 Zürich, Switzerland

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of peri-implant infections, including findings from the peri-implant crevicular fluid, the inflammatory exudate of the peri-implant tissue. Moreover, it discusses the diagnosis and current approaches for the treatment of oral infections.

4.1 Biofilms in the Oral Cavity

The oral cavity consists of both soft mucosal tissue surfaces and hard dental tissues all bathed in constantly secreted saliva. This anatomical niche of the human body constitutes a dynamic ecosystem that is continuously colonized by commensal microorganisms, which are collectively defined as the oral microbial flora. They have evolved along with the host, while their survival is tightly dependent on their capacity to use the available nutrients for their growth and their adaptability to the host's innate and adaptive immune system. It is estimated that the diversity of oral microbiota accounts for more than 700 different species, with at least 100 species populating the oral cavity of a given individual (Aas et al. 2005; Paster et al. 2001). The oral bacteria rarely grow in single planktonic form, but they naturally form biofilm communities with each other on the tooth surface. Biofilms exhibit a very high level of structural and functional bacterial organization, whereby the individual bacterial constituents communicate with each other by finely tuned molecular processes (also defined as "quorum sensing") (Huang et al. 2014). Biofilms demonstrate much more virulent characteristics compared to bacteria in planktonic state, as they exhibit altered gene expression patterns and are less penetrable by neutrophils, antibodies, or antimicrobial factors (Schaudinn et al. 2009), even by a factor of 500 (Costerton et al. 1995). Clinically, the "dental plaque" forming on the tooth surface holds the full properties of a biofilm (Marsh 2003).

Changes in the local microenvironment may cause shifts in the composition of the biofilm microflora, giving leeway to certain bacterial species to overgrow, enhance their virulence properties and eventually become opportunistic pathogens. Such species may be found at low numbers in health and can become pathogenic only when the newly established conditions permit them so. This is the principle drive for the "ecological plaque hypothesis", the predominant theory that explains the etiology of the polymicrobial oral diseases as a disturbance of the relationship between the resident oral microbiota and the response of the host that they populate (Marsh 2003; Marsh and Devine 2011). Dysbiotic biofilms can endure the host response and concomitantly exploit the inflammatory host response, in a manner that propagates the magnitude of the inflammatory tissue destruction, as is the case of bone loss in periodontitis (Hajishengallis and Lamont 2012; Hajishengallis 2014).

4.1.1 Biofilms and Oral Disease

Dental caries and periodontal diseases are the two main and highly prevalent oral diseases, both caused by biofilms growing on the tooth surface. Dental caries manifests essentially as the demineralization of the hard dental tissues (namely enamel and dentine), by acids generated due to the fermentation of dietary carbohydrates by the biofilms grown on the tooth surface. Its incidence has increased with sugar consumption and it is among the most prevalent infectious diseases in the industrialised world. If dental caries remains untreated, the biofilm-associated bacteria can eventually invade into the deeper soft dental pulp tissue, causing pulpitis, and subsequently tooth necrosis. Periodontal diseases are a major group of biofilm-associated oral diseases that destroy the tooth-supporting (periodontal) tissues as a result of excessive inflammatory response of the juxtaposed gingival tissue. Etiologically, they are

attributed to polymicrobial biofilms accumulated on the tooth surface, and particularly under the gingival margin (subgingival). The inflammation can be contained within the gingival tissue (gingivitis), and manifests as swelling and bleeding of the gingiva, symptoms that are easily identifiable by the patient. This condition affects virtually the whole global population, and is reversible once the biofilm is removed from the tooth surface and proper oral hygiene is instilled. Persistence and progression of an exacerbated inflammatory response can destroy the deeper periodontal tissues, namely the periodontal ligament that links the tooth surface to the supporting alveolar bone. The disease has now progressed to the stage of periodontitis which, apart from the loss of supporting bone and periodontal ligament, is also characterised by the formation of deep periodontal pockets. If left untreated, periodontitis will result in exfoliation of the affected tooth, impairing the chewing function and compromising the esthetic appearance. Periodontitis is the main cause of tooth loss in the industrialised world, and is perhaps the most prevalent inflammatory infectious disease in human adults, affecting approximately 1/3 of the population. One of the restorative treatment options for replacing teeth missing due to periodontitis is dental implants.

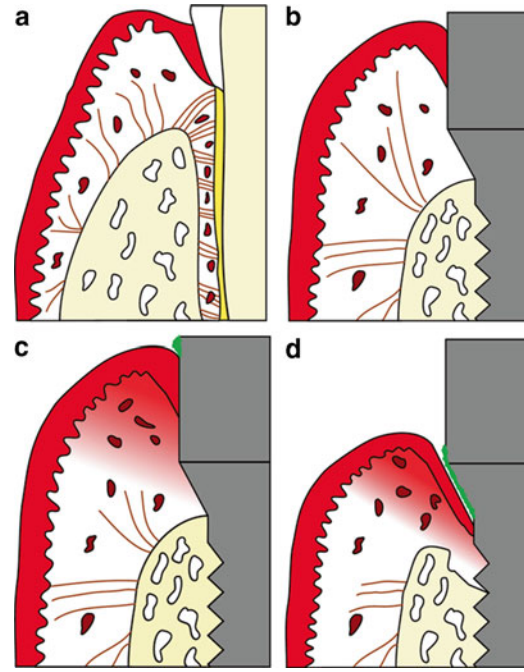

Fig. 4.1 Schematic representation of periodontal (**a**) and peri-implant tissues (**b**). The lack of periodontal ligament from the peri-implant tissues is evident. Accumulation of biofilm (marked *green*) on the implant surface will eventually result in inflammation of the peri-implant mucosa and establishment of peri-implant mucositis (**c**). Progression of this inflammation can lead to the destruction of the peri-implant tissues, including the supporting bone, culminating in peri-implantitis (**d**)

4.2 Dental Implants and Comparison to Natural Teeth

Dental implants are titanium-based screw-like devices that are surgically installed into the jaw bone, in the place of one or more missing teeth. Thereafter, a transmucosal abutment is adapted onto the implant, mediating the connection to the final prosthetic restoration that is visible in the patient's oral cavity. Hence, the patient's functional and esthetic needs are re-established. The titanium surface is biologically "accepted" by the surrounding bone tissue, and forms a connection known as "osseointegration". This titanium-bone relationship possesses the essential functional properties required to support the replacement of a missing tooth.

There is merit at this stage to define the main dissimilarities between periodontal and peri-implant tissues, in order to better understand peri-implant infections, or diseases (Fig. 4.1a, b). In the case of dental implants, the main difference with natural teeth is the absence of periodontal ligament, thus necessitating direct interface between the bone and the implant surface (Heitz-Mayfield and Lang 2010). Instead, the collagen fibers of the submucosal connective tissue are arranged parallel to the implant surface, thus forming a "collar". Consequently, the formed peri-implant crevice is deeper than the gingival crevice of natural teeth, resulting in a weaker physical barrier against bacterial invasion. Apart from very restricted mobility, the lack of the periodontal ligament also means restricted blood supply. Hence, the delivery of cells of the immune system, needed to tackle the early stages of

bacterial infection, is rather compromised. Collectively, these characteristics may render dental implants more susceptible to endogenous infection, than natural teeth. An exposed dental implant surface is also prone to microbial colonization. Thus biofilms can as well form on implants, with potential detrimental effects on the health of the surrounding peri-implant tissues.

4.3 Peri-Implant Infections: Classification and Diagnosis

Failures of dental implant function are classified either as early, or as late ones (Listgarten 1997; Tabanella et al. 2009). The early ones refer to incomplete osseointegration following surgical installation, and may be attributed to early loading, surgical contamination, poor compatibility of the material, or inefficient healing due to systemic disease. In late failures, the normal function of an already osseointegrated implant is disrupted, resulting from chronic infection of the peri-implant tissues.

Peri-implant mucositis is characterized by biofilm-induced inflammation localized on the soft peri-implant mucosa, but with no evidence of destruction of the supporting bone (Fig. 4.1c). Progression of the inflammation may lead to gradual destruction the bone, manifesting as peri-implantitis (Fig. 4.1d). Peri-implant mucositis and peri-implantitis are analogous to gingivitis and periodontitis of natural teeth (Heitz-Mayfield and Lang 2010). Mucositis occurs in approximately 80 % of patients with dental implants, and 50 % of the implants. The prevalence of peri-implantitis has varied reportedly from 28 to 56 % among patients, and 12 to 43 % among implants (Zitzmann and Berglundh 2008).

To-date the diagnosis of peri-implant diseases is based on clinical and radiographic criteria (Mombelli and Lang 1998; Kao et al. 1997). While mucositis is characterized by inflammation, erythema and bleeding of the tissue particularly during examination, peri-implantitis exhibits additionally a peri-implant pocket which is greater than 4 mm, potentially suppuration, and a characteristic "saucer-" or "crater-shaped" bone destruction around the implant, which is revealed radiographically (Heitz-Mayfield 2008). This biological complication around dental implants is characterized by profound inflammation of the tissues surrounding an implant in function with progressive loss of supporting bone (Lindhe and Meyle 2008). Peri-implantitis is becoming a pathological entity of growing concern among clinicians because of its aggressive pattern, and in certain cases rapid reach of the terminal stage i.e. implant loss.

4.4 Peri-Implantitis as a Biofilm-Initiated Disease

Despite the early introduction of the term "peri-implantitis" (Levignac 1965; Lindhe and Meyle 2008), the infectious nature of the disease was documented almost two decades later (Rams and Link 1983; Rams et al. 1991). Treatment with osseointegrated implants was introduced in fully edentulous patients, and in such individuals there was no biological rationale to consider post-osseointegration infections, since periodontitis-associated bacteria were to be automatically 'removed' from the oral cavity together with the extracted teeth. Late implant failure was explained during many years by overloading or excess loading. However, a recent systematic review (Naert et al. 2012) clarified that no association between overload and peri-implant bone loss could be found in the absence of peri-implant inflammation. Indeed, research should address critical questions with regard to the etiopathogenesis of peri-implantitis, which in turn would guide evidence-based treatment.

Given the non-shedding surface of the dental implant in the oral cavity, it is easy to understand why biofilm formation on the implant is an inadvertent process. The mouth provides not only a portal of entry for bacteria but also an inherent environment for bacterial colonization and growth. In a similar fashion to the tooth, by the time the dental implant is exposed in the oral cavity, it is covered by an acquired pellicle layer i.e. an organic stratum mainly consisting of proteins, glycoproteins and lipids. The pellicle triggers early bacterial colonization by providing receptors for the adhesins of specific species of oral

bacteria. Early colonizers are *Streptococcus* and *Actinomyces* species (Nakazato et al. 1989; Li et al. 2004) and create the preconditions for the accumulation of late-colonizing bacteria such as *Fusobacterium* and *Prevotella* species (Hannig 1997; Aas et al. 2005). The bacterial colonization of the surface starts already 30 min after implant insertion, and these early bacterial species can be found as part of the mixed biofilm community on the implant surface even several months later (Furst et al. 2007). The mature biofilm can eventually detach with dispersal and spread further, a critical stage for bacterial dissemination and consequent colonization of deeper tissue sites.

In terms of initial (i.e. 4 weeks) subgingival colonization, the frequency of detection of different species is similar between natural teeth and implants. Nevertheless, the colonization pattern on implants appears to be initially slower than on natural teeth (Quirynen et al. 2005), given the pristine surfaces of the implant and the lack of the desired indigenous microbiota. The bacterial composition of the biofilm formed on implants closely resembles that of the neighboring teeth (Botero et al. 2005; Salvi et al. 2008). This leads us to postulate that the oral microbial flora, and especially that of neighboring natural teeth, acts as a "reservoir" for the biofilms that build-up around implants.

4.4.1 Peri-Implant Microflora Resembling Periodontal Microflora

The peri-implant microflora in health consists mainly of Gram-positive cocci and non-motile bacilli, and a limited number Gram-negative anaerobic species, resembling gingival health (Mombelli et al. 1987; Bower et al. 1989). The switch to peri-implant mucositis is associated with increased presence of cocci, motile bacilli and spirochetes, comparable to gingivitis (Pontoriero et al. 1994). The transition to peri-implantitis is accompanied by emergence of Gram-negative, motile, and anaerobic species that are commonly found in periodontitis (Mombelli et al. 1987; Mombelli and Decaillet 2011). An interesting finding is that the microbial composition of peri-implant pockets in partially edentulous patients resembles that of the neighboring periodontal pockets, while the presence of *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* can be higher in peri-implantitis (Botero et al. 2005; Hultin et al. 2002; Shibli et al. 2008). Hence, the qualitative composition of the microbial flora of peri-implantitis-associated biofilms is in concordance with periodontitis. A representative microscopy image of a submucosal biofilm sample obtained from a site with peri-implantitis is provided in Fig. 4.2, whereby the diversity of morphotypes and taxa can be depicted. Finally, a finding of further interest is that some bacteria retrieved from peri-implantitis biofilms, most often *Prevotella intermedia/nigrescens* and *Streptococcus constellatus*, may display *in vitro* resistance to one or more standard antibiotic treatments (Rams et al. 2013).

4.4.2 Distinct Peri-Implant Microflora

By use of molecular techniques in microbiological analyses we have nowadays appreciated the breadth of microbial diversity in the subgingival/submucosal biofilms. Though it may sound logical that implants and neighboring teeth share similar microbiota since they share a similar ecological niche i.e. interdental space, emerging evidence suggests that they could be microbiologically distinct from each other (Kumar et al. 2012; Dabdoub et al. 2013; Heuer et al. 2012). By use of broad-range PCR techniques (Heuer et al. 2012) and pyrosequencing (Kumar et al. 2012; Dabdoub et al. 2013) it was demonstrated that the peri-implant microbiome was distinct from the periodontal microbiome. Given the distinct topography and immunological characteristics of the implant compared to the tooth, the two ecosystems could be regarded divergent. This could explain why teeth and implants may harbour diverse bacterial lineages.

A number of microorganisms have been identified in peri-implantitis that are less regularly detected in periodontitis. These include, but not

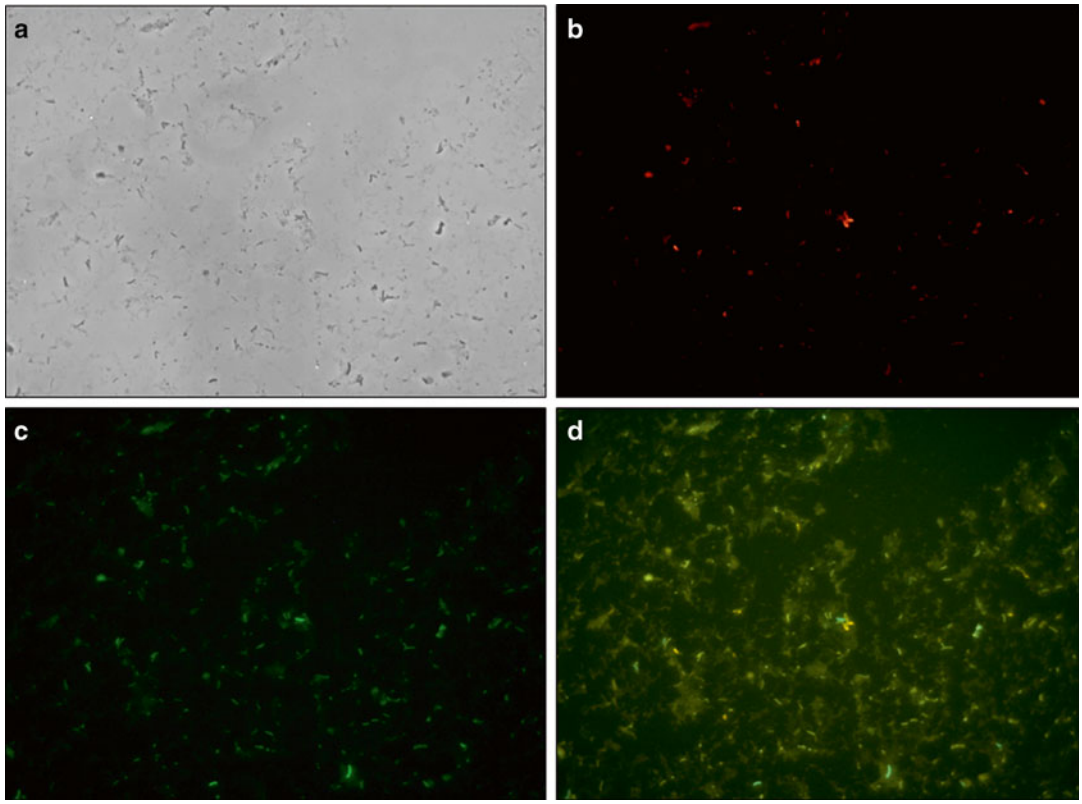

Fig. 4.2 Inverted light microscopy image of a submucosal biofilm sample obtained from a site with peri-implantitis (**a**). Epifluorescence microscopy image of the same field, combined with fluorescence *in situ* hybridization (FISH) using a 16S rRNA-targeted oligonucleotide probe for oral *Spirochaetes* (**b**), oral *Synergistetes* (**c**), or overlap of both (**d**)

restricted to, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterobacter aerogenes*, *Enterobacter cloace*, *Escherichia coli*, *Helicobacter pylori*, *Pseudomonas* spp, as well as *Candida* spp fungi (Renvert et al. 2007; Rams and Link 1983; Persson and Renvert 2014; Belibasakis 2014; Rams et al. 1991; Leonhardt et al. 1999, 2002, 2003). *S. aureus* is a versatile human pathogen discussed extensively in orthopedics as the leading etiologic agent of implant infection and of the associated osteomyelitis (Arciola 2009). It has a wide array of virulence factors, including up to 21 different adhesins or Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) and cytotoxins (Patti et al. 1994; Darouiche et al. 1997; Speziale et al. 2009). For this reason, it has attracted attention over recent years as a specific pathogen for peri-implantitis, distinct to periodontitis. The presence of *S. aureus* shortly after dental implant insertion can be confirmed even 1 year later (Salvi et al. 2008), while a recent microbiological study revealed by checkerboard methodology that significantly higher counts of *S. aureus* and *Staphylococcus anaerobius* were detected at implants with peri-implantitis than healthy implants (Persson and Renvert 2014).

Aerobic Gram negative bacilli (AGNB) include two wide and distinct categories: (i) bacteria that ferment lactose and belong to the large family *Enterobacteriaceae* (i.e. *E. coli. Enterobacter*, *Klebsiella*, *Citrobacter*), and (ii) non-enteric rods that do not ferment lactose (i.e. *Pseudomonas aeruginosa*, *Acinetobacter baumannii*). In a retrospective investigation of peri-implantitis cases (Charalampakis et al. 2012) culture analysis demonstrated the presence

of aerobic Gram-negative bacilli at moderately heavy or heavy growth in 18.6 % of patients with peri-implantitis. However, the microbial burden could not fully correspond to peri-implant disease severity. AGNB have been detected in previous studies both in peri-implantitis cases (Botero et al. 2005; Alcoforado et al. 1991; Leonhardt et al. 1999; Rosenberg et al. 1991), as well as in healthy implants (Nowzari et al. 2008b; Leonhardt et al. 1999; Nowzari et al. 2008a). Thus, the true role of AGNB in the etiology of peri-implant infections remains unclear.

4.4.3 Bacteria in the Implant-Abutment Interface

A bacterial leakage along the implant-abutment interface has been also discussed in the literature (do Nascimento et al. 2008, 2009; Gross et al. 1999; Callan et al. 2005). Given the fact that the interface includes microgaps ranging from 10 to 100 μm, we cannot exclude the scenario that microorganisms of 2 μm or less in diameter penetrate the passive fit between the implant components. Poor fit of attached components, inadequate torque, geometry of the implant platforms between the various implant systems, poor stability and micro-movements in the deeper inner portions of the system may enhance the extent of bacterial leakage (Binon 2000; Aloise et al. 2010). Such microgaps may function as 'nests' for anaerobic or microaerophilic bacteria to be protected form host defense mechanisms and persist for extended periods. However, the hermetic closure of a contaminated small compartment would serve as entombment, creating unfavorable conditions for bacteria to grow. Thus, the risk for peri-implant infection around two-part implant systems because of microbial leakage per se should be considered minimal.

4.4.4 Effect of Implant Surface on Biofilm

Rough implant surfaces were introduced in the dental market in order to enhance the rate of osseointegration. However, the implant surface roughness has a significant impact on the quantity and the quality of the plaque formed. A rough surface structure characterized by grooves and pits may provide the bacteria with 'protected' areas, inaccessible to conventional mechanical removal (Renvert et al. 2008). Other surface characteristics that enhance initial bacterial adhesion are high wetability and great surface free energy (Teughels et al. 2006). A recent *in vivo* study on the biofilm structure formed on titanium discs with different surface characteristics revealed different microbial patterns (Charalampakis et al. 2014). By SEM analysis it was demonstrated that the discs representing the moderately rough surfaces (Osseospeed™, TiOBlast™, Experimental surface) harbored a complex biofilm with tight intercellular bacterial bindings, whereas the discs with the turned surface hosted a biofilm that presented a pattern of spread bacteria forming less clusters. The study concluded that variations in the biofilm pattern may be associated with the different surface characteristics of titanium discs.

However, there is limited and contradictory evidence on the impact of implant surface on peri-implantitis. Some studies have found a positive correlation between smooth surface and peri-implant health (Astrand et al. 2004; Esposito et al. 2007), whereas others failed to find a correlation between type of implant surface and marginal bone loss (Gotfredsen and Karlsson 2001; Wennstrom et al. 2004). Nevertheless, it is also shown that surface characteristics of the abutments may not influence biofilm formation, or the extent and cellular composition of the inflammatory lesion (Zitzmann et al. 2002). Accordingly, no implant system or surface type was found to be superior in terms of marginal bone preservation (Abrahamsson and Berglundh 2009).

Last but not least, like in natural teeth, implant surfaces are immediately populated by salivary mucoproteins, which are required for the adhesion of bacteria (Kolenbrander et al. 2010). These are genetically defined in each individual, and may coat the surfaces of both natural teeth and implants, before being recognized

by the same bacterial species. It is therefore tempting to postulate that potential differences in bacterial adhesion due to surface microstructure may partially be equilibrated by the mediating salivary pellicle (Busscher et al. 2010). Hence, given the inevitable mediation of the pellicle in bacterial adhesion, implant surface characteristics may not notably affect the initial stages of biofilm formation.

4.5 Histopathological Events in Peri-Implant Infections

Like in the case of natural teeth, the development of a biofilm on the implant surface is an igniting factor of the inflammatory response of the surrounding peri-implant tissues. Peri-implant mucositis is characterized by inflammation that is confined to epithelium, connective tissue loss, microvascular changes (Sanz et al. 1990), and increased infiltration of leukocytes (Zitzmann et al. 2002, 2004). The sequence of inflammatory events that take place in peri-implant mucositis is similar to those in gingivitis, but potentially of a larger extent than gingivitis (Ericsson et al. 1992; Berglundh et al. 1991, 1992). The switch to peri-implantitis is accompanied by a further influx of inflammatory cells into the affected area of the peri-implant mucosa, that now expands to reach the bone tissue (Gualini and Berglundh 2003; Talarico et al. 1997; Lindhe et al. 1992), while a large number of osteoclasts form onto the bone surface and initiate bone resorption (Carcuac et al. 2013).

4.5.1 Immune Responses to Biofilm in Peri-Implant Infections

The histopathological events associated with peri-implant infections have been characterized over the past two decades. Nevertheless, there are still pending questions regarding the molecular regulatory events underlying these described processes. Peri-implant mucosal tissue biopsies, as well as inflammatory tissue exudates are suitable biological material to investigate in depth the molecular events associated with peri-implant diseases. In that respect, diseased tissue obtained from peri-implantitis sites is shown to exhibit higher expression of several mediators of inflammation, including pro-inflammatory cytokines interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)-α, compared to healthy or peri-implant mucositis sites (Venza et al. 2010; Konttinen et al. 2006; Duarte et al. 2009b). Although tissue biopsies can give an actual view of the undergoing molecular events within the tissue, the invasiveness of the collection process makes it almost impossible to use this material on a regular basis. In contrast, the collection process of the inflammatory exudate of the peri-implant tissues, namely the peri-implant crevicular fluid (PICF), is much simpler and non-invasive. This topic is discussed further in the next section.

4.5.2 Peri-Implant Crevicular Fluid as a Reservoir of Inflammatory Mediators

The PICF is the inflammatory exudate of the peri-implant sulcus or crevice, which is the tight anatomical depending formed between the implant surface and the peri-implant mucosa. This niche can be converted into a peri-implant pocket as peri-implantitis progresses, fostering a submucosal biofilm. Similarly to gingival crevicular fluid (GCF) which bathes natural teeth, PICF is the outcome of increased permeability of the vessels within the underlying connective tissue, as an inflammatory response to the growing biofilm. Although, the molecular characterization of PICF is at its infancy compared to GCF, it is already known to contain several serum and locally produced molecules, such as tissue breakdown products, inflammatory mediators and antibodies directed against the bacteria of the biofilm (Adonogianaki et al. 1995). Therefore, analysis of the PICF might be suitable to evaluate the inflammatory status of peri-implant tissues, in a quantitative manner (Kaklamanos and Tsalikis 2002; Belibasakis 2014).

In healthy peri-implant tissues, the diffusion of PICF is rather passive and slow. However, its

volume amount is significantly increased at a given site once biofilm-induced inflammation is established (i.e. peri-implant mucositis). Human experimentally induced peri-implant mucositis studies have elegantly showed that both PICF volume and protein content increases by the end of the 3-week period of plaque accumulation protocol (Salvi et al. 2012). Despite the strong clinical similarities between human experimental gingivitis and peri-implant mucositis, the latter is presented with a more pronounced inflammatory response to biofilm accumulation (Salvi et al. 2012; Pontoriero et al. 1994; Schierano et al. 2008). Since the composition of PICF is modified along with the histopathological changes during the course of progressive inflammation, its molecular analysis may support the early detection of clinically undetectable changes.

Pro-inflammatory cytokines have been the primary candidates to be investigated in PICF, due to their central role in triggering the inflammatory process, and the good amount of knowledge that already derives in studies on GCF. It was confirmed that higher concentrations of TNF-α, IL-17 and IL-1β are present in PICF collected from peri-implantitis-affected sites, compared to healthy controls (Ataoglu et al. 2002; Curtis et al. 1997; Darabi et al. 2013; Vieira et al. 2013; Severino et al. 2011), whereas the opposite was the case for anti-inflammatory cytokine IL-10 (Casado et al. 2013). More recently multiplex cytokine arrays have been applied in the analysis of PICF, allowing for a broader simultaneous screening of multiple inflammatory cytokines and chemokines. In one such study, 12 markers were assessed in both peri-implant health and disease, including granulocyte macrophage colony-stimulating factor (GM-CSF), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, interferon (IFN)-γ and TNF-α (Fonseca et al. 2014). In line with previous findings, there were no differences with regards to IL-6, IL-8, IL-10 (Severino et al. 2011), whereas the levels of IL-1β were significantly higher at peri-implantitis, compared to mucositis sites. Further studies also showed that peri-implantitis treatment reduced the PICF levels of IL-1β (Bassetti et al. 2013) and TNF-α (de Mendonca et al. 2009; Duarte et al. 2009a). Studies have also demonstrated that single nucleotide polymorphisms (SNP) in the IL-1 gene may hold an increased risk for the development of peri-implantitis, particularly when combined with smoking (Andreiotelli et al. 2008; Bormann et al. 2010). However, the presence of a specific SNP does not necessarily translate into higher levels of IL-1β in PICF (Lachmann et al. 2007; Jansson et al. 2005; Melo et al. 2012). Although there is good evidence of the involvement of IL-1β as a crucial mediator of the host inflammatory response in peri-implant tissues (Murata et al. 2002; Salvi et al. 2010; Ataoglu et al. 2002), this needs to be complemented with further interventional studies, whereby IL-1β inhibitors are part of the treatment. Moreover, other members of the IL-1 family, such as IL-18, which may display differential regulation from IL-1β, should also be considered in PICF (Hamedi et al. 2009; Bostanci et al. 2009).

Matrix metalloproteinanses (MMPs) are proteolytic enzymes with a strongly documented role in collagen degradation in various disease processes, including periodontitis (Sorsa et al. 2006). Their involvement in peri-implant tissue destruction has also received attention, and a number of studies have demonstrated that MMP levels in PICF from peri-implantitis sites are elevated compared to healthy sites, and that their enzymatic activity increases with disease severity (Kivela-Rajamaki et al. 2003a, b; Ozcakir-Tomruk et al. 2012; Xu et al. 2008; Teronen et al. 1997), while successful treatment results in reduction (Wohlfahrt et al. 2014; Salvi et al. 2012). Moreover, studies to determine whether MMP-8 is useful prognostic marker for peri-implantitis have shown that sites with higher PICF levels of MMP-8 are at greater risk for progressive bone loss (Arakawa et al. 2012).

As bone resorption is the hallmark of peri-implantitis, the regulation of osteoclastogenesis and osteogenesis-associated markers have also been studied in PICF. Similarly to findings in GCF obtained from sites with periodontitis (Belibasakis and Bostanci 2012), there is increasing evidence showing the association of the receptor activator of nuclear factor kappaB

ligand (RANKL) – osteoprotegerin (OPG) system with the occurrence and severity of peri-implantitis (Arikan et al. 2008, 2011; Sarlati et al. 2010; Guncu et al. 2012; Duarte et al. 2009a, b; Rakic et al. 2013). A few studies looking into other markers of bone metabolism, such as osteocalcin, reported that these are higher in PICF from peri-implant mucositis compared to healthy sites, whereas no differences were observed between peri-implantitis and either mucositis or health. Hence, elevated levels of osteocalcin in PICF may reflect increased local bone turnover around implants, rather than severe bone resorption (Murata et al. 2002).

4.6 Treatment of Peri-Implant Infections

The development of peri-implantitis shows comparable features to the development of periodontitis (Heitz-Mayfield and Lang 2010). Clinical treatment of peri-implantitis is performed by various means and there is currently no consensus on an official standard of care. Therapy generally aims at the settlement of inflammatory peri-implant processes and the preservation of hard and soft tissues, as evaluated by reduced bleeding on probing and reduced probing depth or stable radiographic bone level, respectively (Heitz-Mayfield and Mombelli 2014). Four phases of treatment are suggested in order to enable the successful treatment of peri-implantitis: (1) pre-treatment phase (oral hygiene, prosthodontic aspects), (2) surgical access (mucoperiosteal flap, bone substitute with or without membranes), (3) post-operative anti-infective control (systemic antibiotics, chlorhexidine rinses), (4) maintenance care (3–6 months) (Heitz-Mayfield and Mombelli 2014).

Supportive periodontal therapy is seen as means to reduce the likelihood of an onset of peri-implantitis (Salvi and Zitzmann 2014). Presurgical therapy of peri-implantitis should include measures of oral hygiene. These mostly result in a reduction of mucositis, by targeting the disruption of the associated biofilm (Mishler and Shiau 2014). Also air abrasive powders and laser treatment have been applied for the reduction of biofilms on implant surfaces (Mishler and Shiau 2014; Schwarz et al. 2013). Further, the mechanical debridement of the implant surface should be performed, using instruments that cause little trauma to the surface, antiseptics and possibly antibiotics (Lang et al. 1997). Surgical therapy enables the debridement of granulation tissue within a peri-implant defect and the possible performance of an implantoplasty by diamond burs, smoothening implant threads and structured implant surfaces to a polished state. Different surface topographies show differences in their susceptibility for peri-implant inflammation. During surgical therapy, the mechanical implantoplasty modifies the surface state, depending on the implant material (e.g. titanium grade 4) and the prior surface treatment (e.g. sandblasted, acid etched). This causes differences in post-surgical peri-implant bone formation (Albouy et al. 2011).

An additional regenerative step may be the application of bone replacement substances with or without membranes, aiming at peri-implant hard tissue regeneration. The desired aim is the re-osseointegration of a previously biofilm-covered surface. This regenerative therapy may be influenced by the peri-implant defect morphology and the absence or presence of keratinized mucosa (Schwarz et al. 2010). The type of prosthodontic restoration also influences the surgery. The operative site can be easily assessed through the removal of screw-retained implant crowns. In case of cemented crowns, debridement and implantoplasty may be limited to the buccal and approximal areas. A meta-analysis of treatment outcomes identified four surgical procedures, namely (a) access flap and debridement, (b) surgical resection, (c) regeneration with bone grafts, and (d) guided bone regeneration. A reduction in probing depth of 2–3 mm and a mean 2 mm radiographic bone gain is described for regenerative procedures. Most of these analyses have follow-ups of 1–2 years (Chan et al. 2014). Outcomes for regenerative approaches are described to vary the most.

Following surgical therapy, the onset of oral hygiene measures by the patient is important for

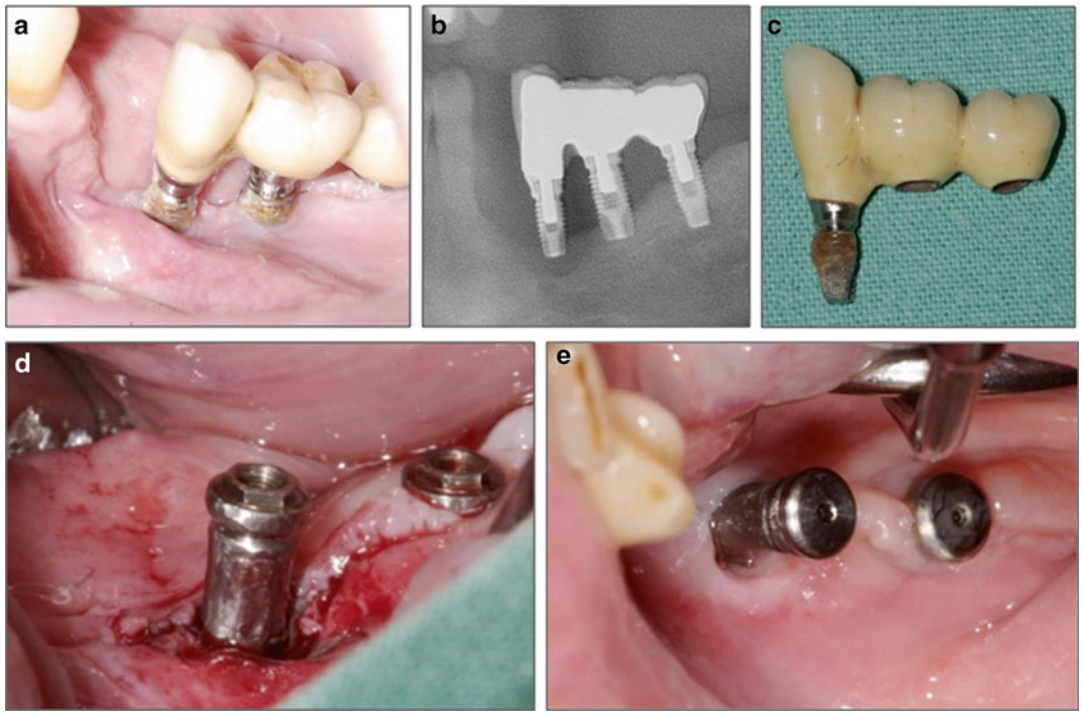

Fig. 4.3 Intraoral images of a patient with peri-implantitis. The implant threads are exposed to the oral cavity due to the destruction of the supporting bone. Large biofilm deposits have been accumulated over time on the implant surface (**a**). Accordingly, the radiographic image of the same site demonstrates severe bone loss around the implants. In fact, the implant on the left has no supporting bone at all (**b**). This implant was explanted (**c**), while the remaining two implants were maintained and underwent treatment that involved removal of the granulation tissue, implantoplasty and surface decontamination (**d**). The remission of inflammation and healing is evident already 2 weeks following the completion of the surgical treatment (**e**). Gingival healing caps were placed on the implants, and later on the prosthetic components were adapted, so that the implant function and esthetics are restored

long-term treatment success. It is important to maintain periodontal care over many years, as peri-implantitis may occur 5–10 years after implant placement and may reoccur at any time after treatment. Bone loss >2 mm post-prosthetic treatment and bleeding on probing could be indicators of peri-implantitis (Klinge 2012). A probing depth threshold of 5 mm without any bleeding on probing may predict the cessation of bone loss and the successful outcome of peri-implantitis therapy (Klinge 2012; Heitz-Mayfield and Mombelli 2014) In case of an onset of bone loss, implant mobility will follow. In such cases implant removal, debridement of the peri-implant defect, tissue regeneration and possibly re-implantation will follow. A clinical example of a peri-implantitis case is provided in Fig. 4.3.

4.7 Concluding Remarks on Peri-Implant Infections

It is evident that dental implant surfaces provide a suitable substrate for the growth of oral biofilms, in a similar manner as natural teeth. This is not without consequences, as the uncontrolled biofilm formation due to inefficient oral hygiene will eventually cause inflammation of the surrounding tissues in the form of peri-implant mucositis, and potentially lead to tissue destruction, manifesting as peri-implantitis. It is clear that the microbiota of the peri-implant biofilms derives from the various micro-ecological niches in the oral cavity, including the neighboring teeth, periodontal pockets, mucosal tissues and saliva. Although in principle the mixed microfolora of

peri-implant infections resembles that of periodontal ones, a number of non-typical oral taxa are more frequently found in peri-implantitis than periodontitis. Such are staphylococci, AGNB (e.g. enterobacteria and *Pseudomonas* spp) and *Candida* spp. The application of metagenomics in the analyses of biofilm samples is also likely to reveal specific microbial signatures in peri-implant infections. With regards to the pathogenesis of peri-implant infections the qualitative composition and sequence of the underlying immunological events resemble those of periodontal infections, but their magnitude is greater, thus resulting in a more aggressive progression of the disease. Hence peri-implant infections are "contemporary" infections caused by oral biofilms. They have emerged along with the continuous application of dental implants in restorative dentistry. While they are currently being treated in a similar philosophy as periodontal diseases, there is a need for reconsideration of their distinctive differences. This could lead to highly specialized therapeutic protocols, optimized for peri-implantitis.

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Microbiological Diversity of Peri-Implantitis Biofilms

5

Marcelo Faveri, Luciene Cristina Figueiredo, Jamil Awad Shibli, Paula Juliana Pérez-Chaparro, and Magda Feres

Abstract

The open-ended microbial diagnostic approaches such as the complete or partial sequencing of the 16S ribosomal gene by Sanger sequencing or by pyrosequencing have provided new insights into the diversity of the oral microbiota. These techniques have recently been used to evaluate the microbiota associated with osseointegrated implants and these results have expanded the knowledge on the diversity of the microbial communities associated with peri-implantitis. Taken together, the results of these studies suggest that the diversity of the microbial community of peri-implantitis and periodontitis might not be as similar as previously thought. Although certain known periodontal pathogens may also be associated with the etiology of peri-implantitis, apparently there were many differences between these two clinical conditions, involving distinct microorganisms. Further investigations on the diversity of peri-implant microbiota would be essential in order to define effective preventive and therapeutic strategies for peri-implantitis. It is also important to standardize laboratory protocols to make the results of the open-ended diagnostic techniques based on PCR amplification more comparable throughout the different research groups.

5.1 Introduction

Peri-implant diseases are characterized by the presence of an inflammatory process that affects the peri-implant tissues under loading (Mombelli and Lang 1998; Heydenrijk et al. 2002). The signs of this infection vary from a mild inflammatory process of the peri-implant mucosa, including bleeding on probing and suppuration, to clinical attachment and bone loss (Heitz-Mayfield 2008;

M. Faveri (✉) • L.C. Figueiredo • J.A. Shibli
P.J. Pérez-Chaparro • M. Feres
Department of Periodontology, Dental Research Division, Guarulhos University, Guarulhos, São Paulo, Brazil
e-mail: mfaveri@prof.ung.br

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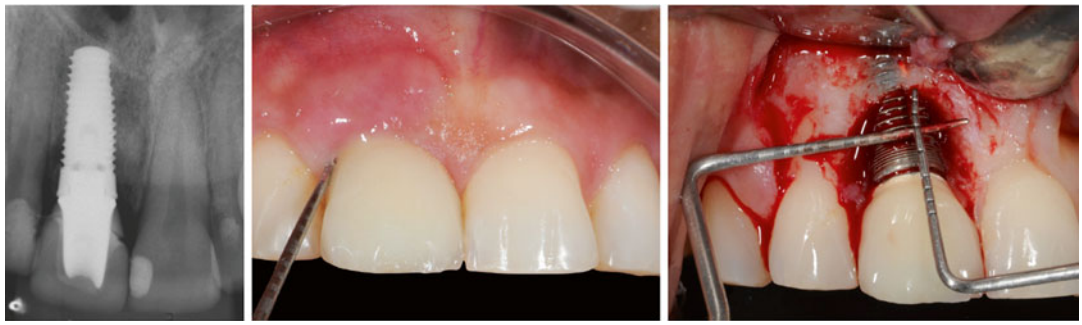

Fig. 5.1 Clinical and radiographic aspects of an implant with peri-implantitis

Zitzmann and Berglundh 2008). Similarly to periodontal diseases, peri-implant diseases result from a disruption in host-compatible/pathogenic microorganisms that may lead to two specific clinical situations: peri-implant mucositis, which is a lesion restricted to the peri-implant soft tissue, and therefore reversible; and peri-implantitis, which affects the soft tissue and the bone tissue adjacent to the osseointegrated dental implant (Mombeli 1999; Zitzmann and Berglundh 2008) (Fig. 5.1). Recent evidence has indicated that peri-implant mucositis may affect 63.4 % of subjects and 30.7 % of implants, and peri-implantitis 18.8 % of subjects and 9.6 % of implants (Atieh et al. 2013).

Microbiological studies have shown that the biofilm associated with implant failures differs substantially from that of healthy implants (Mombelli and Mericske-Stern 1990; Sanz et al. 1990; Leonhardt et al. 1999; Hultin et al. 2002; Quirynen et al. 2006; Renvert et al. 2007; Shibli et al. 2008). In humans, the subgingival biofilm around dental implants with clinically healthy marginal peri-implant tissues have demonstrated a microbiota with high proportions of coccoid cells, low proportions of anaerobic and Gram-negative species and a low prevalence of periodontal pathogens (Mombelli et al. 1987; Lee et al. 1999; Renvert et al. 2007; Shibli et al. 2008). In contrast, a peri-implantitis pocket seems to harbor a microbiota similar to that found in periodontitis, with high levels and proportions of *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans* (Becker et al. 1990; Mombelli 1993; Hultin et al. 2002; Leonhardt et al. 2003a, b; Quirynen et al. 2006; Shibli et al. 2008; Kumar et al. 2012). Therefore, it is assumed that the same pattern of colonization that occurs in healthy periodontal tissues or in periodontal disease may also occur around the subgingival surface of dental implants. This is a relevant piece of information and has direct clinical implications since the treatments proposed for peri-implant diseases have been based on this microbial similarity.

The introduction of open-ended microbial diagnostic approaches in the early 2000s, such as complete or partial sequencing of the 16S ribosomal gene by Sanger sequencing or by pyrosequencing have provided new insights into the diversity of the oral microbiota associated to periodontal health and disease (Paster et al. 2001; Faveri et al. 2008; Shchipkova et al. 2010). These techniques have recently been used to evaluate the microbiota associated with osseointegrated implants and these results have expanded the knowledge on the composition of these microbial communities.

This chapter presents a current overview of the composition of the biofilms associated with peri-implantitis, with focus on current knowledge about the diversity of these biofilms, based on the results of the studies that have used cutting-edge open-ended approaches. In addition, a brief discussion regarding the strengths and weaknesses of these new diagnostic techniques is also presented. This body of information might help to understand the shifts occurring in the composition of peri-implant biofilm structure that may lead to the development of peri-implantitis.

5.2 Microbial Profile in Peri-Implantitis

Several authors have used different microbiological techniques to study the role of bacterial biofilm in peri-implant diseases in humans (Pontoriero et al. 1994; Lee et al. 1999; Hultin et al. 2002; Renvert et al. 2007; Persson and Renvert 2013) and in animal models (Eke et al. 1998; Tillmanns et al. 1998; Shibli et al. 2003; Charalampakis et al. 2014). Most of these studies have demonstrated a clear relationship between some specific bacterial species and peri-implant mucositis or peri-implantitis (Hultin et al. 2002; Renvert et al. 2007; Persson and Renvert 2013). Indeed, after the implant surface has been exposed to the oral cavity a complex subgingival microbiota is established in a 'pristine' peri-implant pocket within 1–2 weeks, and apparently, the stability of the biofilm community is reached after 3 months (Quirynen et al. 2006).

Early studies characterizing the microbiota of healthy implants by dark field microscopy, described coccoid bacteria as the main morphotype, with a low proportion of spirochetes, fusiforms and motile and curved rods (Sanz et al. 1990; Mombelli and Mericske-Sterm 1990; Silverstein et al. 1994). These results were corroborated by culture techniques that described high levels of Gram-positive facultative cocci, *Actinomyces* and *Veillonella* spp., low total anaerobic rods and a low frequency of detection of *Fusobacterium* spp. and "Black-pigmented Bacteroides" (Leonhardt et al. 1999; Hultin et al. 2002). Therefore, these studies indicated that the microbiota colonizing clinically healthy implants was quite similar to that associated with healthy periodontal sites in periodontally healthy subjects.

Several studies have also compared the microbiota of healthy and diseased implants. Mombelli et al. (1987) described that the microbiota of peri-implantitis sites presented much higher levels of motile rods, spirochetes and fusiforms than that of healthy implants. In another study, subgingival biofilm samples taken from implants with peri-implantitis in 37 subjects, and from healthy implants in another 51 subjects were compared using culture methods (Leonhardt et al. 2003a). The authors analyzed the prevalence of *P. intermedia*, *A. actinomycetemcomitans*, *P. gingivalis* and *Staphylococcus* ssp. In the group not affected by peri-implantitis, *P. intermedia* was detected in 26 % of subjects and *P. gingivalis* in 2 %, as opposed to 66 and 31 % of the subjects presenting peri-implantitis. The prevalence of *A. actinomycetemcomitans* was low in both groups, around 3 %. In addition, *Staphylococcus* spp. were only found in peri-implantitis, in 17 % of the subjects.

Checkerboard DNA-DNA hybridization has been also used to examine the microbial profiles of supra- and subgingival biofilms in subjects with and without peri-implantitis (Shibli et al. 2008). Higher mean counts of *P. gingivalis*, *Treponema denticola* and *T. forsythia* (red complex species) were found in supra- and subgingival biofilms of subjects with peri-implantitis. In addition, the proportions of the pathogens from the red complex were elevated, while host-compatible beneficial microorganisms, such as the *Actinomyces* species, were reduced in diseased compared with healthy implants (Fig. 5.2). The microbiological profiles of supra- and subgingival environments did not differ substantially within healthy or diseased implants. Persson and Renvert (2013) analyzed the levels of 78 bacterial species from 166 implants with peri-implantitis and 47 healthy implants. Nineteen bacterial species were found at higher counts in peri-implantitis, including *A. actinomycetemcomitans*, *Campylobacter gracilis*, *Campylobacter rectus*, *Campylobacter showae*, *Helicobacter pylori*, *Haemophilus influenzae*, *P. gingivalis*, *Staphylococcus aureus*, *Staphylococcus anaerobius*, *Streptococcus intermedius*, *Streptococcus mitis*, *T. forsythia*, *T. denticola* and *Treponema socranskii*. The authors suggested that a cluster of seven bacterial species could be associated with peri-implantitis. The total bacterial load for these seven species (*T. forsythia, P. gingivalis, T. socranskii, S. aureus, S. anaerobius, S. intermedius*, and *S. mitis*) was approximately four times higher in implants with peri-implantitis (6.5×10^5) than in the healthy ones (1.8×10^5).

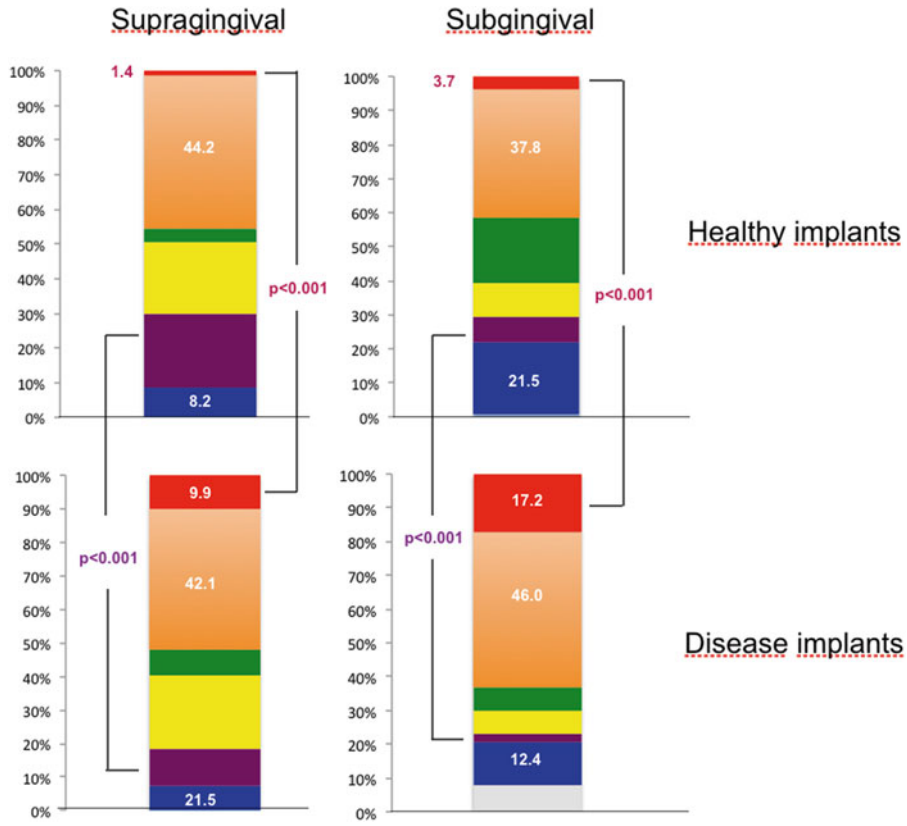

Fig. 5.2 Bar stacks of the mean proportions of each microbial complex in supra-and subgingival plaque samples taken from 22 subjects with a healthy implant and 22 subjects with peri-implantitis. The percentage of DNA probe counts for each species was determined at each site and then across subjects in each group. Species in the complexes were summed and the proportions of which each complex were comprised were determined. The colors represent the different complexes described by Socransky et al. (1998). The *grey color* represents species that do not fall into any complex. The significance of differences in mean proportions was sought using the Mann–Whitney *U*-test (Data adapted from Shibli et al. 2008)

Overall, the available data on the composition of subgingival biofilm associated with peri-implantitis indicate elevated levels of certain bacterial species previously associated with periodontitis. However, other microorganisms not commonly implicated as etiological agents of periodontal diseases have also been detected in samples from peri-implantitis lesions, such as *Enterobacter aerogenes*, *Enterobacter cloace*, *Escherichia coli*, *H. pylori*, *Peptostreptococcus micra*, *Pseudomonas* spp, *Candida* spp, *S. aureus* and *Staphylococcus epidermidis* (Leonhardt et al. 1999; Mombelli and Décaillet 2011; Persson et al. 2010; Persson and Renvert 2013; Rams et al. 2013; Heitz-Mayfield and Lang 2010). Furthermore, *in vitro* studies have demonstrated that *S. aureus* has an affinity for titanium surfaces (Harris et al. 2007; Hudetz et al. 2008), which might indicate a specific role of this species in the etiology of peri-implantitis.

In summary, the overall results of the studies using culture and targeted molecular diagnostic techniques suggest that peri-implantitis is associated with a specific mixed microbiota that presents several similarities with the microbial profile associated with periodontal infections, as well as some other microorganisms not commonly associated with the etiology of periodontitis, such as *Staphylococcus spp.* and *Enterobacteriaceae*.

5.3 The Role of Open-Ended Molecular Diagnostic Tests in the Study of Oral Biofilm Diversity

During the last decade, a great progress has been made as regards the application of novel molecular microbiological methods in the studies of the oral microbiota. The cutting-edge open-ended molecular techniques allow for genome mapping of the entire microbial spectrum in a sample, and provide comprehensive characterization of both the cultivable and not-yet-cultivable microbiota associated with periodontal health and disease. These techniques allow an overview of the microbial communities as a whole, which represents an important advantage over culture and even over other molecular targeted methods, such as specific-specific polymerase chain reaction (PCR), DNA probes and microarrays (Hiyari and Bennett 2011; Wade 2011). The large body of information derived from these sequencing techniques has revealed new species that could act as pathogens in several oral infections (Paster et al. 2001; Faveri et al. 2008; Kumar et al. 2012), including peri-implantitis (Kayanagi et al. 2010, 2013; da Silva et al. 2014; Kumar et al. 2012). To date, the microbial diversity of peri-implantitis has been investigated using PCR-Denaturing Gradient Gel Electrophoresis (DGGE)-Sanger sequencing, PCR-cloning-Sanger sequencing and Next generation sequencing technologies, more specifically, pyrosequencing.

From 2001 to 2010, Sanger sequencing was the most widely used DNA sequencing method for studying the microbial diversity of the oral biofilm (Paster et al. 2001; Kumar et al. 2006; Faveri et al. 2008; Shchipkovaet al. 2010). Several studies published in the 1990s indicated that sequencing of the small ribosomal subunit gene (16S *rDNA*) could be useful for microbial identification (Weisburg et al. 1991; Green and Giannelli 1994; Cilia et al. 1996). This gene is the most common molecular marker used for identification and classification of prokaryotes due to its essential function, ubiquity and evolutionary properties (Case et al. 2007). It allows the detection and identification of a microorganism at a species level, or below, which is a crucial step while trying to understand etiology and treatment of human infections. Therefore, the construction and analysis of ribosomal gene libraries is a very important tool for studying microbial ecology.

Sanger sequencing is considered a *chain-terminator* method because DNA fragments of varying lengths are synthesized by incorporating nucleotides and dideoxy terminators (deoxyribonucleotide triphosphates [dNTPs] and dideoxynucleotide triphosphates [ddNTPs], respectively). Random incorporation of the ddNTPs causes chain termination that produces DNA fragments of every possible length. In a more recent modification, each ddNTP (A, C, T, or G) carries a unique fluorescent molecule, so that the extension products are both terminated and labeled with the appropriate fluorophore (Sanger et al. 1977; França et al. 2002). Terminated products must be purified from unincorporated ddNTPs, and the fragments are subsequently separated by size using capillary electrophoresis, in which the terminal nucleotide of each fragment is detected by fluorescence at wavelengths unique to each of the terminators (Prober et al. 1987). The read lengths for Sanger sequencing have increased in length, and 500–800 base reads can now be achieved routinely (França et al. 2002).

Although a large body of phylogenetic data for microbial identification has been generated via Sanger sequencing, new sequencing technologies that offer a series of additional benefits have emerged recently. One of these new sequencing technologies, pyrosequencing, is faster and more cost-effective than Sanger sequencing (Rastogi et al. 2013; Harrington et al. 2013) and allows thousands to hundreds of thousands of sequence reads to be generated in a single run (Harrington et al. 2013). For this method, specific genetic targets, such as hypervariable regions within bacterial *16S rDNA*-genes may be amplified by PCR and subjected to DNA pyrosequencing. Sequencing by synthesis occurs through a DNA polymerase-driven generation of inorganic pyrophosphate, with the formation of ATP and ATP-dependent conversion of luciferin into oxyluciferin. The generation of

oxyluciferin causes the emission of light pulses, and the amplitude of each signal is directly related to the presence of one or more nucleosides (Petrosino et al. 2009). Pyrosequencing is fundamentally different from Sanger sequencing in that bioluminescence results from strand elongation in real time, whereas, with Sanger sequencing, fluorescence is detected as a separate step after chain termination (Harrington et al. 2013). At present, pyrosequencing technology produces the longest reads of the next-generation sequencing platforms at approximately 700 pb (Harrington et al. 2013).

Sanger sequencing and pyrosequencing are powerful methods for evaluating oral biodiversity; however, DNA extraction and PCR amplification have been reported to be potential sources of biases associated with these techniques (Diaz et al. 2012; Abusleme et al. 2014). The understanding of possible limitations, intrinsic bias and inherent variability of the different diagnostic methods is crucial for the proper evaluation and interpretation of the results of the various studies. Diaz et al. (2012) evaluated the possible bias of DNA isolation and PCR amplification of 454-sequencing of 16S *rDNA* gene (Fig. 5.3). The authors used three different laboratory-created samples (mocks) of seven bacterial species (*Streptococcus oralis, Streptococcus mutans, Lactobacillus casei, Actinomyces oris, Fusobacterium nucleatum, P. gingivalis* and *Veillonella* sp.). Mock 1 contained equal numbers of *16S rDNA* molecules, mock 2 equal numbers of cells and mock 3 unequal numbers of cells of these seven bacterial oral species. In theory, no difference in the number of reads of these species would be expected in mock 1, since they comprised equal amounts of genomic DNA for each species. On the other hand, mocks 2 and 3 could potentially be affected either by some bias of the PCR or sequencing processes or the cell lysis procedures. However, mock 1 did not show the estimated results, as *F. nucleatum* produced a higher number of reads and *A. oris* and *L. casei* a lower number of reads than expected. In addition to being under-represented in mock 1, *A. oris* and *L. casei* were also under-represented in mocks 2 and 3, which could be due to some PCR bias. Both *S. mutans* and *P. gingivalis* were shown in lower abundance than expected only in mocks 2 and 3, suggesting that these species were less effectively lysed. Other research groups have also observed some of these biases associated with the Sanger or pyro-sequencing techniques (de Lillo et al. 2004; Abusleme et al. 2014).

The results of the above-mentioned studies suggest that although pyrosequencing is a powerful technique for investigating the oral microbial diversity, the abundance of species is subject to empirical bias introduced through the methods used for DNA isolation and amplification. Investigators should be aware of these limitations in order to minimize technical errors by accounting for them while designing the studies and evaluating their data.

5.4 A Current Overview on the Microbial Diversity of Peri-Implantitis

Kayanagi et al. (2010) were the first to explore the microbial diversity of the subgingival biofilm around dental implants with different clinical conditions, by *16s rDNA* PCR-cloning-Sanger sequencing. The authors selected three subjects that presented at least one healthy implant and one with peri-implantitis, as well as teeth with periodontitis. A total of 112 different taxa were identified from 335 sequenced clones sequenced. Among these taxa, 46 % (51 phylotypes) were not-yet cultivable and 20 % (22 phylotypes) were novel. The number of species detected in the subgingival biofilm of peri-implantitis, periodontitis and periodontally healthy sites was 77, 57 and 12, respectively. Some bacterial phyla, such as Chloroflexi, Tenericutes and Synergistetes were only detected at peri-implantitis sites, together with some species belonging to the *Firmicutes* phyla, such as *Parvimonas micra*, *Peptostreptococcus stomatis*, *Pseudoramibacter alactolyticus* and *Solobacterium moorei*. Interestingly, some bacterial species that have previously been associated with peri-implantitis, such as *P. gingivalis* and *A. actinomycetemcomitans*

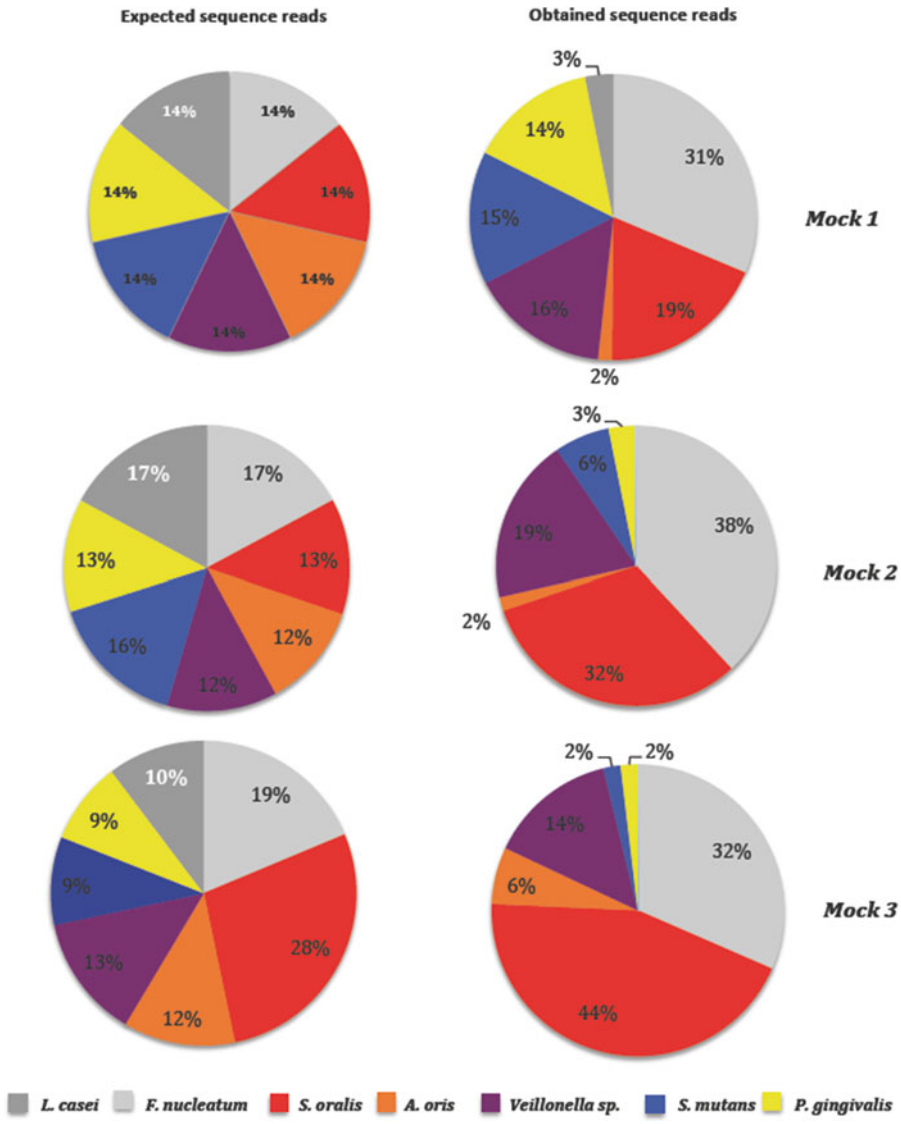

Fig. 5.3 Pie charts of the accuracy of 16S ribosomal RNA (rRNA) amplification followed by 454-pyrosequencing in estimating abundance of species. Mock 1 contained equal numbers of *16S rRNA* molecules, mock 2 equal numbers of cells and mock 3 unequal numbers of cells of these seven bacterial oral species. Expected numbers of sequence reads for mocks 2 and 3 were normalized according to the number of 16S rRNA copies in the genome of each organism (Data adapted from Diaz et al. 2013)

were found in low proportions in this study. However, due to the small sample size this study was unable to establish any type of association between taxa detected and clinical status. More recently, these authors (Kayanagi et al. 2013) continued to explore the microbial diversity of sites with periodontitis and peri-implantitis by adding three new subjects to the previously conducted study (Kayanagi et al. 2010). After screening 799 clones, a total of 333 species were identified, 63 % were not-yet cultivable and 23 % were novel. One hundred and ninety two species were detected in peri-implantitis and 142 in periodontitis. The most abundant phyla in both clinical groups were *Firmicutes* and *Bacteroidetes*, while *Chloroflexi* and *Deferribacteres* were

only detected in peri-implantitis. *Dialister* spp., *Eubacterium* spp. and *Porphyromonas* spp. were more prevalent at peri-implantitis than periodontitis sites. According to the previous publication (Kayanagi et al. 2010), *P. micra*, *P. stomatis*, *P. alactolyticus* and *S. moorei* were limited to peri-implantitis sites and the most abundant species found among all samples was *F. nucleatum*. Interestingly, the authors described that the microbial composition of peri-implantitis was more diverse than that of periodontitis.

Recently, our research group used cloning and Sanger sequencing (da Silva et al. 2014) to investigate the microbial diversity of healthy implants placed in a group of subjects who had no diseased implants (Control group; n = 10) with that of healthy and diseased implants from another group of subjects (Test group; n = 10). The phylogenetic identity of 1387 *16S rRNA* gene clones was determined. Uncultivated phylotypes accounted for an average of 32.1 and 35.8 % of the taxa recovered from healthy implants in the Control and Test groups, respectively, and of 41.2 % from peri-implantitis. Higher proportions of some recognized periodontal pathogens from the orange complex (Socransky et al. 1998), such as *F. nucleatum*, *P. micra*, *P. intermedia* and *C. gracilis* were found in peri-implantitis sites (Table 5.1). Moreover, these sites presented significantly higher percentages of clones from de genera *Desulfobulbus*, *Dialister*, *Filifactor*, *Fusobacterium*, *Mitsuokella* and *Porphyromonas* in comparison with healthy implants. The biofilm associated with peri-implantitis harbored more pathogenic bacterial species from the orange complex, and other "unusual" putative periodontal pathogens, such as *Filifactor alocis*, *Dialister invisus* and *Mitsuokella* sp. HOT 131 in comparison

Table 5.1 Mean number ± standard deviation of top-20 species/phylotypes in the Control and Test group

Experimental groups			
	Control	Test	
Species/phylotype	Healthy implant	Healthy implant	Peri-implantitis
Prevotella oris	0.61 ± 0.35	0.91 ± 0.21	0.78 ± 0.47
Streptococcus mitis	0.86 ± 0.48	0.71 ± 0.64	0.29 ± 0.47**
Veillonella parvula	0.49 ± 0.46	0.75 ± 0.46	0.50 ± 0.50
Fusobacterium nucleatum	0.47 ± 0.44	0.48 ± 0.47	0.68 ± 0.56*#
Capnocytophaga gingivalis	0.41 ± 0.39	0.50 ± 0.49	0.47 ± 0.43
Capnocytophaga granulosa	0.61 ± 0.54	0.29 ± 0.54*	0.09 ± 0.29#
Actinomyces naeslundii	0.25 ± 0.55	0.42 ± 0.55	0.26 ± 0.34
Parvimonas micra	0.38 ± 0.42	0.18 ± 0.29	0.47 ± 0.54#
Streptococcus sanguinis	0.34 ± 0.41	0.22 ± 0.49	0.36 ± 0.50
Prevotella intermedia	0.54 ± 0.61	0.00 ± 0.01**	0.26 ± 0.45#
Gemella haemolysans	0.17 ± 0.52	0.46 ± 0.52	0.11 ± 0.22#
Streptococcus mutans	0.42 ± 0.46	0.25 ± 0.34	0.36 ± 0.40
Actinomyces gerencseriae	0.16 ± 0.26	0.48 ± 0.54	0.25 ± 0.34
Capnocytophaga sputigena	0.48 ± 0.45	0.24 ± 0.41	0.16 ± 0.26
Campylobacter gracilis	0.00 ± 0.00	0.31 ± 0.51	0.35 ± 0.52*
Veillonella atypica	0.40 ± 0.37	0.30 ± 0.42	0.16 ± 0.35
Selenomonas sputigena	0.23 ± 0.37	0.21 ± 0.27	0.33 ± 0.54
Villonella dispar	039 ± 0.42	038 ± 0.42	0.00 ± 0.00** #
Dialister invisus	0.00 ± 0.00	0.00 ± 0.00	0.65 ± 0.42 ** ##
Streptococcus anginosus	0.15 ± 0.31	0.43 ± 0.38	0.16 ± 0.26

The significance of differences between groups was assessed using Mann-Whitney *U*-Test (*$p < 0.05$; **$p < 0.01$, indicate significant differences between samples of the Test group with Control group)
The significance of differences within subject in the Test group was assessed using Wilcoxon Test (# $p < 0.05$; ## $p < 0.01$ indicate significant differences between Healthy implant and Disease implant)

with the healthy implants. Al-Radha et al. (2012) also found higher proportions of some species from the orange complex, such as *Fusobacterium* spp. and *Prevotella* ssp. in peri-implantitis sites using PCR-DGGE followed by Sanger sequencing. The authors also described that these species were more prevalent in the early stages of disease, whilst an increased diversity of species was present during the more advanced stages of disease.

Studies using these culture-independent techniques have also suggested that the *Archaea* domain might be associated with some oral infections, including periodontitis (Lepp et al. 2004; Matarazzo et al. 2011) and endodontic infection (Vianna et al. 2006). Therefore, it has been hypothesized that this domain could also have some association with the etiology of peri-implantitis. In 2011, we (Faveri et al. 2011) studied the prevalence and levels or *Archaea* in a group of 50 subjects presenting only healthy implants (Control, n = 25) or both healthy implants and peri-implantitis (Test, n = 25). In the peri-implantitis group, *Archaea* were detected in 48 %, 16 % and 8 % of diseased implants, healthy implants and teeth, respectively. Implants with peri-implantitis presented a significantly higher prevalence of *Archaea* in comparison with healthy implants and natural teeth. *Methanobrevibacter oralis* was the most prevalent phylotype and was detected in all *Archaea* positive samples, representing 92 % of the clones identified in the Control group, and 95.3 % of those identified in the Test group. The results of this study suggested an increased prevalence of *Archaea* in peri-implantitis sites, mainly the species *M. oralis,* in comparison with the healthy implants. Although these data do not necessarily denote that the *Archaea* domain has a direct function in tissue destruction, they suggest a possible role of this domain in the etiology of peri-implantitis. One possibility is that species from the *Archaea* domain may alter the ecosystem to a more anaerobic environment, which in turn would stimulate the further growth of strict anaerobes species, represented not only by methanogens but also by the members of the red complex, *T. forsythia*, *T. denticola* and *P. gingivalis*, as well as species of the orange complex.

Some authors have used the pyrosequencing technology to study the structure of the bacterial community associated with peri-implant health and disease (Kumar et al. 2012). Kumar et al. (2012) allocated 40 subjects in 4 clinical groups of 10 subjects each, as follows: peri-implantitis, healthy implants, chronic periodontitis, and periodontal health. The sequences represented 370s-OTUs and 84 genus level OTUs that were catalogued into the phyla *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Spirochaetes*, Candidatus Saccharibacteria (Syn. Candidate division TM7), Candidate division Sulphur River 1 (OP11) and *Synergistes*. Uncultivated phylotypes accounted for an average of 52.6 % 44.6 %, 77.8 % and 48.4 % of the taxa isolated from biofilms associated with periodontal health, periodontitis, healthy implants and peri-implantitis, respectively. The genera *Anaerococcus*, *Anaerovorax*, *Anaerofilum*, *Exiguobacterium* and *Burkholderia* were detected only in the peri-implantitis biofilm. A higher degree of similarity was observed between healthy and diseased implants than between healthy and diseased teeth. In addition, the biofilm associated with peri-implantitis showed statistically significant lower richness than healthy implants or diseased teeth, using the Shannon index to compare the microbial diversity. These data are somehow contradictory to those reported in the aforementioned studies (Kayanagi et al. 2010, 2013; da Silva et al. 2014). For the first time, authors also reported that there was greater abundance of Gram-negative anaerobes in the biofilm collected from healthy implants than that from peri-implantitis or periodontitis, as opposed to findings reported by other studies (Kayanagi et al. 2010, 2013).

More recently, Dabdoud et al. (2013) also used 16S rDNA pyrosequencing to explore the degree of congruence between adjacent peri-implant and periodontal microbiota in health and disease. The authors collected subgingival and peri-implant biofilm samples from 81 partially edentulous subjects with periodontal and peri-implant health and disease. Overall, the data revealed the presence of 12 phyla, 110 genera and 523 species. The predominant microorganisms were aerobes, evenly distributed between Gram-positive (194) and Gram-negative (148) species, followed by Gram-positive (47) or

Gram-negative (99) anaerobes, and microorganisms that have not previously been identified (34). *Staphylococcus* and *Treponema* genera were statistically significantly associated with implant infection, but not with periodontal infection. Sixty percent of subjects shared fewer than 50 % of all species between their periodontal and peri-implant biofilms. In addition, 85 % of subjects shared fewer than 8 % of the most abundant species between tooth and implant. Also, the red complex pathogens were found in the peri-implantitis biofilm in only 37 % of the cases. Although these data corroborate the results of previous studies suggesting that certain periodontal pathogens may be present in both diseased teeth and implants (Mombelli et al. 1995; Rutar et al. 2001; Tabanella et al. 2009), the majority of the species, especially the most abundant types, showed distinct differences between periodontitis and peri-implantitis.

5.5 Concluding Remarks

The studies on the composition of the biofilm associated with peri-implantitis started in the late 1980s and from the beginning, the main focus of these studies has been the search for already known periodontal pathogens. A considerable amount of data from studies using culture and molecular targeted techniques supported the notion that most of the periodontal pathogens were also found in higher levels and proportions in peri-implantitis. Thus, at the end of the 2000s it was widely accepted that there was a great similarity between the composition of the subgingival biofilms of peri-implantitis and periodontitis. In the last few years, the use of cutting-edge open-ended diagnostic techniques to study the diversity of peri-implantitis microbiota has brought new insights on this subject. The overall results of these studies suggest that the structure of the microbial community of peri-implantitis might not be as similar to the subgingival microbiota of periodontitis, as previously thought. In addition, putative pathogens other than those associated with periodontal diseases may play a role in the onset and progression of peri-implant infection. However, it is important to note that these findings come from a limited number of studies evaluating relatively reduced numbers of samples. Therefore, further investigations on the diversity of peri-implant microbiota would be helpful in order to establish a better comparison between periodontal and peri-implant biofilms and could greatly contribute to define more effective preventive and therapeutic strategies for peri-implant diseases.

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Anaerobes in Biofilm-Based Healthcare-Associated Infections

6

Claudia Vuotto and Gianfranco Donelli

Abstract

Anaerobic bacteria can cause an infection when they encounter a permissive environment within the host. These opportunistic pathogens are seldom recovered as single isolates but more frequently are involved in polymicrobial infections, together with other anaerobes or aerobes. Nowadays it's known that some anaerobic bacteria are also able to grow as biofilm even if this feature and its role in the healthcare-associated infections (HAIs) are still poorly characterized. As consequence, the involvement of biofilm-forming anaerobic bacteria in infections related to healthcare procedures, including surgery and medical devices implantation, is underestimated.

The current knowledge on the role of biofilm-growing anaerobes in HAIs has been here reviewed, with particular reference to respiratory, intestinal, intra-abdominal, wound, and urogenital tract infections. Even if the data are still scarce, the ability to form biofilm of opportunistic anaerobic species and their possible role as causative agents of HAIs should alert even more clinicians and microbiologists on the need to search for anaerobes in clinical samples when their presence can be reasonably assumed.

6.1 Introduction

The increased recovery of anaerobes from clinical samples has led to a greater appreciation of their role in infections at virtually all body sites, including mouth, lung, gastrointestinal tract, urogenital tract, bloodstream, skin and soft tissue, and of their involvement in a variety of clinical presentations, such as abscess formation, foul-smelling pus, and tissue necrosis (Finegold 1995a).

In principle, anaerobic bacteria can cause an infection, becoming opportunistic pathogens, when they encounter a permissive environment within the host, subsequent to breakdown of the common barriers as a result of surgery, injury, blood vessel disease or shock. In fact, tissue destruction (necrosis) or poor blood supply can favour the growth of anaerobic bacteria because of the resulting low oxygen conditions (Finegold 1995b). Other predisposing factors include malignancy,

C. Vuotto (✉) • G. Donelli
Microbial Biofilm Laboratory,
Fondazione Santa Lucia IRCCS, Rome, Italy
e-mail: c.vuotto@hsantalucia.it

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immunodeficiency, diabetes and presence of foreign bodies (Castillo et al. 1999).

These opportunistic pathogens are seldom recovered as single isolates, such as *Finegoldia magna* that is quite often isolated in pure culture (Wildeboer-Veloo et al. 2007), but more frequently are involved in polymicrobial infections, being isolated together with other anaerobes or aerobes (Nichols and Florman 2001; Brook 2002; Dryden 2010). Usually, many infections are initially caused by aerobic bacteria and then worsened by anaerobes that become predominant when the tissue microenvironment turn out to be anaerobic.

In a study by Mikamo and co-workers covering the years 1994–2003, it has been demonstrated that the most often isolated strains in polymicrobial infections are Gram-positive anaerobic cocci (25–30 %), followed by *Prevotella spp.*, *Bacteroides fragilis* group, *Clostridium spp.*, *Veillonella spp.*, *Fusobacterium spp.* and *Porphyromonas spp.* (Mikamo et al. 2011). The major role of gram positive anaerobic cocci in mixed infections has been recently confirmed by other authors (Murphy and Frick 2013).

Other than the high number of endogenous anaerobic species commonly inhabiting our body and possibly causing infections, there are few anaerobes, first of all *Clostridium difficile*, able to cause endogenous or exogenous infections (Spigaglia et al. 2011; Wiegand et al. 2012; Knight and Surawicz 2013).

The three major virulence factors supporting anaerobes in host adhesion and invasion are: (i) the production of toxins or enzymes such as superoxide dismutase, catalase, immunoglobulin proteases (Mastrantonio et al. 1996); (ii) the surface structures such as the lipopolysaccharide or the capsular polysaccharide, that are often expressed only in chronic infections (Brook et al. 1991), (iii) the ability to adhere to or invade epithelial surfaces (Hofstad 1989; Brook and Frazier 1993; Brazier 2006).

Besides the above mentioned virulence factors, nowadays it's known that some anaerobic bacteria are also able to grow as mono- (Fig. 6.1) or dual-species biofilms (Donelli et al. 2012) even if this feature and its role in the healthcare-associated infections (HAIs) are still poorly characterized.

In fact, although it has been well demonstrated the close association between biofilm-forming anaerobic species and oral diseases, such as peri-implantitis, the involvement of biofilm-forming anaerobic bacteria in infections related to surgery, devices implantation or other healthcare procedures is underestimated.

The current knowledge on the role of biofilm-growing anaerobes in HAIs will be here reviewed, with particular reference to respiratory, intestinal, intra-abdominal, wound, and urogenital tract infections.

6.2 Lower Respiratory Tract Infections

According to the American Thoracic Society and Infectious Diseases Society of America (2005), the lower respiratory tract infections, including hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP), are the most common HAIs in acute care hospitals, causing significant morbidity and mortality in hospitalized patients (Chroneou et al. 2007; Werarak et al. 2010).

In healthy people, thanks to the cleansing action of the ciliated epithelium, the lower respiratory tract (trachea, bronchi, and pulmonary tissues) is virtually free of microorganisms that are pushed upward and removed by coughing, sneezing, swallowing, etc.

When mechanical or chemical injuries to the ciliated epithelium affect the normal mucus removal, the patient may become susceptible to infection by pathogens, including *Streptococcus pneumoniae* and several nosocomial multidrug resistant bacteria, such as the aerobes *Acinetobacter baumannii*, *Haemophilus influenzae*, *Klebsiella pneumoniae* and the facultative anaerobes *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Ferrara 2006; Spronk 2007).

However, most of the lower respiratory tract infections are polymicrobial and some of them, such as aspiration pneumonia or ventilator-associated pneumonia, include also strictly anaerobic bacterial flora. The predominant anaerobic bacteria were the pigmented *Prevotella* spp., other than *Actinomyces*, *Bacteroides*, *Peptostreptococcus*, *Veillonella*, *Propionibacterium*, *Fusobacterium*

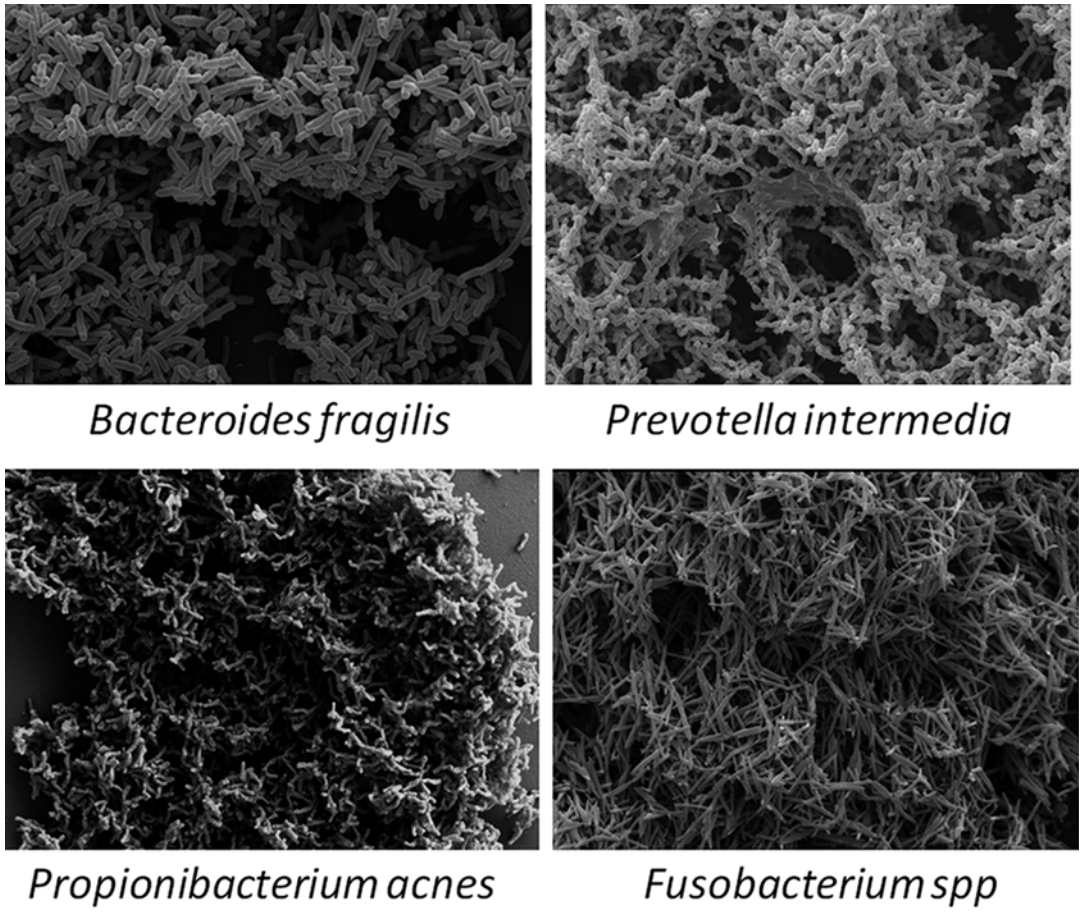

Fig. 6.1 Field Emission Scanning Electron Microscopy (FESEM) of *Bacteroides fragilis*, *Fusobacterium spp.*, *Prevotella intermedia* and *Propionibacterium acnes* strains (5,000×) growing as single-species biofilms for 48 h

spp. and *Porphyromonas* spp. (Marik and Careau 1999; Robert et al. 1999; Brook 2004a; Bahrani-Mougeot et al. 2007; Bartlett 2012).

Particularly, VAP is the most common intensive care unit (ICU)-acquired infection in patients requiring prolonged mechanical ventilation and it occurs in 8–28 % of patients (Agbaht et al. 2007; Choudhuri 2013). Furthermore, VAP, from the economic side, is associated with higher costs due to the longer hospital stay (Gould 2013).

The endotracheal tube (ETT) is considered one of the major risk factors for VAP, altering the patient's ability to clear secretions by coughing, thus allowing their passage into the airways, and acting as a reservoir for potentially infecting microbes and as a bridge between the oropharyngeal environment and the sterile bronchoalveolar space (Koerner 1997).

One of the most important mechanisms implicated in the development of VAP is the biofilm formation on the ETT surfaces. In fact, shortly after intubation, the ETT may represent a source of pathogens by providing inner and outer luminal surfaces to which microbes can adhere and form biofilms, thus contributing to pathogenesis and persistence of colonization (Bauer et al. 2002; Zur et al. 2004; Pneumatikos et al. 2009; Zolfaghari and Wyncoll 2011; Vandecandelaere et al. 2012).

Once biofilm on ETT is formed, there are several mechanisms by which it can infect the lungs: microbial clusters may be dispersed and

passively moved towards the lungs, cell aggregates can be aerosolized and aspirated in the airways and individual cells in contact with liquids can be transferred deeply into the lungs (Luna et al. 2009).

Even if the bronchoalveolar lavage cultures are considered the 'gold standard' for the identification of respiratory pathogens causing VAP, this procedure is not able to identify all the potential pathogens constituting the ETT biofilm. In fact, two independent groups (Perkins et al. 2010; Cairns et al. 2011) recently explored the bacterial community adherent to the ETT surfaces through molecular techniques, both of them evidencing the presence of an anaerobic component of the oral cavity.

Perkins and co-workers examined eight ETTs recovered after intubation periods between 12 h and 23 days from patients admitted in a surgical and a medical intensive care unit. To identify and quantify the fastidious/non culturable organisms present within the multi-species biofilm of the investigated ETTs, 16S rRNA gene survey and quantitative polymerase chain reaction (qPCR) were performed. The results showed that, on a number of 1263 near full-length 16S rRNA gene sequences from the diverse bacterial communities, the second most frequent genus identified corresponded to the anaerobe *Prevotella* spp. (179/1263), with the highest relative concentrations for the ETT tubes with short intubation periods. This study firstly demonstrated the presence of anaerobic oral bacteria directly within the ETT biofilms (Perkins et al. 2010). Afterwards, Cairns and coworkers also demonstrated that oral obligate and facultative anaerobic bacteria form part of the ETT biofilm. In fact, by using species specific PCR, the obligate anaerobe *Porphyromonas gingivalis* and the facultative anaerobe *Streptococcus mutans* were detected within the polymicrobial biofilm grown on the ETTs of 9 out of 20 patients (Cairns et al. 2011).

The detection of oral anaerobic species has demonstrated that oral biofilm may play a considerable role in lower respiratory tract infections. Oral decontamination, i.e. by the use of chlorhexidine-based oral hygiene, in conjunction with VAP prevention bundle, is effective in reducing the incidence of VAP by 30 % and the duration of mechanical ventilation in patients in the surgical ICU (Genuit et al. 2001; Chlebicki and Safdar 2008).

Considering the ability of oral anaerobic species to form a polymicrobial biofilm (Marsh 2004; Kolenbrander et al. 2010; Roberts and Mullany 2010; Zijnge et al. 2012) and the increasing number of studies suggesting a potential role of anaerobes in respiratory diseases, particularly in cystic fibrosis (Costerton 2002; Worlitzsch et al. 2009; Ulrich et al. 2010; Su and Hassett 2012), the direct involvement of anaerobes in the pathogenesis and persistence of these biofilm-based HAIs has to be taken into consideration and further investigated.

6.3 Intestinal Infections

The gastrointestinal (GI) tract of the normal healthy humans harbours a complex indigenous flora, mostly anaerobes within the colon, that plays a crucial role in the maintenance of normal metabolic and immunologic homeostasis (Savage 1977).

These mucosal communities are characterized by a sessile mode of growth, rather than a non-adherent planktonic state, with different fermentation profiles and enzymatic activities significantly higher in sessile-growing bacteria (Probert and Gibson 2002; Zoetendal et al. 2002; Macfarlane and Macfarlane 2006).

Microscopic investigation of the colonic mucosa by using specific 16S rRNA fluorescence in situ hybridization (FISH) probes, has confirmed that mucosal bacteria, including enterococci, bacteroides and bifidobacteria, are distributed throughout the mucus layer and occur extensively in microcolonies. Live/dead staining of these structured communities showed that most of the bacteria were living, particularly those closest to the mucosal surface. These findings suggest that the bacteria are actively growing in the mucus layer, and that their presence is not a result of passive transfer of the cells from faecal material along the gut lumen (Macfarlane and Dillon 2007; Macfarlane et al. 2011).

Due to their proximity to the epithelial surface, mucosal bacteria growing in biofilm may be

important in modulating the host's immune system and possibly contributing to some inflammatory bowel diseases (ulcerative colitis, Crohn's disease), in which exists a dysbiosis in microbial community structure, with a reduction in putatively protective mucosal microorganisms such as bifidobacteria (Macfarlane et al. 2011).

Sproule-Willoughby and coworkers studied selected representatives from the human colonic microbiota by using mucosal bacterial communities from the human colon and allowing them to grow in a surface-adherent mode of growth. The resulting biofilms were complex, multi-species communities, stable in composition over an extended period. This model is useful for investigating the effects of exogenous microbial, environmental and pharmaceutical influences on bacterial community structure and function in the intestine (Sproule-Willoughby et al. 2010).

Different intestinal anaerobic isolates, belonging to the species *Bacteroides*, *Clostridium*, *Fusobacterium*, *Finegoldia*, *Prevotella*, and *Veillonella* have been demonstrated to be able to in vitro adhere, to develop as mono-species biofilms, and to interact with each other giving rise to dual-species biofilms (Donelli et al. 2012).

The healthcare-associated intestinal infections are mainly related to the increased use of broad-spectrum antibiotics that are able to promote abnormal gut colonization by resistant pathogens. In fact, the disruption of the anaerobic flora has been shown to be a key factor for the gut colonization by the anaerobic-facultative vancomycin-resistant *Enterococcus sp* (Donskey et al. 2000) and *C. difficile* (Lo Vecchio and Zacur 2012), both species being strongly associated with hospital outbreaks and invasive infections.

Many strains of *Enterococcus faecium* subpopulation belonging to the clonal complex 17 (CC-17) contain a putative pathogenicity island encoding a variant of enterococcal surface protein (Esp). Esp expression depends on growth conditions like temperature and anaerobiosis, this protein being found in half of these strains grown at 37 °C under anaerobic conditions. Furthermore, amounts of surface-exposed Esp was shown to correlate with initial adherence to polystyrene ($R(2) = 0.7146$) and biofilm formation ($R(2) = 0.7535$). These data indicate that *E. faecium* senses and responds to the change of environmental conditions, which might play a role in the early stages of infection when bacteria transit from oxygen-rich conditions at room temperature to anaerobic conditions at body temperature (Van Wamel et al. 2007).

Regarding the most insidious intestinal HAI, i.e. the *Clostridium difficile* infection (CDI), different research groups have started to study *C. difficile* biofilm. Donelli and coworkers first demonstrated the in vitro ability of a *C. difficile* clinical strain to grow as biofilm, alone or synergistically developing together with a *F. magna* strain (Donelli et al. 2012).

Afterwards, the hypervirulent strain R20291 was revealed to grow as biofilm and a possible link between sporulation and biofilm formation was suggested, by putting into evidence a reduction of biofilm formation in a *spo0* mutant (Dawson et al. 2012).

A deeper analysis on the strain 630 and the hypervirulent strain R20291 conducted by Đapa and coworkers confirmed the ability of the *C. difficile* hypervirulent strain to form biofilm and, employing isogenic mutants, authors showed that the virulence-associated proteins, Cwp84, flagella, and a putative quorum-sensing regulator, LuxS, are all required for a maximal biofilm formation. It has been also demonstrated that bacteria in clostridial biofilms are more resistant to high concentrations of vancomycin, a drug commonly used for CDI treatment (Đapa et al. 2013; Dapa and Unnikrishnan 2013).

On the whole, the above mentioned data suggest that biofilm formation by *C. difficile* (Figs. 6.2 and 6.3) is a complex multifactorial process, modulated by several different factors, that could play a key role in gut colonization and bacterial survival, thus possibly affecting its pathogenesis and persistence, and contributing to recurrence of CDI.

6.4 Intra-Abdominal Infections

Healthcare-associated intra-abdominal infections affect a spectrum of adult patients receiving cares in acute hospitals or residing in chronic care settings.

Cultures of the peritoneal cavity of patients affected by peritonitis due to a post-operative

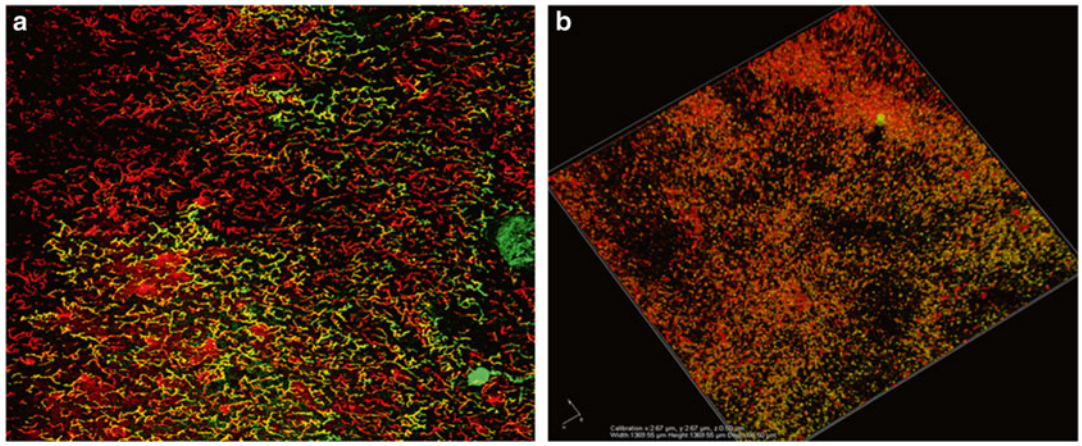

Fig.6.2 Bidimensional (**a**) and three-dimensional (**b**) images of *C. difficile* in vitro biofilm obtained by using Confocal Laser Scanning Microscopy (CLSM). Fluorescent staining propidium iodide (PI) has been used to detect red bacterial cells and A -conjugated fluorescein isothiocyanate (Con A-FITC) to stain in green the glycocalyx matrix

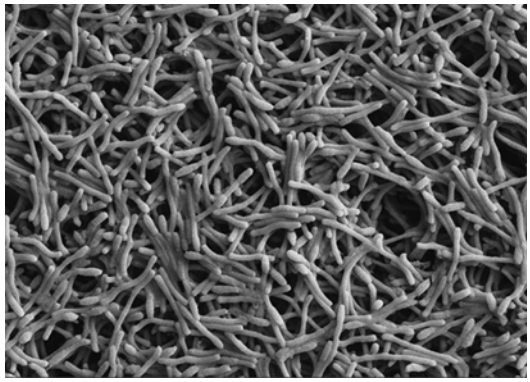

Fig. 6.3 FESEM micrograph (5,000×) of a biofilm formed in vitro by a clinical strain of *C. difficile* isolated from explanted biliary stent

complications, anastomotic leaks or device-related infections, such as continuous ambulatory peritoneal dialysis (CAPD)-related peritonitis and infected ventriculoperitoneal shunt, allow to identify the polymicrobial nature of this infection (Brook and Frazier 2000; Marshall 2004; Mazuski and Solomkin 2009).

In fact, patients more often at risk for infection with multidrug resistant (MDR) bacteria, are typically infected with *Escherichia coli*, *P. aeruginosa* and *Acinetobacter* spp., extended spectrum beta-lactamase (ESBL)–producing *Klebsiella* spp., *Enterobacter* spp., *Proteus* spp., and *Enterococci*. In addition, the involvement of anaerobes in these infections has been also demonstrated (Sartelli et al. 2012).

In case of post-operative complications, after an initial stage of infection caused by aerobes and characterized by preliminary disruption of intra-abdominal hollow viscera and decrease in the oxidation-reduction potential of the oxygenated tissue, the anaerobic *B. fragilis* starts to predominate in one third to one half of these infections (Goldstein and Snydman 2004; Marshall 2004; Brook 2008).

On the contrary, in CAPD-associated peritonitis the typical spectrum of microorganisms include gram-positive (67 %) and gram-negative (28 %) aerobic bacteria, and a low percentage of anaerobic microorganisms (2.5 %) (Troidle and Finkelstein 2006; Chao et al. 2013). A polymicrobial biofilm as cause of this infection has been deeply analyzed (Verger et al. 1987; Ward et al. 1992; Gorman et al. 1994 Dasgupta and Larabie 2001; Dasgupta 2002; Hanlon et al. 2004; García-López et al. 2012; Nessim et al. 2012; Martins et al. 2013) and the presence of an anaerobic component has been revealed in some investigated peritoneal catheters (Troidle and Finkelstein 2006; Pihl et al. 2013).

Troidle and coworkers examined microbial biofilms grown on ten peritoneal catheters removed from eight patients because of peritonitis and from other two patients because no longer

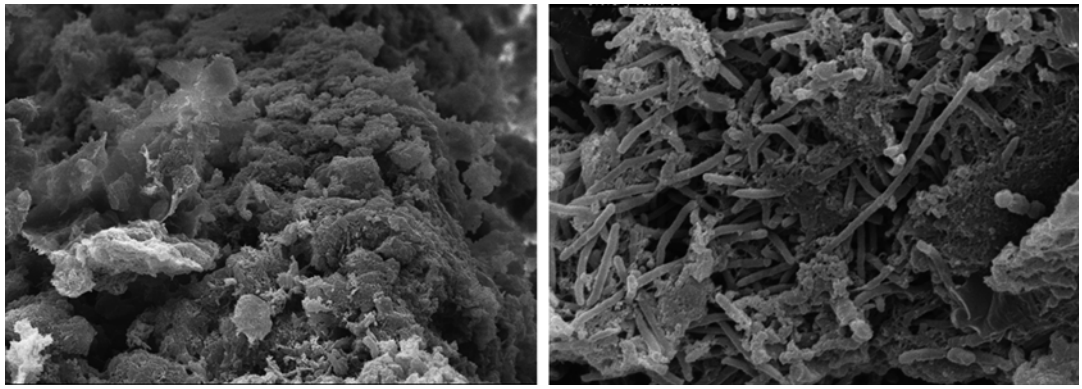

Fig. 6.4 Multi-species biofilms observed by FESEM at low (2,000×) and high magnification (10,000×) on the inner surfaces of an explanted biliary stent

needed. Among the microorganisms identified within the biofilm, *B. fragilis* represented the most frequently isolated anaerobe (Troidle and Finkelstein 2006).

Literature data have demonstrated the ability of *B. fragilis* to form in vitro biofilm (Weinacht et al. 2004) and to grow on and colonize mucin surfaces (Macfarlane et al. 2005) as well as its capability to enhance bacterial co-aggregation under bile salt exposure by overexpressing several *bmeB* efflux pumps and the outer membrane protein *Omp* (Pumbwe et al. 2007).

These findings support the hypothesis that biofilm-growing *B. fragilis* could contribute to cause intra-abdominal infections due to the insertion of peritoneal catheters.

By using standard microbiology methods and 16S rRNA gene sequencing, Pihl and coworkers set out to identify the range of aerobic and anaerobic bacterial species on CAPD catheters from patients with or without infections. Bacteria were found heterogeneously spread on catheters, both as single microorganism or mixed microbial communities. The most common colonizer was *Staphylococcus epidermidis*, followed by the anaerobic species *Propionibacterium acnes*, the latter being widely spread over the surface of colonized catheters (Pihl et al. 2013).

Another biofilm-based infection has been related to the failure of biliary decompression after endoscopic insertion of a plastic stent in patients suffering from obstructive jaundice. In fact, the device occlusion (Fig. 6.4) has been first described as consequence of the deposition of biliary sludge (McAllister et al. 1993; Weickert et al. 2001) while, later, it has been reported that microbial biofilm plays a pivotal role in the clogging process (Jansen et al. 1993; Hoffman et al. 1994; Sung 1995; Brant et al. 1996; Leung et al. 1998; Zhang et al. 2002; van Berkel et al. 2005; Donelli et al. 2007; Weickert et al. 2009).

Leung and coworkers demonstrated for the first time the presence of anaerobic bacteria, especially *Clostridium perfringens*, *Clostridium bifermentans* and *B. fragilis*, in the biliary stents' biofilm, and their contribution in initiating stent blockage in patients who had received antibiotic prophylaxis against gram-negative bacterial infection (Leung et al. 2000).

Afterwards, several species of strictly anaerobic bacteria were observed in a couple of papers. Particularly, Scheithauer and coworkers evidenced *Fusobacterium spp.* and *Veillonella spp.* (Scheithauer et al. 2009) while Guaglianone and co-workers have reported the isolation of strictly anaerobes from 57 % of the investigated biliary stents, *Bacteroides spp.* and *Clostridium spp.* being the most represented anaerobic species, followed by *Prevotella spp.*, *Veillonella spp.*, *Fusobacterium spp.* and *Peptostreptococcus spp.* (Guaglianone et al. 2010).

6.5 Wound Infections

Infected wounds are damaged area of the body colonized by bacteria or other microorganisms that, depending on their pathogenicity and inoculum size, overwhelm the body's immune defences, producing either a delay in wound healing or deterioration of the wound.

The onset of chronic wounds, that are lesions failed to proceed through an orderly and timely restore to health, is the main cause of the delay in the healing process (Edwards and Harding 2004; Thomson 2011).

The occurrence of chronic wounds has been related to the presence of highly persistent biofilm communities enabling microbial escape from host immune system and resistance to antibiotic treatment (James et al. 2008; Wolcott et al. 2008; Percival et al. 2012).

Infections that occur in the wound site at the end of an invasive surgical procedure are generally referred to as surgical site infections (SSIs). They represent about a fifth of all HAIs and are an important cause of morbidity and mortality, over one-third of postoperative deaths being related, at least in part, to SSIs (Mangram et al. 1999; Bansal et al. 2005; Kiernan 2012).

Viable biofilms have been associated with both monofilament and braided infected sutures, and associated reactive soft tissue (Kathju et al. 2009; Edmiston et al. 2013).

Microorganisms causing SSIs are usually derived from the patient (endogenous infection), but also exogenous infection may occurs when microorganisms from the instruments and external environment contaminate the operative site (Bowler et al. 2001). Even if *S. aureus* is the microorganism most commonly cultured from SSIs, wounds are very often infected by a whole range of microorganisms (*National Collaborating Centre for Women's and Children's Health*. Clinical Guideline, October 2008). In fact, it is considered that also other aerobic or facultative pathogens such as coagulase-negative staphylococci, *P. aeruginosa*, *E. coli*, *Klebsiella spp*., *Enterobacter spp*., *Enterococcus spp*. and beta-hemolytic *Streptococci*, as well as *Candida spp*., are the primary causes of delayed healing and infection in chronic wounds, especially the surgical ones (Mangram et al. 1999).

Furthermore, Wolcott and coworkers specified that over 60 % of the bacteria in the evaluated SSIs were anaerobic bacilli while the previous literature data indicates that aerobic cocci predominate in such wounds (Wolcott et al. 2009).

Also the healthcare-associated pressure ulcers, defined as localized injury to the skin and underlying tissue usually as a result of pressure occurring in immobilized patients, are often associated to infections caused by polymicrobial biofilms, with no single bacterial species exclusively colonizing the wounds (Ebright 2005; James et al. 2008; Smith et al. 2010).

Today, approximately 20 % of long-term care patients suffer from infected pressure ulcers (Zulkowski et al. 2005; Donelli and Vuotto 2014).

A multi-faceted approach constituted by 16S rRNA pyrosequencing, epifluorescence microscopy, FISH, and quorum sensing analysis, is today available to identify the entire spectrum of bacterial species and to fully characterize the microbial complex nature of chronic wounds (Han et al. 2011).

In fact, three separate 16S-based molecular amplifications followed by pyrosequencing, shotgun Sanger sequencing, and denaturing gradient gel electrophoresis have allowed to survey the whole biofilms-forming bacterial populations in pressure ulcers. Results showed that obligate anaerobes represented 62 % of the investigated microbial populations (Dowd et al. 2008).

Consistent results have been obtained by Smith and colleagues in 2010, many of the analyzed wounds being predominated by what are either facultative or obligate anaerobic bacteria with only 36 % of aerobes. The most frequently isolated strict anaerobe was *F. magna* (32/49 decubitus ulcer samples), followed by *Anaerococcus vaginalis* (23/49), *Anaerococcus lactolyticus* (20/49), *Peptoniphilus indolicus* (20/49), *Peptoniphilus harei* (18/49), *Peptoniphilus ivorii* (17/49), *Peptoniphilus lacrimalis* (13/49), *Porphyromonas somerae* (13/49), *Prevotella buccalis* (12/49). The anaerobic species *B. fragilis*, *Porphyromonas* spp., and *Prevotella bivia* were isolated in lower number (Smith et al. 2010).

The development of these aerobic-anaerobic populations is facilitated by the low oxygen tension (hypoxia or anoxia) and the reduced redox potential of the wound environment (Gerding 1995).

The high prevalence of anaerobic bacilli detected today suggest that the complexity of bacterial communities in wounds has historically been underestimated and that these bacterial species may be leading contributors to the aetiology of biofilm-related chronic wound infections.

6.6 Urogenital Infections

Urinary tract infections (UTIs) are the most common HAIs in the intensive care units (Shuman and Chenoweth 2010).

It has been recently estimated (Cek et al. 2014) that around 10 % of hospitalized urological patients are at risk to develop UTIs often caused by multiresistant uropathogens, such as enteric Gram-negative bacilli, enterococci, Candida species, and *P. aeruginosa*, *E. coli* being the most frequent isolate (544 of 1,371 isolates; 39.7 %).

Persistent or recurrent UTIs predominantly occur in patients with indwelling urinary catheters prone to be colonized by different bacteria, catheter-associated urinary tract infections (CAUTIs) accounting for approximately 40 % of all HAIs (Chenoweth and Saint 2011). Multidrug resistant microorganisms are able to colonize the inner and outer surfaces of indwelling or temporary catheters and to form polymicrobial biofilms (Frank et al. 2009) that persist on bladder epithelium, despite the removal of catheter, and resist antibiotic penetration (Blango and Mulvey 2010). The biofilm-forming pathogen most commonly implicated in urogenital infections is *E. coli* (Wang et al. 2010).

The involvement of fastidious anaerobic bacteria in many different types of urinary tract infections, including para- or peri-urethral cellulitis or abscess, acute and chronic urethritis, cystitis, acute and chronic prostatitis, pyelonephritis, renal abscess, and other infections, has been highlighted even if little attention has been paid so far (Brook 2004b). In a quite recent study, on 1,449 urine specimens examined both by culture and by PCR, the anaerobic bacteria detected only by using PCR (22.43 %) were *Bacteroides spp*., pigmented *Prevotella spp*., *Porphyromonas* sp., *F. magna*, *Peptostreptococcus vaginalis*, and *Bifidobacterium* spp. (Imirzalioglu et al. 2008).

Back in 1973, it was demonstrated that patients with indwelling urethral catheters had a high incidence of anaerobes recovered from urine (Alling et al. 1973) and later it was reported that patients with indwelling Foley catheters showed anaerobes along with aerobes and facultative organisms in urine samples (Sapico et al. 1976).

Nevertheless, even if strictly anaerobic bacteria have been found in the bladder urine of some patients with indwelling urethral catheters, no other specific studies have been published so far on their role in the initiation and perpetuation of CAUTIs and on their contribution in forming a polymicrobial biofilm on the urinary catheter surfaces.

6.7 Prosthetic Joint Infections

Infection processes, although uncommon, are the most serious complications occurring after prosthetic joint surgery. In fact, orthopaedic implants, surgically implanted into sterile areas of the body, can be colonized as a consequence of a transient sepsis, thus requiring additional surgery for revision arthroplasty (Sendi and Zimmerli 2011; Cobo and Del Pozo 2011). According to epidemiological data, prosthesis-related infections take place in 0.8–1.9 % of knee arthroplasties (Jämsen et al. 2009) and in 0.3–1.7 % of hip arthroplasties (Del Pozo and Patel 2009).

As increasingly reported in the recent years, single and multi-species biofilms are recognized as the main responsible for these implant-associated infections that are highly resistant to antibiotic treatment, due to poor penetration of antimicrobial molecules through the biofilm matrix, and to the host immune responses (McDowell and Patrick 2005; Song et al. 2013).

Most of the orthopaedic implants-associated infections are caused by staphylococci (about four out of five), particularly *CoNS* species (30–43 %)

and *S. aureus* (12–23 %), followed by Streptococci (9–10 %), Enterococci (3–7 %), gram negative bacilli (3–6 %), and anaerobes (2–4 %). Polymicrobial infections are observed in about 10–11 % (Zimmerli and Moser 2012).

However, one of the main problems encountered in determining the severity and the rate of infection is the difficulty to isolate biofilm-forming bacteria from prosthetic surfaces, especially anaerobes and microorganisms in viable but nonculturable state. This problem can be overcome with the use of molecular identification procedures, such as PCR combined with cloning, immunofluorescence microscopy (IFM) and FISH (Høgdall et al. 2010), with specific transport media for fastidious and robust aerobes and anaerobes (Tano and Melhus 2011) or with the placing the implants in an anaerobic jar directly after surgical removal (Tunney et al. 1998).

In this regard, Dempsey and co-workers have demonstrated that conventional identification techniques led to the detection of biofilm-forming bacteria on surfaces of the hip prosthesis in only the 22 % of cases compared to a detection rate of 72 % using molecular identification methods based on 16S rRNA. In the same study authors were able to identify also anaerobic species, such as *B. fragilis* (Dempsey et al. 2007).

Furthermore, molecular methods have also increased the sensitivity of *P. acnes* detection, thus becoming evident that many cases of 'aseptic' prosthesis loosening might due to *P. acnes* infections. In fact, the number of delayed joint prosthesis infections caused by this microorganism has been so far significantly underestimated (Tunney et al. 1999).

More recent studies have confirmed that *P. acnes* is an important cause of invasive infections related to prosthetic joint surgery, this anaerobe being isolated at a relative frequency comparable to many other pathogens (Lutz et al. 2005; Zeller et al. 2007; Zappe et al. 2008; Portillo et al. 2013) and being able to form biofilm both in vitro and in vivo (Ramage et al. 2003; Bayston et al. 2007; Coenye et al. 2007; Tunney et al. 2007).

Even if the ability of *P. acnes* to form biofilm is now firmly established, the regulation of biofilm production and the differences in biofilm formation by diverse clinical strains are still poorly explored. Holmberg and co-workers examined a large collection of *P. acnes* isolates and showed that strains collected from deep infections related to foreign material produce more biofilm in vitro with respect to the isolates from skin of healthy individuals. This finding provides evidence that *P. acnes* biofilm production is affected by the isolation site, genes encoding biofilm components being subjected to environmental influences. This phenomenon is important for a better understanding of delayed joint prostheses infections caused by this microorganism (Holmberg et al. 2009).

It has been also demonstrated that the presence of human plasma in solution, or at the plastic surface, inhibits *P. acnes* biofilm formation, which could explain why it primarily infect the plasma-poor environments of joint prostheses (Levy et al. 2008; Holmberg et al. 2009).

The management of severe joint prostheses infections caused by *P. acnes* involves a combination of antimicrobial treatment and surgical intervention for the device removal. Intravenous penicillin G and ceftriaxone are the first choice for these serious infections, with vancomycin and daptomycin as alternatives, and amoxicillin, rifampicin, clindamycin, tetracycline, and levofloxacin for oral treatment (Portillo et al. 2013).

Clostridium spp., easily isolated from the human intestinal tract, has been also recognized as potential pathogen of prosthetic joint infections, penetrating trauma and hematogenus spread, a concomitant systemic infection being considered the most important source of infection. Although *Clostridium spp*. has been isolated in different orthopaedic infections, data about pathogenesis, natural history, and treatment of these infections are scarce (McCarthy and Stingemore 1999; Lazzarini et al. 2004).

A better understanding of biofilm formation mechanisms of *P. acnes* and *Clostridium spp.* and their role in the polymicrobial biofilm formation could help to set up innovative strategies to counteract delayed joint prostheses infections.

6.8 Bloodstream Infections

Central venous catheters (CVC) are among the most frequent causes of healthcare-associated bloodstream infections (Mermel et al. 2001; Zingg et al. 2009). Catheter-related bloodstream infections (CRBSIs) in ICU patients are associated with sepsis in the 28 % of cases (Alberti et al. 2002), the intravascular portion of the device being rapidly coated, after the CVC insertion, by a rich layer of host-derived proteins that promotes adherence and biofilm formation of both blood-borne microbes and those introduced during the catheter insertion (Passerini et al. 1992). The biofilm remains in the tract also after catheter removal, rendering the patient susceptible to chronic establishment of biofilm and increasing the risk of continuous hematogenous bacterial spread (Donelli 2006).

The presence on the CVC surfaces of anaerobic species growing as biofilm, alone or within a polymicrobial biofilm, has been poorly investigated. In fact, the first evidence dates back to 1988 when Haslett and co-workers isolated *Clostridium spp*. and *Propionebacterium spp*. directly from indwelling central intravascular catheters (Haslett et al. 1988). After that, just few studies have demonstrated the presence of anaerobic species in microbial biofilms causing CRBSIs.

Although current guidelines for the management of CR-BSIs include *Propionibacterium* spp. as a potential infectious agent, *P. acnes* is rarely reported as cause of intravascular colonization or CR-BSI (O'Grady et al. 2002).

Martín-Rabadán and coworkers in 2008 have advanced the hypothesis that the low rate of catheter colonization and CR-BSI by *Propionibacterium* spp. reported in the medical literature is a consequence of an inappropriate laboratory detection methodology, the chances of detecting *Propionibacterium* bacteremia being reduced by including automatic detection of growth without terminal subcultures, reduction of incubation times, and elimination of anaerobic bottles. Authors demonstrated that *P. acnes* frequently colonize vascular catheter tips and suggested sequential aerobic-anaerobic processing as a simple procedure to analyze catheter tips by the roll-plate method (Martín-Rabadán et al. 2008).

Also a recent study for the detection of colonization and CR-BSI include *Propionibacterium* spp. as a potential cause, being isolated by anaerobic processing of catheter-cultures in the 8 % of cases (Guembe et al. 2012).

The potential of the anaerobic biofilm former *P. acnes* as a cause of catheter-related bacteremia deserve further studies.

6.9 Conclusions

Most of the infections caused by anaerobes are considered opportunistic infections, arising from microorganisms of the normal flora that take advantage of generalized or localized defects in defence mechanisms to damage the host.

These anaerobic opportunistic pathogens are difficult to isolate and thus are frequently missed when clinical samples are cultured, their isolation requiring appropriate methods of collection and transport as well as cultivation of specimens in properly equipped clinical microbiology laboratories.

In fact, there is growing interest in a more accurate routine identification of anaerobes, for example by applying matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) or 16S rRNA gene sequencing (La Scola et al. 2011; Jamal et al. 2013).

Furthermore, even if highly virulent anaerobes should be considered for testing their antibiotic resistance as individual isolates (Brook et al. 2013), the slow growth, the often polymicrobial nature as well as the increasing antimicrobial resistance over time of these microorganisms, make not routinely performed the in vitro susceptibility testing (Nagy 2010).

Therefore, the treatment of these infections is mostly empirical and based on the administration of antimicrobial agents with known efficacy against anaerobes.

However, it's important to take into account that the spectrum of antibiotic resistance among anaerobes is significantly changed during the last decades and, nowadays, it includes also carbapenems and nitroimidazoles. In fact, these

drugs, once considered universally active, now exhibit a variable efficacy depending on the geographical area. For this reason, the CLSI recommends periodic monitoring of resistance trends of clinically relevant anaerobes to select the best empirical antimicrobial therapy (Wybo et al. 2014).

Just to give an example, the best selection of antibacterial drugs against both facultative and strictly anaerobic bacteria in respiratory infections are β-lactams with β-lactamase inhibitors, clindamycin, cephamycins and carbapenems, since the rates of β-lactamase production are low for *Peptostreptococcus* spp. and *Fusobacterium* spp., while are high for *Prevotella* and *Bacteroides*; the drug resistance rates to ampicillin are high in *Prevotella* spp. and *Bacteroides spp*., while the rate to piperacillin is moderate in Bacteroides. By contrast, the drug resistance rates to combinations of these drugs, i.e., piperacillin and tazobactam (TAZ/PIPC), for all the most insidious anaerobic bacterial species, are low (Japanese Society of Chemotherapy Committee on guidelines for treatment of anaerobic infections 2011).

According to the whole findings reported in the last decades, the demonstrated ability to form biofilm of opportunistic anaerobic species and their possible role as causative agents of HAIs should alert even more clinicians and microbiologists on the need to search for anaerobes in clinical samples, when their presence can be reasonably assumed, and carefully verify their antibiotic susceptibility.

In fact, the desirable availability in clinical microbiological laboratories of appropriate facilities for isolation of anaerobes could add significant information on the possible contribution of anaerobic species to biofilm-based polymicrobial infections and, thus, drive the antimicrobial therapy in the right direction.

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Microbial Biofilm Development on Neonatal Enteral Feeding Tubes

7

Noha A. Juma and Stephen J. Forsythe

Abstract

Neonates in intensive care units often require supporting medical devices and antibiotic treatment. The intensive care treatment combined with their immature immune system, the increased permeability of mucosa, and the undeveloped microflora of the gut may render the neonates highly vulnerable to colonisation and subsequent infections when exposed to opportunistic pathogens. These infections may not only be local gastrointestinal infections, but also systematic following translocation from the gastrointestinal system. This could be particularly alarming considering that common antibiotics may not be effective if the causative strain is multi-drug resistant.

This chapter reviews our information on the microbial colonization of neonatal feeding tubes. The range of organisms which have been recovered are wide, and while primarily bacterial, fungi such as Candida have also been found. The bacteria are principally *Staphylococcus* spp. and Enterobacteriaceae. The *Enterobacteriaceae* isolates are predominantly *Enterobacter cancerogenus*, *Serratia marcescens*, *Enterobacter hormaechei*, *Escherichia coli* and *Klebsiella pneumoniae*. Many of these isolates encode for antibiotic resistance; *E. hormaechei* (ceftazidine and cefotaxime) and *S. marcescens* strains (amoxicillin and co-amoxiclav).

7.1 Outlook on Neonates and Enteral Feeding

Neonates in intensive care units often require supporting medical devices and antibiotic treatment (Westerbeek et al. 2006). The intensive care treatment combined with their immature immune system, the increased permeability of mucosa, and the undeveloped microflora of the gut

N.A. Juma
Department of Medical Microbiology and Parasitology, Faculty of Medicine, King Abdulaziz University, Jeddah, KSA

S.J. Forsythe (✉)
Pathogen Research Group, School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham, UK
e-mail: Stephen.forsythe@ntu.ac.uk

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(Greenough 1996; Mehall et al. 2002b) may render the neonates highly vulnerable to colonisation and subsequent infections when exposed to opportunistic pathogens. These infections may not only be local gastrointestinal infections, but also systematic following translocation from the gastrointestinal system. This could be particularly alarming considering that common antibiotics may not be effective if the causative strain is multi-drug resistant.

As a predisposing factor for various infections (Dima et al. 2007; Jerassy et al. 2006; Thongpiyapoom et al. 2004), invasive devices may facilitate the acquisition and transmission of organisms which are able to attach and form biofilms on abiotic surfaces (Donlan and Costerton 2002). These biofilms are likely to develop at the point where the medical device is inserted or on its surface as a result of local colonization, hematogenous spread from a distant site, or infusion of contaminated material such an enteral feed. Enteral feeding is a well-known standard practice used for nutritional provision to patients who have a functional gastrointestinal system but cannot ingest food orally (Matlow et al. 2003; Roberts 2007). In contrast to parenteral feeding, enteral nutrition is generally associated with fewer complications (Kirby et al. 1995), as well as being cost effective (Tucker and Miguel 1996), and helping to maintain the structure and the function of the gastrointestinal system (Abou-Assi et al. 2002; Kalfarentzos et al. 1997; Gupta et al. 2003). However, bacterial contamination of the enteral formula is one of the recognized disadvantages of this method of nutritional support. In conjunction with this, enteral feeding tubes have been identified as a reservoir for microbial colonization (Hurrell et al. 2009b; Matlow et al. 2003; Mehall et al. 2002a; Roy et al. 2005).

The World Health Organization (2007) have recommended avoiding the ambient temperature storage of the reconstituted feed in order to minimize the risks of neonatal infections. This recommendation is not followed in all countries and is impractical when preparing small volumes of feed and fortifying breast milk (Holy and Forsythe 2014). This proposition also does not take into account the time enteral feeding tubes are kept at body temperature inside the nasogastric tract of the neonate, which may pose a greater risk in allowing bacterial proliferation than during the actual feeding which could last less than 30 min (Hurrell et al. 2009b). The nutritional feeding regime for infants in the neonatal intensive care units involves feeding every 2–3 or 4 h via enteral feeding tubes (Hurrell et al. 2009b; Mehall et al. 2002b; Mitchell et al. 2001; Washington et al. 1999), which can be in place for more than 48 h to 7 days (Hurrell et al. 2009b; Mehall et al. 2002b). During this time, bacterial proliferation and biofilm formation occurs and can act as loci for repeated microbial contamination of subsequent feeds due to bacteria in the lumen of the same enteral feeding tube entering the neonate's stomach and intestines (Hurrell et al. 2009b).

7.2 Microbial Flora of Enteral Feeding Tubes

In a previous study by Hurrell et al. (2009b) 76 % of 129 enteral feeding tubes collected from neonatal intensive care units were found to contain biofilms and the cell counts were up to 10^7 CFU/tube. A scanning electron microscope image of a neonatal enteral feeding tubing revealed a diverse range of bacterial morphotypes and yeast cells with budding points (Fig. 7.1) (Hurrell et al. 2009b). The residual liquid in the lumen of the tubes harbored up to 10^7 *Enterobacteriaceae* CFU/mL (Hurrell et al. 2009b). As the biofilm ages, the bacterial cells will begin to disperse in the form of clumps, and this cellular aggregation will be protected from the low stomach acidity (pH ~ 4.0) (Hurrell et al. 2009b) due to the bacterial capsular material. Consequently, bacterial biofilm formation may pose a risk to neonatal health since the bacterial cells could evade the detrimental effects of the acidity and transit into the intestinal tract (Kim et al. 2006).

The Hurrell et al. (2009b) study revealed a complex microbial flora colonising the enteral feeding tubes. This included members of the *Enterobacteriaceae*, *Pseudomonas fluorescens*, *Pseudomonas luteola*, staphylococci, lactic acid bacteria as well as fungi such as *Candida albicans*.

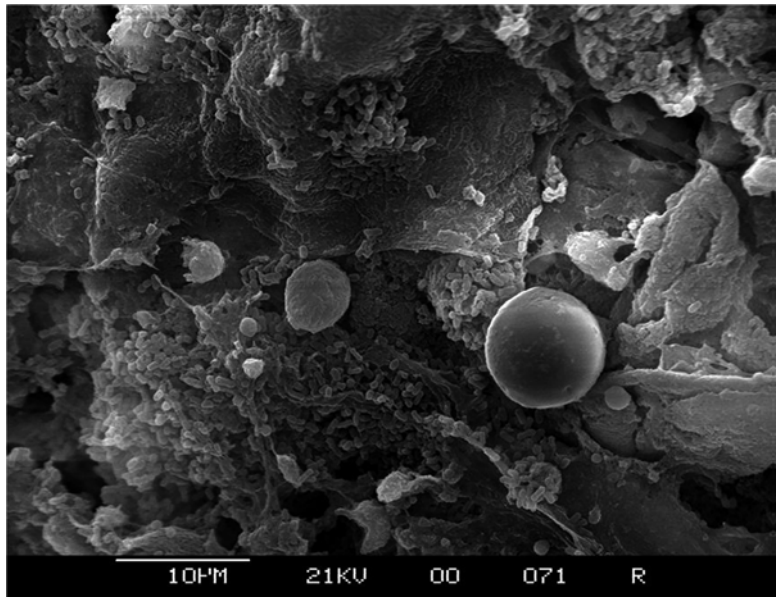

Fig. 7.1 Electron microscopy of enteral feeding tube inner wall from neonate fed breast milk and reconstituted PIF with added thickener. *Bar* indicates 10 µm size marker (Hurrell et al. 2009a, copyright retained)

The *Enterobacteriaceae* were predominantly *Enterobacter cancerogenus* (41 %), *Serratia marcescens* (36 %), *Enterobacter hormaechei* (33 %), *E. coli* (29 %) and *Klebsiella pneumoniae* (25%). All *Enterobacteriaceae* isolates were susceptible to gentamicin, ciprofloxacin and meropenem. However a quarter of the *E. hormaechei* were resistant to third generation cephalosporins (ceftazidine and cefotaxime). All *S. marcescens* strains were resistant to amoxicillin and co-amoxiclav.

Less frequently, but of considerable significance was the isolation of *Cronobacter sakazakii* from these feeding tubes. This organism and pseudomonads are well known for their ability to produce capsular polysaccharides which could entrap other organisms less able to colonise the tubing wall leading to the multiorganisms biofilm formation. In addition, *C. sakazakii* has been associated with neonatal infections (necrotizing enterocolitis and meningitis) through the ingestion of contaminated reconstituted infant formula (Holy and Forsythe 2014). However not all cases have been linked to formula contamination and the organism in this study was isolated from the tube of neonates who had not been fed infant formula, but breast fed and ready-to-feed formula.

E. coli K1 strains were also isolated from the used neonatal nasogastric tubes. This *E. coli* pathovar is of high significance as it is associated with neonatal meningitis. The *E. coli* K1 strains recovered from the tubes of 12 neonates over a 3 week period were indistinguishable according to pulsed-field gel electrophoresis indicating a common source of exposure and were sequence type 95 (unpublished results). This colonization of neonatal feeding tubes by organisms recognized as bacterial pathogens which can cause highly severe infections of neonates has not been further investigated.

The source of the organisms in the enteral feeding tubes is unclear since 81 % of the neonates were receiving sterile ready-to-feed formula directly from the jar. These products are sterile and have tamper-proof lids to indicate any bacterial growth before use. An alternative source of the enteral tube flora is the throat due to gastro-esophageal reflux. This is common in preterm neonates, occurring three to five times per hour, when the lower oesophageal sphincter relaxes. This may increase the exposure of the feeding tube to the throat flora.

A follow up laboratory-based study by Hurrell et al. (2009a) examined the extent to which

Table 7.1 Capsule production and biofilm formation of *Enterobacteriaceae* on enteral feeding tubes, and silver-impregnated tubing (Modified from Hurrell et al. 2009a)

	Polyvinylchloride (log10 cfu/cm)			Polyurethane (log10 cfu/cm)			Silver-impregnated (log10 cfu/cm)		
Organism	Average	Max	Min	Average	Max	Min	Average	Max	Min
C. sakazakii 7 (ST1)	6.3	6.4	5.8	5.6	5.9	5.3	7.4	7.7	7
C. sakazakii ATCC 12868 (ST3)	4.6	4.8	4.3	6.3	6.7	5.6	6.4	6.5	6.1
C. sakazakii 25 (ST3)	5.5	5.7	4.9	5.9	6.1	5.5	7	7	6.5
C. sakazakii 14 (ST4)	5.6	5.8	5.3	5.5	5.6	5.1	5.3	5.4	5.1
C. sakazakii 424 (ST8)	5.9	6.2	5.2	6.4	6.9	5.7	6.4	6.9	5.7
C. sakazakii NCTC 11467^T (ST8)	5.7	6.3	4	6.2	6.6	5.2	6.3	6.8	5.1
C. sakazakii 23 (ST134)	5.3	5.5	4.4	5.9	6.1	5.7	6.1	6.5	4.6
C. sakazakii 716 (ST14)	6.2	6.6	5.5	6.9	7	6.6	6.9	7.2	6.5
Salm. Anatum	6.6	6.9	5.3	6.6	6.8	6.3	6.7	7.1	5.9
Salm. Give	5.3	5.7	5	6.9	7.3	6.3	6.7	7	6.3
Salm. Kedougou	5.7	5.9	5.2	6	6.2	5.5	5.6	5.7	5.2
E. coli K12	4.4	4.7	3.9	4.6	4.6	4.6	4.4	4.5	4.2
E. hermanii	6.3	6.6	6.1	6.7	6.8	6.6	6.7	6.9	6.1
E. vulnerus	6.4	6.6	5.9	6.4	6.8	5.8	6.5	6.9	6
C. freundii	5.1	5.4	4.4	5.9	6.1	5.5	5.9	6.3	5.4
C. koseri	5.1	5.3	4.8	6	6.2	5.8	6	6.1	5.7
E. cloacae	5.1	5.5	3.4	5.9	6	5.6	3.7	3.9	3.3
E. hormaechei	5.7	6	4.9	6.4	6.7	5.9	5.6	5.7	5.5
H. alvei	4.9	5	4.7	5.1	5.2	4.8	5.6	5.8	5
K. oxytoca	5.2	5.5	4.7	5.4	5.8	4.9	6	6.5	5.4
K. pneumoniae	5.6	5.7	5.2	5.2	5.4	5	5.9	6.2	5.7
Pantoea spp.	5.6	5.9	5.2	5.6	5.6	5.4	6.2	6.3	6.2
S. marcenscens	5.8	4.1	6.2	5.9	4	6.4	6.2	6.4	5.4

strains of *C. sakazakii*, *Salmonella* serovars, and other *Enterobacteriaceae* can adhere and grow on enteral feeding tubes composed of polyvinyl chloride, polyurethane, and silver-impregnated flexelene. The latter being expected to have antibacterial activity. Three of these strains (*C. sakazakii*, *E. hormaechei*, and *S. marcescens*) were originally isolated from a PVC enteral feeding tube that had been in use at a neonatal intensive care unit. To simulate hospital practices, 37 °C incubated PVC enteral feeding tubes were gently flushed with fresh sterile formula every 2 h, and the lumen contents collected. The number of bacteria on the tube wall and the residual liquid in the tube lumen was determined using the impedance method. All the strains grew as biofilms on the three types of tubing. The highest biofilm levels were recorded with two *C. sakazakii* strains on the flexelene (silver-impregnated) tubing at 7.7 $\log_{10}$ CFU/cm (Table 7.1). *Salm*. Anatum, *Salm*. Give and *Salm*. Kedougou produced biofilms on the three types of tubes at levels similar to *C. sakazakii*; ca.10^5–10^7 CFU/cm. Of the remaining *Enterobacteriaceae*, *E. coli* K12 had the lowest level (10^4 CFU/cm) of biofilm formation on all three types of tubing (Table 7.1). *E. hermanii* and *E. vulneris* had biofilm levels approximately 100-fold higher at 10^6 CFU/cm. In general, across all the bacterial species, the level of biofilm formation on the silver-impregnated tubing was higher than that on the PVC and PU enteral feeding tubes (Table 7.1). In terms of the time course, bacterial numbers on the tubing wall were 10^4–10^5 CFU/cm by 8 h, and 10^4–10^6 CFU/cm after 24 h. The organisms also multiplied in the fresh liquid feed of the tube lumen to 10^7 CFU/

mL by 8 h, 10^8–10^9 CFU/mL with 24 h and had a doubling time of 22 min.

7.3 Influence of Enteral Formula Feeding on Bacterial Adherence and Biofilm Development

Adhesion characteristics of bacterial cells cultivated on innate surfaces can change as a response to the nutrient state (James et al. 1995). Infant formula is rich in nutrients and in such environments attached bacterial cells may migrate across the surface, which is known as the spreading maneuver (Lawrence and Caldwell 1987). A similar type of spreading behavior has been observed during surface growth of *Pseudomonas* (Marshall 1988). Although this dispersive surface colonization allows expansion of the population over a surface, these weakly attached cells may detach more easily due to the liquid flow of subsequent enteral feeds (James et al. 1995). The non-uniform disruption of adhered cells due to the sheer forces of the liquid stream may account for the fluctuation in the biofilm density during the feeding tube placement time-course (unpublished data). An additional reason could be irregular colonization of the tube surface. This inconsistency of the biofilm is of clinical importance as it underlines the fragile nature of the biofilm inside the enteral feeding tube and the frequent dispersion of bacterial clumps in fresh feeds as a consequence.

Biofilm formation is influenced by the components of the medium in which the biofilm develops (Gerstel and Romling 2001; Hood and Zottola 1997; Kim et al. 2006; Stepanovic et al. 2003). This is due to changes in the characteristics of the bacterial cell surface (Kim et al. 2006). The biofilm production by organisms such as the *Cronobacter* spp. is higher under nitrogen-rich conditions (whey-casein) than under carbohydrate-rich conditions (Dancer et al. 2009). Infant formulas are rich in milk protein and contain various nitrogen sources (casein, whey, and soy). The biofilm density of *Cronobacter* however appears to be similar in skimmed milk containing casein to that with added whey (Dancer et al. 2009).

7.4 Risk Factors of Infections to Enterally-Fed Neonates

The risk of the increased and repeated exposure to bacterial cells from the colonized enteral feeding tube is not only due to the state of the neonates immune system and the lack of a competitive intestinal microflora, but also due to the ability to resist host defenses and antibiotics (Fux et al. 2004; Lee et al. 2008). Biofilm cells can grow and thrive in the presence of large concentrations of antibodies directed against epitopes on their surfaces, and can withstand the attack of activated phagocytes (Jensen et al. 1990). These cells can also be less sensitive to acidic conditions (Hurrell et al. 2009b) which are rarely sustained in the neonate stomach as the pH tends to be above 4.0 for prolonged periods of time due to frequent feeding (Hurrell et al. 2009b; Mitchell et al. 2001; Washington et al. 1999). Frequent and short intervals in feeding do not allow enough time for the pH to decrease to levels similar to that in the adult stomach. The long maintenance of the high pH levels with the highest reached at feeding times (Sondheimer et al. 1985), when the organism in the contaminated feed is ingested, may offer a greater chance of bacterial survival, colonization, and potentially subsequent infections in the neonate. Although some pathogens lose their viability to a certain extent, they are not completely killed or eradicated at such pHs (Edelson-Mammel et al. 2006; Koutsoumanis et al. 2004; Koutsoumanis and Sofos 2004). Infants may receive histamine two receptor blockers in order to suppress the production of acid in the stomach as a prophylactic or a therapeutic option (Kuusela 1998). This has also been proved to permits the colonization of this area by opportunistic pathogens (Garrouste-Orgeas et al. 1997; Zavros et al. 2002).

7.5 Biofilms on Enteral Feeding Tubes and Antimicrobial Resistance

Cells growing in biofilms are generally highly resistant to antimicrobial agents (Lee et al. 2008). The enhanced rates of horizontal gene transfer

and recombination between bacterial cells within biofilm communities facilitate the spread of antibiotic resistance (Bergogne-Berezin and Towner 1996; Donlan and Costerton 2002). This will provide bacterial cells within biofilms with a very high level of genomic diversity where it is expected that at least a small proportion of cells will withstand the impact of any antibiotic (Boles et al. 2004). Antibiotic pressure might also select for antibiotic resistant strains, which are capable of forming biofilms. The problem with the high levels of antibiotic resistance is that it may not only be challenging in terms of the choice of treatment and the difficulty to sometimes clear the infection but also lies in the possibility of severe consequences to vulnerable neonates. This could be caused as a result of the delay in recognizing that the bacterium is actually resistant to the antibiotic of choice and hence the delay in using the effective treatment which takes between 24 and 48 h for culturing and susceptibility testing. Of interest also is that even silver-impregnated enteral feeding tubes which were expected to have an antibacterial activity or prevent biofilm development did not appear to have an effect on the ability of strains including *Salmonella* serovars, *C. sakazakii*, other *Enterobacteriaceae* and *Acinetobacter* spp. to grow and form biofilms (Hurrell et al. 2009a).

The acquisition of antimicrobial-resistant organisms such as vancomycin-resistant *Enterococcus* (Matlow et al. 2003; Mehall et al. 2002a; Weinstein et al. 1996), methicillin-resistant *Staphylococcus aureus* (MRSA) (Mehall et al. 2002a), and extended-spectrum beta lactamase encoding *E. coli* and *Klebsiella* spp. (Wiener et al. 1999) are not unusual via the enteral feeding route. Antibiotic-resistant nosocomial bacteria have not only been reported to establish themselves in enteral feeding tubes but also spread to other infants and subsequently cause clinical infections (Mehall et al. 2002a). Transmission from an infected infant to a feeding tube in another patient has also been found (Mehall et al. 2002a). The most likely vehicle was believed to be the hands of staff (Schreiner et al. 1979). Epidemiological and microbiological links between enteral nutrition and feeding intolerance, abdominal distention, aspiration pneumonia, diarrhoea, necrotizing enterocolitis, and even systemic infections such as sepsis caused by bacterial contamination have been reported (Anderson et al. 1984; Anderton 1993; Freedland et al. 1989; Wagner et al. 1994).

7.6 Prevention of Neonatal Infections Caused Be Contaminated Enteral Feeding

Although the intrinsic susceptibility of the neonates may not be controlled, what can be done to prevent potential neonatal infections is to reduce the risks of exposure to opportunistic organisms. Colonization of enteral feeding tubes may occur not only during placement but also as a result of the administration of contaminated feeds. Powdered infant formula can be intrinsically contaminated (Cawthorn et al. 2008; Marino et al. 2007; Miled et al. 2010) where the organisms are likely to survive owing to their desiccation resistance capacity (Wagenvoort and Joosten 2002). Therefore, to reduce this risk, the microbiological safety of neonatal feeds should be ensured during the general preparation and handling of formula and feeding systems. Of particular importance is the temperature at which the formula is reconstituted and stored (if needed). Bacterial overgrowth due to possible temperature abuse of reconstituted feed have accounted for three previous *Cronobacter* spp. outbreaks (Caubilla-Barron and Forsythe 2007; Himelright et al. 2002; Van Acker et al. 2001). For safe consumption of powdered infant formula, the guidelines issued by the Codex Alimentarius Commission (2008) state the use of sterilized equipment for the preparation of the powdered infant formula feed and that feed should be rehydrated at 70 °C or above and only be prepared once needed. Feeds should never be stored in the fridge for longer than 24 h and must also be thrown away if not consumed within 2 h. The time the feed is left at room temperature needs to be minimized to avoid bacterial overgrowth. The prolonged nasogastric placement of the

enteral feeding tube may require reconsideration although it can distress the neonate and should be minimized. Replacing these tubes very frequently can be costly and therefore the risk control aim has to be the quality of the feeds given enterally. Feeding procedures and practices such as the Hazard Analysis Critical Control Point system (Bryan 1990) need to be monitored (Anderton 1995). The closed ready-to hang enteral nutrition delivery system is believed to be an effective method for contamination-free enteral feeding (Chan et al. 1994; Curtas et al. 1991; Wagner et al. 1994).

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Voice Prostheses, Microbial Colonization and Biofilm Formation

8

Matthias Leonhard and Berit Schneider-Stickler

Abstract

Total laryngectomy is performed in advanced laryngeal and hypopharyngeal cancer stages and results in reduced quality of life due to the loss of voice and smell, permanent tracheostoma and occasionally dysphagia. Therefore, successful voice rehabilitation is highly beneficial for the patients' quality of life after surgery. Over the past decades, voice prostheses have evolved to the gold standard in rehabilitation and allow faster and superior voicing results after laryngectomy compared to esophageal speech. Polyspecies biofilm formation has become the limiting factor for device lifetimes and causes prosthesis dysfunction, leakage and in consequence pneumonia, if not replaced immediately. Although major improvements in prosthesis design have been made and scientific insight in the complexity of biofilm evolution and material interaction progresses, the microbial colonization continues to restrict device lifetimes, causing patient discomfort and elevated health costs. However, present scientific findings and advances in technology yield promising future approaches to improve the situation for laryngectomized patients.

8.1 Head and Neck Cancer and Oncological Surgery

Head and neck cancer is the sixth most common type of cancer with about 650,000 new cancer cases and 350,000 cancer death worldwide. It involves cancer of the oral cavity, the pharynx, and the larynx. In more than 85 % of the cases it is a squamous cell carcinoma. The major risk factors are alcohol and tobacco consumption.

Beside other risk factors, such as male gender, Human Papilloma Virus (HPV) infection, oral health situation and periodontal disease are discussed to play an accompanying factor in the development of head and neck cancer.

Despite of wide information campaigns on early symptoms, such as persistent hoarseness, laryngeal pain, dyspnea, dysphagia and enlarged cervical lymph nodes, more than 40 % of laryngeal/

M. Leonhard • B. Schneider-Stickler (✉)
Department of Otorhinolaryngology and Head and Neck Surgery, Medical University Hospital Vienna, Vienna, Austria
e-mail: berit.schneider-stickler@meduniwien.ac.at

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hypopharyngeal tumors can only be diagnosed in advanced tumor stages. Radiation, radiochemotherapy and surgery in combination with primary or adjuvant radiotherapy and possibly chemotherapy represent standard treatment options.

Whereas lower tumor stages of laryngeal cancer (T1–2) can be treated by radiation therapy or partial laser tumor resection with preservation of voice function, advanced stages (T3–4) often require total extirpation of the larynx (laryngectomy) and additional radiation/chemotherapy with the loss of voice function. Total laryngectomy is the surgical procedure to remove the larynx, create an anastomosis between a neopharynx and the esophagus and redirect the trachea to the skin in form of a permanent tracheostoma. Figure 8.1a illustrates the anatomical changes due to extirpation of the larynx. An additional dissection of cervical lymph nodes and postoperative radiation therapy is often necessary depending on tumor staging.

Laryngectomy confronts the patient with a radical and crippling therapy option with intimidating loss of life quality due to the disability to produce voice, loss of sense of smelling, a permanent tracheostomy, dysphagia, and appalling cosmetic results. After tumor therapy a successful voice and speech rehabilitation is of great importance in order to reintegrate a patient in the social life.

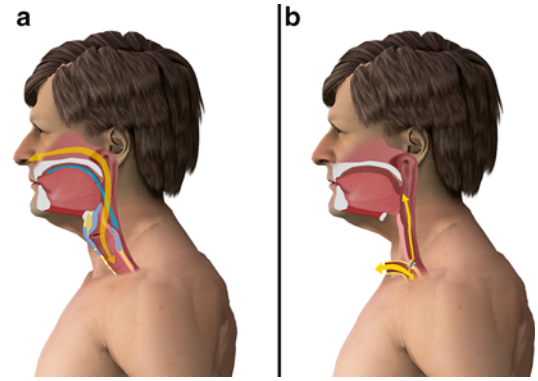

Fig. 8.1 Comparison of the aerodigestive tract before (**a**) and after (**b**) laryngectomy. (**a**) The larynx is the anatomical junction and functional barrier between the airways (*yellow arrow*) and the digestive tract (*blue arrow*). It prevents aspiration during deglutition and produces an individual and highly characteristic personal voice at expiration. (**b**) The surgical removal of the larynx results in the anastomosis of the neopharynx to the esophagus and the redirection of the trachea to the body surface superior of the sternum (permanent tracheostoma). The airway and the digestive tract are separated. The tracheoesophageal fistula unites the esophagus and the trachea with a unidirectional valve system

8.2 Voice Rehabilitation After Laryngectomy

Among the options of voice rehabilitation after laryngectomy the use of voice prostheses has become the gold standard with superior success rates between 50 and 90 % than esophageal speech (30 %) (Hotz et al. 2002; Lam et al. 2005; Bozec et al. 2009; Op de Coul et al. 2000).

Esophageal speech is accomplished by swallowing of small volumes of air (80 ml) into the lower esophagus. Upon regurgitation, a burping sound is produced, which can be used for phonation of short sentences. However, this requires a considerable time of training, and the gastric can be inflated with air.

The success of voice prostheses is explained by a fast regaining of voice after surgery and the ability to use the whole lung capacity for phonation enabling the patient to form longer sentences with better auditory quality. Thus, an increase in numbers of laryngectomees provided with voice prostheses could be observed from 5.6 % (1990) to 60 % (2001) in Germany. In the Netherlands a number of even 90 % is estimated.

8.3 Voice Prostheses

Modern voice prostheses are small polymer tubes with a unidirectional valve mechanism inside and soft polymer flanges at both ends to keep the device in place between the esophagus and the trachea. The location of the tracheoesophageal fistula and the implanted voice prosthesis are shown in Fig. 8.1b.

The fistula is created by a tracheoesophageal puncture (TEP) primarily during laryngectomy or secondarily at least weeks after tumor therapy. The procedure of tracheoesophageal puncture was described first by Maves and Lingeman and has been refined since then (Maves and Lingeman 1982). The valve mechanism of the voice prosthesis

blocks the passage of esophageal contents and prevents aspiration, but allows the patient to transfer inhaled air from the lungs into the esophagus to induce a sonant vibration of the neopharynx. For esophageal phonation, the tracheostoma/the tracheal cannula has to be closed by a finger or another valve system mounted on the tracheostomy tube ("hands free speech"). The small valve inside the voice prosthesis has to protect the lower airway against aspiration by blocking saliva, food and beverages. Due to pressure changes in the esophagus during swallowing, the opening pressure of the valve needs to have a certain resistance in order to only open during phonation.

However, precision and durability of the valve opening and closure are not the only technical challenges in the design of voice prosthesis. The device has to be inserted with minimum trauma to the mucosa of the fistula in order to avoid local infection and widening. This means that at least one flange has to be made of a soft foldable material to fit the diameter of the fistula. Most prostheses are made of silicone or a soft polymer material and have two flexible flanges, which allow repositioning, if the prosthesis is inserted to far ("overshooting"). A radiopaque structure is also needed in order to be able to find a dislocated prosthesis on an x-ray image. To simplify the insertion, each manufacturer provides individual insertion equipment (gel cap or inserter tube).

8.4 History of Voice Prostheses

The basic principle to create a functional shunt for speech rehabilitation using a prosthesis was published first by Mozolewski in 1972 (Mozolewski 1972). The lack of polymer mass manufacturing processes prevented the spreading of the new concept. In 1979 Blom and Singer introduced the duckbill prosthesis with a slit valve in the USA. In 1980 another slit valve prosthesis was developed by Nijdam in Groningen (Groningen Button). In 1981 a "voice button prosthesis" was described by Panje, which comprised a valve mechanism that could be manually cleaned in situ. Later on, a slit valve prosthesis was developed in 1986 in Germany by Herrmann. The Eska-Herrman prosthesis is still in use in cases, where the patient individually wants to decide whether this form of voice rehabilitation is suitable. Due to the comparably small diameter of the fistula (5.5 mm), spontaneous closure in cases of unsatifactionary results is possible (Schuldt et al. 2012). These so called "non-indwelling" prostheses could be withdrawn, cleaned and reinserted by the patient himself. A part of the prosthesis or a twine was used to retrieve it.

However, the necessary manual dexterity for daily atraumatic reinsertion was not met by most of the laryngectomized patients and led to the development of the "indwelling" prosthesis type. It was implanted and replaced transorally with a guiding wire by pulling it from the esophageal side into the fistula. This "retrograde" procedure was stressing to the wake patients, still the concept was introduced successfully with the Provox 1 prosthesis in 1988. The milestone was the leap from a slit valve to a flap valve system, which improved aerodynamic properties of the voice prosthesis and the patients could phonate with less effort due to smaller airway resistance. The high phonation resistance shifted the preference of patients towards valve flap systems (Harms et al. 2011).

In the next step, the transoral replacement technique was superseded by a more comfortable and simple "anterograde" technique through the tracheostoma. An insertion tube was used to orchestrate the folded silicone prosthesis into the fistula. Upon withdrawal of the insertion tube, the flanges of the prosthesis unfolded and secured it in place. The concept was realized in the Provox 2 and proved to remain the leading design of further development of modern voice prostheses. The indwelling time of Provox 1 (mean 224 days) dropped with the Provox 2 (mean 96 days) (Op de Coul et al. 2000; Schäfer et al. 2001). This could be explained by the required softer polymer material and by the patients' better compliance to have the prosthesis reinserted with the new anterograde approach.

With the indwelling prostheses, the cleaning of the valve system by the patient was narrowed down to intraluminal brushing and the problem of device failures due to biofilm formation emerged. It was identified to limit device lifetimes and to

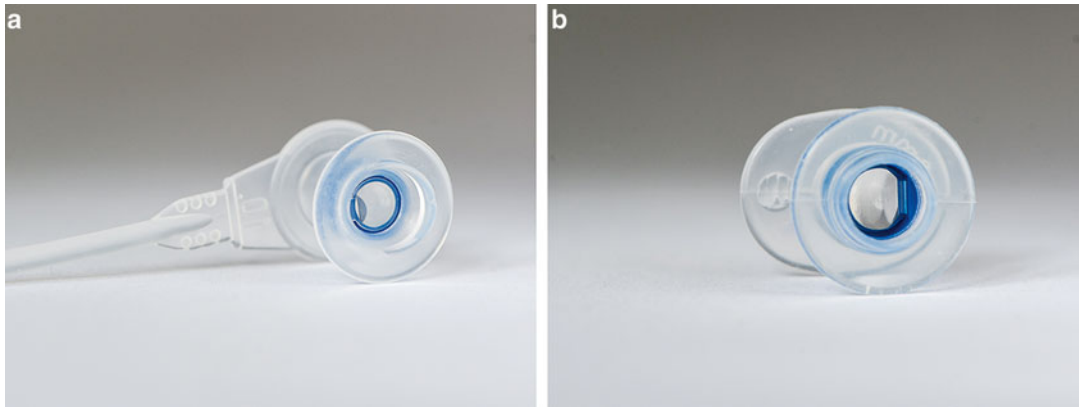

Fig. 8.2 Standard Provox prostheses: Provox 2 (**a**) and Provox Vega (**b**)

cause device failures (Van Weissenbruch et al. 1997). Moreover, structural biological damage to the silicone material could be assessed. The issue was addressed by modifications in prosthesis design and material. A valveless design (Van Den Hoogen et al. 1996), a metal ball valve design (Tracoe, Voicemaster prosthesis, 1998), a polyurethane material (Traissac, Newvox voice prosthesis, 2007) and a thermoplastic elastomer (Leonhard et al. 2010) did not succeed in improving the standards (Van Den Hoogen et al. 1996; Schouwenburg et al. 1998; Traissac et al. 2007; Leonhard et al. 2010). Optimization of aerodynamic properties and resistance to biofilm colonization remained the two leading product development strategies. Today, two companies are established as dominating distributers on the global market: InHealth Technologies (Blom Singer Prostheses, Carpinteria, USA) and ATOS Medical (Provox Prostheses, Hörby, Sweden, Europe). The following prostheses can be seen as current standard in voice rehabilitation of laryngectomized patients.

8.5 Standard Prostheses in Clinical Practice

8.5.1 Provox Voice Prostheses

Modern Provox systems comprise Provox 2 (size: 22.5 French, Fig. 8.2a) and Provox Vega (sizes: 17, 20 and 22.5 French, Fig. 8.2b) and represent the standard voice prosthesis types used especially in Europe. The shaft of the Provox 2 prosthesis is endured by a radiopaque flouroplastic tube, which also forms a solid valve seat. The prosthesis is inserted anterograde through the tracheostoma.

It is superseded by Provox Vega with optimized aerodynamic properties, a standardized valve opening pressure to prevent unwanted openings during swallowing, an easy to cut safety strap and a novel insertion tool to simplify the replacement procedure (Smart Inserter).

8.5.2 Blom-Singer Classic Voice Prosthesis

The Blom-Singer Classic is available in the sizes 20 French and 16 French diameter with optional wide flanges (Fig. 8.3).

This voice prosthesis is entirely made of silicone. The esophageal flange with the valve mechanism can be folded into a water-soluble gel cap and inserted atraumatically into the fistula. The patient then drinks warm water and the esophageal flange unfolds when the gel cap dissolves. With the atraumatic anterograde insertion technique, optional extra wide flanges and oversized shaft lengths the prosthesis is suitable to manage difficult anatomies and is standard in the USA.

Fig. 8.3 Blom Singer Classic voice prosthesis

Fig. 8.4 The Provox ActiValve prosthesis with a flouroplastic valve flap and a magnetic closure mechanism

8.5.3 Modified Prosthesis Designs for Improved Biofilm Resistance

8.5.3.1 Provox ActiValve

The Provox ActiValve prosthesis is specifically designed to endure heavy cases of biofilm infestation and reduce events of unintended valve openings due to negative esophageal pressure during swallowing and inhalation (Fig. 8.4).

This is achieved by an improved valve system with seating and flap both made of flouroplastic. The valve closure is supported by a magnetic force and is available in three different strengths (light, strong, extra strong). The rest of the prosthesis is similar in design and application to the Provox 2. This prosthesis was announced as "problem solver" and has shown significantly increased device lifetimes in clinical trials (Hilgers et al. 2003; Soolsma et al. 2008). Although good performance could be confirmed in multiple studies, this more expensive prosthesis remains limited to patients who cannot be sufficiently provided for with the standard prostheses mentioned above.

8.5.3.2 Blom Singer Advantage and Blom Singer Dual Valve

The Blom Singer Advantage prosthesis comprises a valve flap with 7 % silver oxide incorporated in the silicone material (Fig. 8.5a).

The use of silver has been reported beneficial in antimicrobial coatings of medical devices, such as urinary catheters and tympanic tubes and was adopted to voice prostheses with this model (Politano et al. 2013). A clinical trial showed an improvement in biofilm resistance compared to the classic model (Kress et al. 2006). The Blom Singer Dual Valve adds a second flap valve with silver oxide at the tracheal end of the prosthesis shaft (Fig. 8.5b, c). It is supposed to prevent leakage in cases of valve failure on the esophageal end and therefore should prolong device lifetime. Although equipped with two valves, the silicone prosthesis is still flexible enough to be inserted with the gel cap method.

8.6 Limited Device Lifetimes of Voice Prostheses Due to Biofilm Formation and Device Malfunction

A review of literature shows average device lifetimes of 4–6 months for standard voice prostheses and nearly 10 months for the Provox ActiValve (Op de Coul et al. 2000; Harms et al. 2011; Tićac et al. 2010). A critical review on clinical performance of current prosthesis models based on 749 replacements was published by Kress et al. (2013). However, problem solving voice prostheses remain unaffordable for larger patient groups due to present and future economic pressure on national health care systems. Clinical trials on the

Fig. 8.5 Blom Singer Advantage (**a**) and Blom Singer Dual Valve (**b**). The black valve flap material contains 7 % silver oxide to reduce microbial colonization. The second valve on the tracheal side of the prosthesis works as a backup in cases of leakage of the esophageal valve flap. A metal wire was inserted in the prosthesis shaft to illustrate to opened valves (**c**)

performance of novelties in voice prostheses are invaluable for adequate clinic decisions in voice rehabilitation. Other parameters than device life times have also been proven to impact life quality of laryngectomees.

8.7 Communication-Related Outcome After Laryngectomy

Gaining a new voice is one of the major aims after total laryngectomy. Over the past 25 years there has been significant improvement in the rehabilitation of laryngectomized patients, with speech restoration that has dramatically altered and improved their quality of life.

Although most recently published research promises future laryngeal replacement with an artificial larynx after total laryngectomy (as shown by Debry et al. on a patient who was able to talk in a whispering fashion while the tracheostomy was temporarily closed), voice rehabilitation with insertion of voice prostheses for tracheo-esophageal voice production is superior to other rehabilitation methods like esophageal speech and electrolarynx (Debry et al. 2014; Simpson et al. 1997; Moukarbel et al. 2011).

The assessment of quality of life after voice rehabilitation with voice prostheses was performed by Tisch et al. using the standardized "Quality of Life Questionnaire" of the European Organisation for Research and Treatment of Cancer (EORTC QLQ–30) (Tisch et al. 2003). Seventy two percent of the patients evaluated their ability to communicate with "good" and "very good".

Actual reports on voice parameters like maximum phonation time, pitch, and speed of speaking are relatively sparse. Terada et al. reported on a mean maximum phonation time of 15.1 s (range: 8–20 s) in patients with Provox 2 voice prostheses (Terada et al. 2007). A comparison of Provox 2 and Provox Vega by Lorenz et al. showed mean phonation times from 11.3 to 15.3 s and mean dynamic ranges between 21.9 dB and 25.7 dB (Lorenz and Maier 2010).

Successful voice rehabilitation consider intelligibility, pitch, and speed of the voice, both from the perspective of the patient and when measured objectively.

In 1991 de Maddalena et al. measured the intelligibility by using post-laryngectomy-telephone-test (PLTT). The intelligibility of the laryngectomees equipped with voice prostheses and using tracheo-esophageal voice is significantly higher than the intelligibility of patients with esophageal voice or electrolarynx (de Maddalena et al. 1991).

Only limited information is available whether the different types of modern voice prostheses differ concerning communication-related outcome measures. In 1998, Delsupehe et al. reported on very similar voice quality, lifetime, and patient satisfaction in 52 patients randomly selected to receive either Blom Singer or Provox prostheses (Delsupehe et al. 1998). They concluded, that given the equal and good results in

terms of voice quality, other factors (e.g., costs, surgery-related factors, maintenance, patient preference) should be taken into account when deciding which type of tracheoesophageal voice prosthesis to use.

8.8 Complications of Voice Prostheses

The success of voice rehabilitation with voice prostheses highly relies on correct selection and fitting to the individual patient. Some patients require multiple attempts with different prosthesis models and insertion techniques until a satisfying solution is found. This should encourage clinicians to stay informed on technical advances in the field. Known complications include granulation tissue surrounding the tracheoesophageal fistula, prosthetic dislocation, leakage, trauma and inflammation of the fistula tissue, distortion of the fistula due to scar tissue and the loss of function due to blocking of the tube lumen. These complications lead either to inability to phonate or aspiration, the latter with the risk of aspiration pneumonia and higher mortality rates. The functioning of the prosthesis is crucial to the patient and therefore each patient should be aware of the consequences in delaying the inspection by a trained ENT specialist. In most cases refitting and replacement of the prosthesis can resolve the problems. Sometimes resection or cauterization of granulation tissue, systemic antibiotic treatment or closing of the fistula is necessary.

8.9 Causes for Early Device Failures

In clinical trials as well as in clinical routine, large variations in device lifetimes can be observed even in the same patients after years of using the same type of prostheses. Sometimes a patient stays equipped with a new prosthesis for several months, sometimes she/he returns after several days and a leakage can be verified. The reason for early onset valve leakage is mainly attributed to inconsistent out-of-the-box valve opening pressures. This means, that identical prosthesis models can vary in the valve flap restriction forces. Standardization of these parameters has been taken up and implemented by the manufacturers in new model series (Blom Singer Advantage, Blom Singer Dual Valve, Provox Vega, Provox ActiValve).

The main reason for late onset device dysfunction is associated with biofilm formation on the valve structures causing improper valve closure and transprosthetic leakage. Formation of granulation tissue, local inflammation and widening of the tracheoesophageal fistula are also speculated to be associated with the microbial colonization in situ.

8.10 Biofilm Formation on Voice Prostheses

An inserted voice prosthesis is an exogenous surface in a warm and humid environment. A salivary flow from the oropharynx is rich of proteins to form a conditioning film on the polymer material and facilitates further microbial adhesion. It also transports microbes from the oropharyngeal microbiome to the esophageal surfaces of the voice prostheses. Radiotherapy reduces the secretion of the salivary glands and changes saliva composition. This leads to xerostomia and a shift in microbial population. Patients often suffer from oral candidiasis and a deteriorating dental health status as side effect of postoperative radiotherapy (Fig. 8.6).

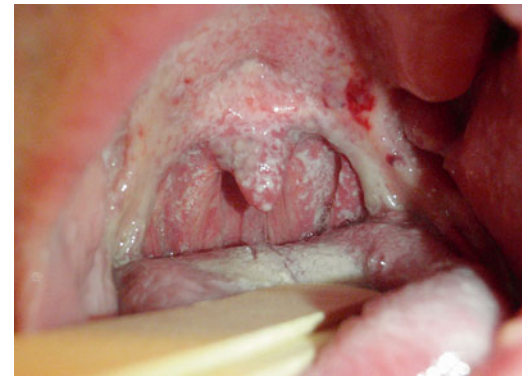

Fig. 8.6 Oral candidiasis after radiotherapy as a commonly observed complication

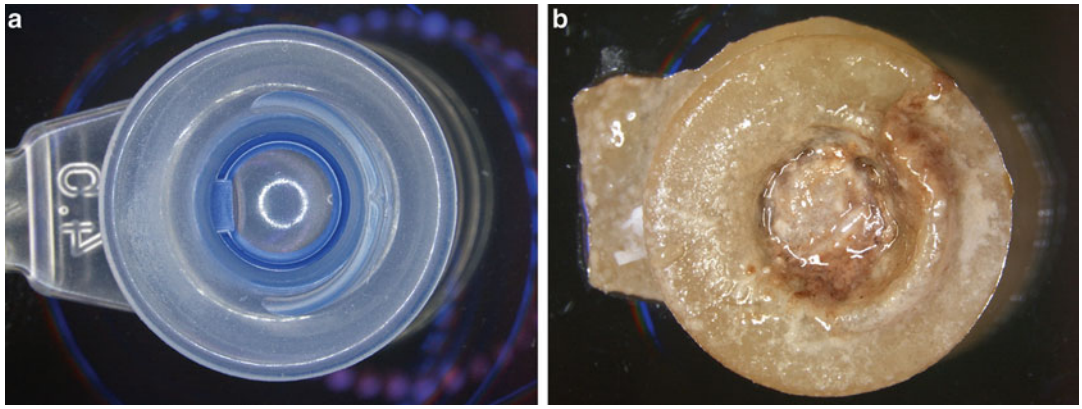

Fig. 8.7 Microbial colonization of a Provox 2 voice prosthesis: out-of-the box view on the esophageal valve and flange (**a**), after 5 months in vivo, the surface is covered with biofilm (**b**)

Nearly 75 % of the healthy population carries resident Candida species in the oral cavity (Ruhnke 2006). *Candida albicans* is the most common commensal human fungal pathogen, which can cause severe systemic and local infections in immune compromised and elderly patients (Pfaller and Diekema 2007). Oropharyngeal bacteria and yeasts seem closely associated to the microbial colonization of voice prostheses (Bertl et al. 2013). Local environmental conditions are affected by nutritional components, humidity, the immune response of the patient and pH-value. Gastric reflux after laryngectomy is estimated over 40 % and has been also discussed to be associated to better: fistula complications and prosthetic dysfunction (Garrido et al. 2007; Smit et al. 1998). The exact mechanism is not clear, but impact on tissue and microbial colonization seems plausible (Pattani et al. 2009; Lorenz et al. 2010; Cocuzza et al. 2012; Boscolo-Rizzo et al. 2008). Notably, on voice prostheses Candida infestation is always mixed with bacteria in form of polyspecies biofilms. Additionally to the reduced perfusion of the scar tissue of the fistula, biofilms take advantage to retreat on the exogenous surface to evade the hosts' immune response.

8.10.1 Macroscopy

Within days after implantation macroscopically microbial deposits appear on the esophageal surfaces of valve parts, inside niches and on the flanges (Fig. 8.7). Progression of the microbial colonization can be observed on explanted voice prostheses. The biofilm deposits finally overgrow the entire esophageal surfaces and sometimes even the whole prosthesis.

Small brushes and syringes are provided to flush and clean the prostheses, but even if performed daily, the deposits begin to bond tightly to the polymer materials and withstand brushing. Sometimes a single colony grows on the valve seating and causes dysfunction few days after implantation. More often a thick microbial mat covers the valve structures and prevents proper movement of the valve flap within weeks or months after insertion.

8.10.2 Microscopy

Microscopic evaluation is performed with (fluorescent) light microscopy, scanning electron microscopy and confocal laser microscopy. Preparation of specimen needs special attention to preservation of the extracellular matrix, in which the cells are embedded. The microbial deposits show a large wide variety in cell density and microbial growth forms. This mirrors the heterogeneity of the biofilm forming species, which work in concert to colonize the polymers. The deposits reveal a dense microbial network of coexisting microconsortia embedded in the

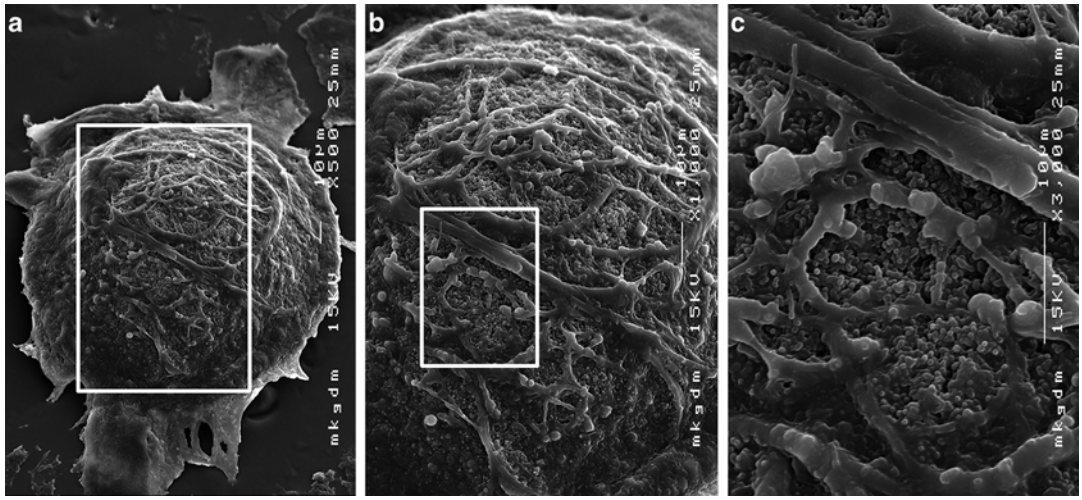

Fig. 8.8 Biofilm deposit on a Provox 2 voice prosthesis (**a**) Inside the EPS matrix is a net of candida hyphae (**b**) and in between bacterial agglomerations (**c**)

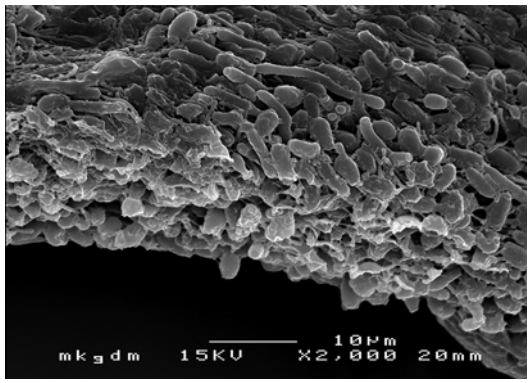

Fig. 8.9 In vivo isolated biofilm detached from a voice prosthesis. The EPS matrix is dehydrated and shrinks during sample preparation for scanning micrography. A net of fungal hyphae and bacteria emerges and shows the close community structure inside biofilms

typical extracellular polysaccharide matrix (EPS) (Fig. 8.8). The matrix is interwoven with yeast hyphae, which stabilize the deposits and bacteria in between (Fig. 8.9). Following the basic concepts of biofilm formation and functioning as dynamic heterogenous microbial microconsortia helps to understand the clinically observed processes.

Although it is speculated that nutrition and life habits might have impact on the biofilms, there is little scientific evidence to support the theory. Though often addressed by studies, the impact of liquor, coke, coffee, tobacco, yoghurt consumption, daily amount of speech, dental health, mouthwashes, etc. could not be verified yet in clinical trials. However, the effect of probiotic bacteria proved to reduce microbial colonization and surface deterioration on voice prostheses in vitro (van der Mei et al. 2000; Rolien 2014). Interestingly, the effect was also associated to the different prostheses, which indicates surface or material cofactors. Nevertheless, the principle appears plausible and laryngectomee support groups as well as internet forums keep discussing the consumption of buttermilk as method to prolong device lifetimes.

8.10.3 Microbiology

Data on microbes most often isolated from explanted voice prostheses comprises mainly *Staphylococcus* spp., *Streptococcus* spp., *Escherichia* ssp., *Enterobacter* spp., *Proteus* spp., *Pseudomonas* spp. and *Candida* spp. (Van Weissenbruch et al. 1997; Tićac et al. 2010; Bauters et al. 2002; Kania et al. 2010). *C. albicans* and Candida subspecies (*Candida tropicalis*, *Candida glabrata*, *Candida krusei*) are consistently emphasized to play a dominant role in biofilm formation on implantable medical devices (Ramage et al. 2006). At least one species

of Candida was assessed in all studies with collections of specimen from voice prostheses. As our knowledge on biofilm functioning increases, a dynamic evolutionary process with constant ecologic pressure, metabolic competition and complex signaling (*quorum* sensing) between the involved species is portrayed. It is still unclear, if unobtrusive species, which are regularly identified in small quantities, do not have more impact on the biofilm formation and maturation process than suspected.

8.10.4 Material Degradation of Voice Prostheses

Continuous biofilm colonization leads to material degradation processes. Candida first adheres to the polymer material in planktonic cell form with specialized surface binding adhesins. As a reaction to adherence and by sensing of environmental changes (such as serum, N-acetylglucosamine, neutral pH, high temperature, starvation, CO_2), the fungus can proliferate in a budded yeast form or a filamentous hypheal form (Biswas et al. 2007). The latter is considered as the more virulent growth form, which can penetrate tissue and prosthetic polymer materials. Virulence is also increased by secretion of proteases, phospholipases and lipases which improve the penetration (Mayer et al. 2013; Naglik et al. 2003). The processes of biodegradation of synthetic polymers include fouling, degradation of leaching components, corrosion by lytic enzymes, hydration, hypheal penetration and discoloring by microbial pigments (Flemming 1998). Excavations of silicone, hypheal infiltration and dense cell colonies deforming the silicone matrix impressively demonstrate the process of material deterioration on voice prostheses (Leonhard et al. 2009) (Figs. 8.10, 8.11, and 8.12).

8.11 Strategies to Avoid Biofilm Formation on Voice Prostheses

The research for biofilm inhibition on voice prostheses has started with the identification of the problem. Since then many different approaches have been investigated and discussed. Possible targets are the prevention of microbial adhesion to the prosthetic surface by coatings and surface modifications, inhibition of further microbial proliferation, containing the biofilm forming species by non-pathogenic probiotic species, disruption of the shielding EPS matrix structure and interception of cell communication. An extended literature review on investigated methods to achieve the mentioned principles including metal coatings, plasma surface treatments, adsorption of flouroplastics to increase hydrophobicity, quaternary ammonium silane coatings, bulk surface modifications, the use of biosurfactants, probiotics and antifungal agents and synthetic salivary peptides, was published 2007 by Rodrigues et al. (2007). Surprisingly, most of the proposed approaches have been evaluated as feasible and effective, but none were implemented in prosthetic design yet. This raises the question why promising research data fails to be realized by industry and the possible reasons should be discussed.

Most of the data was assessed by in vitro methods, which do not necessarily display the same properties as in vivo biofilms. In fact, the complexity of in vivo biofilms remains unmatched in regard to numbers of involved species, functional and community shifts and very often short observation periods. Studies comprising only monospecies biofilms to test efficacies have to be considered as unrealistic for voice prostheses, which are placed in highly heterogenous microbial environments. It has been proved that, microbial adhesion of a single species to surfaces can vary greatly due to co-aggregation of other species and an in vitro medium not necessarily supports growth of both species. It is tentative to speculate that there are microbial consortia, which synergistically can pioneer almost every surface – just as in nature; almost no surfaces remain free of microbial colonization.

Observation times of biofilm formation test protocols for voice prostheses should also be dimensioned similar to in vivo biofilm observations. Although microscopic biofilm layers evolve within days of incubation in vitro, they are

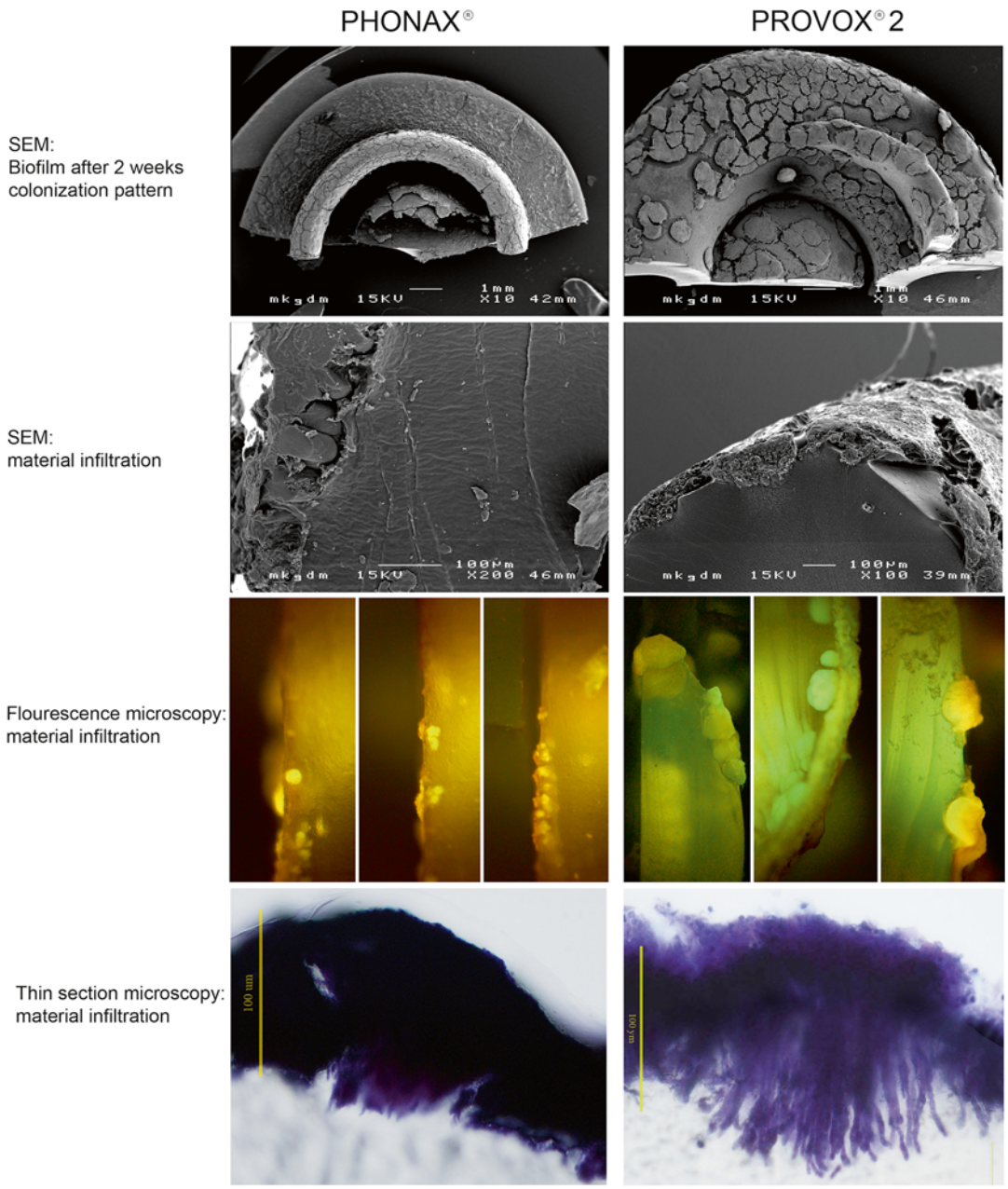

Fig. 8.10 Biofilm material degradation of voice prostheses illustrated by different examination methods. Excavations filled with dense cell agglomerations and hypheal infiltration can been seen on Phonax (thermoplastic elastomer) and Provox 2 (medical grade silicone) voice prostheses (Courtesy of Head & Neck – Leonhard et al. 2010)

not the observed cause of prosthesis dysfunction. These typical biofilm deposits appear in vivo after 15–20 days and show signs of distinctive infiltration after about 30 days. Many in vitro models are terminated after 10–14 days and can hardly display the effects of long-term biofilm colonization. Due to these limitations of in vitro assessed data, clinical trials are compulsory to test the effects in vivo. The use of antifungal and antibiotic substances in coatings or as chronic treatment can be effective, but also bears the risk of long-term side effects (e.g. on the microbiome of the digestive tract) or selection of resistant strains due to genetic transfers between species facilitated in biofilm communities. New materials and surface alterations also have to be tested for biocompatibility to the epithelial tissue in order not to cause irritation. This especially applies to nano scale modifications, which seem promising, but need to be tested thoroughly before used on implantable devices (Panacek et al. 2009; Singh et al. 2011). This points to the probably most important reason preventing realization of the proposed methods. The expenses for manufacturing processes of effective but complex material improvements and testing for biocompatibility often exceed the economic potential of advanced more durable prostheses. Seen from this angle, simple solutions, such as the proposed promising use of probiotics are more likely to find a way in clinical application and require confirmation in clinical studies (van der Mei et al. 2000; Rolien 2014). However, scientists should continue to search for approaches to improve biofilm resistance of implantable materials. Voice prostheses are the current gold standard of voice rehabilitation, but standards improve as well. Recently, the artificial larynx has been published as possible next generation implant, which might change the current views on the issue in future decades (Debry et al. 2014).

Fig. 8.11 Thin section with crystal violet staining of a used Provox 2 voice prosthesis. The valve flap shows infiltrating biofilm deposits over nearly the whole flap surface. The deformation of the flap form can cause insufficient valve closure

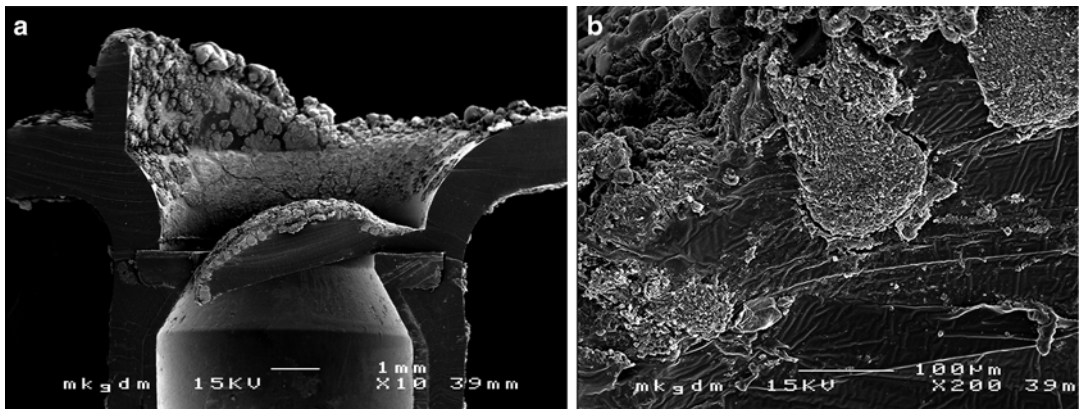

Fig. 8.12 (**a**) Scanning electron micrography of a Provox 2 voice prosthesis 48 weeks in situ. The prosthesis was cut in two pieces in the midline through the valve hinge. Heavy biofilm colonization on the esophageal flange and the valve flap is displayed. (**b**) The silicon material shows multiple excavations filled with dense cell formations

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9 Microbial Composition and Antibiotic Resistance of Biofilms Recovered from Endotracheal Tubes of Mechanically Ventilated Patients

Ilse Vandecandelaere and Tom Coenye

Abstract

In critically ill patients, breathing is impaired and mechanical ventilation, using an endotracheal tube (ET) connected to a ventilator, is necessary. Although mechanical ventilation is a life-saving procedure, it is not without risk. Because of several reasons, a biofilm often forms at the distal end of the ET and this biofilm is a persistent source of bacteria which can infect the lungs, causing ventilator-associated pneumonia (VAP). There is a link between the microbial flora of ET biofilms and the microorganisms involved in the onset of VAP. Culture dependent and independent techniques were already used to identify the microbial flora of ET biofilms and also, the antibiotic resistance of microorganisms obtained from ET biofilms was determined. The ESKAPE pathogens play a dominant role in the onset of VAP and these organisms were frequently identified in ET biofilms. Also, antibiotic resistant microorganisms were frequently present in ET biofilms. Members of the normal oral flora were also identified in ET biofilms but it is thought that these organisms initiate ET biofilm formation and are not directly involved in the development of VAP.

9.1 Introduction

9.1.1 Clinical Relevance of Biofilms

Biofilms are defined as structured communities of microbial cells enclosed in a self-produced polymeric matrix, attached to a surface (Costerton et al. 1999; Stoodley et al. 2002). Biofilms are often unwanted and can cause problems in industrial facilities such as waste water treatment plants (Flemming 2002). Also, the presence of biofilms in medical settings has some major consequences and it has been estimated that up to 80 % of all infections worldwide are biofilm-related (Coenye and Nelis 2010).

Typically, high cell densities are achieved within a biofilm e.g. up to 10^9 cfu are present in a *Staphylococcus aureus* biofilm, grown in a 96-well microtiter plate (Wanner et al. 2011; Vandecandelaere et al. 2014). The shedding of

I. Vandecandelaere • T. Coenye (✉)
Laboratory of Pharmaceutical Microbiology, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium
e-mail: Tom.Coenye@UGent.be

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dense clumps of bacterial cells can lead to the ingestion or inhalation of a high infective dose (Hall-Stoodley and Stoodley 2005). Detachment has traditionally been considered as a passive process, typically caused by shear stress (Hall-Stoodley and Stoodley 2005; Kostakioti et al. 2013). However, dispersal is also an active process in which bacteria colonize new environments (Hall-Stoodley and Stoodley 2005) and cause infections in surrounding tissue or in new niches. Alterations in nutrient availability, oxygen pressure or toxic products concentration can trigger biofilm dispersal (Kostakioti et al. 2013). In addition, the presence of small secreted molecules promotes detachment (Oppenheimer-Shaanan et al. 2013). Importantly, high cell densities within a biofilm activate quorum sensing systems leading to the production of virulence factors (Tegmark et al. 1998). For example, the production of phenol-soluble modulins is regulated by the *agr* system in staphylococci (Peschel and Otto 2013). Biofilms also contribute to the development of infections by allowing the persistence of pathogens within the host (Ramsugit et al. 2013). Biofilms consist of heterogeneous populations of cells in which a subset may be particularly virulent (Hall-Stoodley and Stoodley 2005). A clinical important example is the development of small colony variants of *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients over time; representatives of this phenotype exhibit an enhanced cytotoxicity and an increased resistance towards antibiotics (von Gotz et al. 2004). Biofilms form on living tissue, but also attach to inert implanted surfaces such as voice prostheses, replacement joints, prosthetic heart valves, catheters and endotracheal tubes (Lindsay and von Holy 2006). Biofilms on medical devices are hard to remove and constitute a source of chronic localized and/or disseminated infections (Lindsay and von Holy 2006; Agarwal et al. 2010). Moreover, antibiotic treatment often fails due to the increased antibiotic resistance of bacterial cells within a biofilm (Costerton et al. 1999, 2011; Donlan and Costerton 2002; Agarwal et al. 2010; Roberts 2013). A biofilm is a suitable environment for bacteria to exchange plasmids, carrying genes encoding antimicrobial resistance (Wang et al. 2002; Savage et al. 2013).

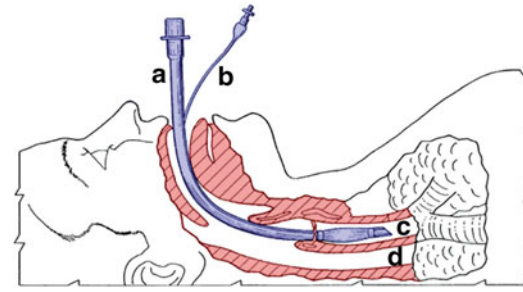

Fig. 9.1 Schematic overview of the insertion of an endotracheal tube in the airways of a mechanically ventilated patient. (**a**) endotracheal tube; (**b**) cuff inflation tube; (**c**) trachea; (**d**) oesophagus

Biofilm formation on medical devices leads to deterioration, blockage and loss of function and removal of the devices is often the only solution (Lindsay and von Holy 2006).

9.1.2 Biofilm Formation on Endotracheal Tubes

In some patients, breathing can be impaired e.g. critically ill patients with a decreased consciousness need to be mechanically ventilated. Therefore, an endotracheal tube (ET), connected to a ventilator, is placed in the lower airways (Fig. 9.1). Although mechanical ventilation is a life-saving procedure, it is not without risk for the patient (Pneumatikos et al. 2009). Firstly, the ET impairs the natural host-defense mechanisms including the cough reflex (Pneumatikos et al. 2009; Abu Samra et al. 2013; Mietto et al. 2013). Also, the introduction of the ET in the airways causes pressure on the tracheal wall, thereby decreasing the mucosal integrity and mucociliary clearance (Deem and Treggiari 2010). Consequentially, tracheobronchial secretions (i.e. mucus) accumulate at the distal end of the ET (Mietto et al. 2014). Secondly, the presence of an ET in the airways allows the transfer of bacteria from the highly colonized oropharynx to the sterile tracheobronchial tree (Inglis et al. 1989; Cheung et al. 2007; Deem and Treggiari 2010; Perkins et al. 2010; Abu Samra et al. 2013). These bacteria can also originate from the gastrointestinal tract (Inglis et al. 1989; American Thoracic Society 2005). The cuff of the ET acts as a seal between

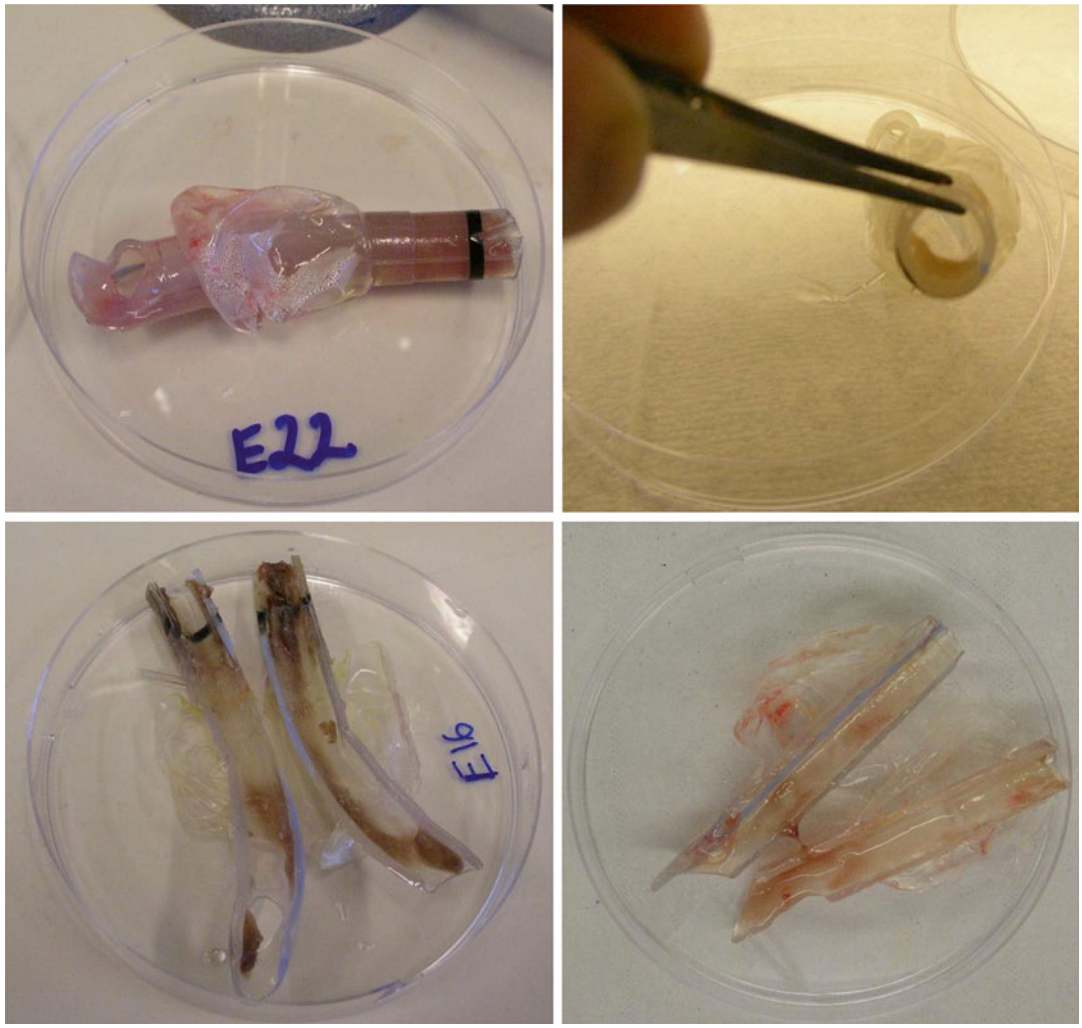

Fig. 9.2 Pictures of the distal end of ET, showing biofilm formation

the sterile lungs and the upper, highly colonized airways (Fig. 9.1). However, the presence of folds along the inflated cuff impairs sealing (Lorente et al. 2007; Pneumatikos et al. 2009; Zanella et al. 2011; Shiotsuka et al. 2012). As a result, secretions containing bacteria leak in the subglottic area, leading to tracheal colonization (Deem and Treggiari 2010; Mietto et al. 2014). Altogether, the accumulation of mucus, the impairment of host-defense mechanisms and the introduction of bacteria in the sterile airways favor the development of a biofilm on the distal part of the ET and, indeed, biofilm formation on the ET is frequently observed (Inglis et al. 1989; Feldman et al. 1999; Pneumatikos et al. 2009; Perkins et al. 2010; Zolfaghari and Wyncoll 2011; Gil-Perotin et al. 2012) (Fig. 9.2). Moreover, there appears to be a link between the presence of a biofilm on the ET and the development of ventilator-associated pneumonia (VAP) (Sottile et al. 1986; Inglis et al. 1989; Wilson et al. 2012). For example, Adair et al. (1999) reported that in 70 % of VAP patients an identical bacterial population was found in the infected lungs and in the ET biofilms. Several mechanisms by which ET biofilms can infect the lungs were already suggested. Biofilm pieces might be dispersed and be moved to the lungs (Inglis et al. 1995; Perkins et al. 2010; Wilson et al. 2012) and also, parts of the biofilm might be aerosolized

during ventilation and individual cells in contact with liquids can be transferred deeply into the lungs (Luna et al. 2009). The entry of biofilm pieces and biofilm cells in the sterile lungs can lead to infection and pneumonia (Dhanireddy et al. 2006; Lung and Codina 2012). The attributable mortality due to VAP is not exactly known but numbers reported range from 24 to 76 % (American Thoracic Society 2005). It should be noted that the overall mortality rate is of course heavily influenced by the severity of the underlying medical conditions (Papazian et al. 1996; Chastre et al. 1998; Bregeon et al. 2001).

Besides VAP, biofilm formation on the ET can also lead to (partial) obstruction of the ET (Feldman et al. 1999; Shah and Kollef 2004; Mietto et al. 2014), subglottic stenosis (Suzumura et al. 2000) and bronchopulmonary dysplasia (Gibbs and Holzman 2012).

9.1.3 Investigating the Microbial Diversity and Antibiotic Resistance of the ET Biofilm Flora

Sampling of microorganisms in ET biofilms is not easy as it is advised not to replace the ET during the course of mechanical ventilation (Torres et al. 1995). Therefore, identification of members of the ET biofilm is only possible at the time of extubation (Torres et al. 1995; Perkins et al. 2010; Vandecandelaere et al. 2012). Alternatively, endotracheal aspirates can be used to identify the microbial flora of ET biofilms (Flanagan et al. 2007; Luna et al. 2013). In fact, different kinds of surveillance cultures (SC; e.g. nose and throat swabs) were already used to identify bacteria present in ET biofilms of VAP patients (Depuydt et al. 2008; Vandecandelaere et al. 2013). It has even been suggested to rely on the 'local flora' (as identified by SC) to guide initial antimicrobial therapy in order to treat VAP (American Thoracic Society 2005). Several studies have reported a good correlation between pathogens present in SC and those identified when VAP is diagnosed (Depuydt et al. 2008; Jung et al. 2009; Pirracchio et al. 2009; Gursel et al. 2010). In contrast, Hayon et al. (2002) demonstrated that there is a low correspondence between bacteria in SC and those involved in the pathogenesis of VAP. Although there is still controversy, most clinical microbiologists consider SC as a valuable tool to study ET biofilms and to identify bacteria involved in the development of VAP.

Most studies assessing the microbial diversity of ET biofilms, relied on culture dependent techniques (Adair et al. 1999; Gil-Perotin et al. 2012). However, cultivation of bacteria from biofilms is not straightforward and culture-based approaches often fail to recover all biofilm cells (Trampuz et al. 2007; Esteban et al. 2008; Wolcott and Ehrlich 2008; Esteban et al. 2010). In general, culture dependent methods allow the recovery of only a small subset of the microbial diversity (Tyson and Banfield 2005; Lasken 2012). Traditional culturing methods lack both sensitivity and specificity and as such, many bacteria can be missed and/or misidentified (Bittar and Rolain 2010; Parahitiyawa et al. 2010; Sibley et al. 2012). In contrast, culture independent techniques offer a more complete overview of the diversity of a microbial population (Petti 2007). Importantly, the DNA extraction method used can have an impact on the downstream molecular analyses (Lazarevic et al. 2013). Especially, the isolation of *Staphylococcus* DNA is hampered by its rigid cell wall (Willner et al. 2012). For example, Zhao et al. (2012a) observed that a standard lysis method resulted in a significantly lower detection rate of *Staphylococcus* spp. but in higher levels of overall community diversity, richness and evenness compared to the method using a lysis buffer amended with lysostaphin and lysozyme.

Culture independent methods were already used in ET biofilm studies and include denaturing gradient gel electrophoresis (Cairns et al. 2011), construction of 16S rRNA gene clone libraries (Perkins et al. 2010) and next-generation sequencing (Vandecandelaere et al. 2012). Thus far, only the latter three studies reported the identification of ET biofilms by culture independent methods. It can be expected that studies in which data coming from culture independent techniques are described, will increase during the next years

as next-generation sequencing will find its way into clinical laboratories (Palmer et al. 2011).

Antibiotic resistance of members of the ET biofilm flora was investigated by the disk diffusion method and the determination of the minimal inhibitory concentration (MIC) and minimal biofilm eradicating concentration (MBEC) (Adair et al. 1999; Singhai et al. 2012; Vandecandelaere et al. 2013). The most used practice to report the antibiotic susceptibility of microorganisms is the determination of the MIC (Adair et al. 1999; Antunes et al. 2010). However, MIC methods rely on the use of pure cultures while most biofilms are composed of multiple interacting species (Wolcott et al. 2013). Moreover, this method tests planktonic cells instead of biofilm cells (Antunes et al. 2010). The antibiotic susceptibility of sessile cells can be tested either by determining the minimal biofilm inhibitory concentration (MBIC) or the MBEC (Antunes et al. 2010; Oettinger-Barak et al. 2013). In general, higher MBEC were reported compared to the MIC, and this difference was more evident for isolates with moderate to strong biofilm forming capacity (Antunes et al. 2010).

9.2 Identification of Microorganisms in ET Biofilms

9.2.1 Presence of Aerobic Nosocomial Pathogens in ET Biofilms

The ESKAPE pathogens (i.e. *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) play an important role in the development of VAP and it has been reported that up to 80 % of all VAP episodes are correlated with the presence of ESKAPE pathogens (Sandiumenge and Rello 2012). Although the exact role of *S. epidermidis* in VAP is not yet clear, it was frequently identified in ET biofilms. We have chosen to include *S. epidermidis* in the discussion below. Several studies have identified bacteria which are not usually considered as nosocomial pathogens (Perkins et al. 2010; Vandecandelaere et al. 2012). The role of these organisms in the development of VAP is unknown and they are not discussed in this review.

9.2.1.1 *Staphylococcus aureus*

S. aureus is one of the most important nosocomial pathogens worldwide (Chatterjee and Otto 2013). Infections caused by *S. aureus* range from skin infections to life-threatening infections such as bacteraemia (Becker and von Eiff 2012). The virulence of *S. aureus* is associated with its ability to produce virulence factors and to form biofilms (Otto 2012, 2014). Especially, the presence of methicillin resistant *S. aureus* (MRSA) in medical settings is a major problem (Haddadin et al. 2002). This resistance is due to the presence of the *mecA* gene encoding the penicillin-binding protein, which has a decreased affinity for β-lactams and which catalyses effective cell wall synthesis even in the presence of penicillins (including cephalosporins and carbapenems) (Moellering 2012). About 40 % of the nosocomial *S. aureus* isolates in the USA are resistant to methicillin and in central and Southern Europe, more than 25 % of bacteraemia cases are caused by MRSA (Haddadin et al. 2002; Moellering 2012).

S. aureus is the most common cause of nosocomial pneumonia and plays a prominent role in the development of VAP (American Thoracic Society 2005; Park 2005; Bahrani-Mougeot et al. 2007; Weber et al. 2007; Dickson et al. 2008; Hidron et al. 2008; Jones 2010; Joseph et al. 2010; Becker and von Eiff 2012; Park et al. 2012).

S. aureus is frequently isolated from ET biofilms by cultivation (Inglis et al. 1995; Adair et al. 1999; Feldman et al. 1999; Berra et al. 2012; Vandecandelaere et al. 2012; Liu et al. 2013) and also, the use of culture independent methods resulted in the identification of *S. aureus* in ET biofilms (Perkins et al. 2010; Cairns et al. 2011; Vandecandelaere et al. 2012). As *S. aureus* is part of the human nasal flora (Becker and von Eiff 2012), it can be assumed that *S. aureus* in ET biofilms originate from nasopharyngeal secretions (Feldman et al. 1999; Safdar et al. 2005;

Perkins et al. 2010). Subsequently, parts of the ET biofilm (containing *S. aureus*) can be dispersed to the lungs and this causes VAP (Otto 2013b).

Methicillin resistance was also detected among *S. aureus* isolates obtained from ET biofilms (Berra et al. 2012; Gil-Perotin et al. 2012; Vandecandelaere et al. 2013). Intrinsically, MRSA strains are also resistant to cephalosporins and carbapenems and MRSA resistant to cefotaxime and cefuroxime have already been isolated from ET biofilms (Adair et al. 1999). Furthermore, *S. aureus* isolates obtained from ET biofilms often show resistance to antibiotics from other classes including tobramycin (Adair et al. 1999).

9.2.1.2 *Pseudomonas aeruginosa*

P. aeruginosa is an important nosocomial pathogen causing a variety of infections in immunocompromised patients and infections range from pneumonia (Damron and Goldberg 2012), chronic wounds (Mulcahy et al. 2014), festered burn injuries (Bielecki et al. 2008) to septicemia (Cornelis and Dingemans 2013). The large genome of *P. aeruginosa* harbors a broad array of antimicrobial defenses and virulence factors (Bjarnsholt and Givskov 2007). The emergence of multidrug resistant *P. aeruginosa* is of major concern (Bukholm et al. 2002; Kallen et al. 2010; Master et al. 2013) and this high degree of resistance is due to a combination of *P. aeruginosa*'s intrinsic resistance to many antibiotics (e.g. caused by efflux pumps and low permeability of the cell membrane) and its ability to acquire resistance via mutations and plasmids (Livermore 2002; Strateva and Yordanov 2009). So far, resistance to penicillins, cephalosporins, aminoglycosides, fluoroquinolones, polymyxins and carbapenems was reported (Hancock and Speert 2000; Bonomo and Szabo 2006; Strateva and Yordanov 2009).

Importantly, *P. aeruginosa* plays a role in the development of VAP (Park 2005; Weber et al. 2007; Jones 2010; Joseph et al. 2010; Gibbs and Holzman 2012; Zhao et al. 2012b; Mietto et al. 2013); for example, Chastre and Fagon (2002) reported that *P. aeruginosa* was the most common pathogen in the lungs of 1,689 VAP patients. Moreover, the presence of *P. aeruginosa* in bronchoalveolar lavages is correlated with poor prognosis of VAP. In general, *P. aeruginosa* is the most common cause of fatal episodes of VAP (Rello et al. 1993; Chastre and Fagon 2002). Flanagan et al. (2007) showed that *P. aeruginosa* became the most dominant organism in the lungs of VAP patients in function of time, despite appropriate antibiotic treatment (i.e. treating *P. aeruginosa* with an antibiotic for which *P. aeruginosa* is sensitive to).

P. aeruginosa has also been frequently identified in ET biofilms by cultivation (Inglis et al. 1989; Adair et al. 1999; Feldman et al. 1999; Berra et al. 2012; Gil-Perotin et al. 2012; Liu et al. 2013). Cairns et al. (2011) detected *P. aeruginosa* by species-specific PCR and also direct sequencing of ET biofilm samples resulted in the identification of *P. aeruginosa* (Perkins et al. 2010; Vandecandelaere et al. 2012). Analysis of the microbial diversity of ET biofilms revealed that the number of microbial species decreased when *P. aeruginosa* was present in that particular biofilm (Perkins et al. 2010; Vandecandelaere et al. 2012). This significant loss in microbial diversity due to the presence of *P. aeruginosa* was also observed in the lungs of cystic fibrosis patients although other factors such as age and antibiotic resistance also played a role (Klepac-Ceraj et al. 2010; Pittman et al. 2010; Zhao et al. 2012b).

As the ET biofilm is a reservoir of pathogens, residing *P. aeruginosa* isolates can disperse from the ET biofilm into the lungs and cause infection. Iron uptake strategies (Cornelis and Dingemans 2013), rhamnolipids (Alhede et al. 2014), oxidative stress responses (Bielecki et al. 2008) and protease secretion systems (Bleves et al. 2010) contribute to the onset of *P. aeruginosa* infections. Multidrug resistant *P. aeruginosa* isolates were obtained from ET biofilms. Adair et al. (1999) isolated *P. aeruginosa* resistant to tobramycin, cefotaxime and cefuroxime while more recently, resistance to ciprofloxacin and meropenem was detected (Vandecandelaere et al. 2013).

9.2.1.3 *Acinetobacter* spp.

Although *Acinetobacter* spp. is not frequently found in healthy humans, it can cause severe infections in critically ill hospitalized patients (Munoz-Price and Weinstein 2008; Guerrero et al. 2010; Mortensen and Skaar 2012). *Acinetobacter baumanii*, *Acinetobacter calcoaceticus* and

Acinetobacter lwoffii are the *Acinetobacter* spp. most frequently involved in the onset of infections (Munoz-Price and Weinstein 2008) and *A. baumannii* and *A. calcoaceticus* represent 80 % of all *Acinetobacter* infections (Hartzell et al. 2007). Infections range from skin and soft tissue infections to nosocomial pneumonia (Doyle et al. 2011).

The ability of *Acinetobacter* spp. to survive environmental dessication for weeks, helps to initiate infections (Munoz-Price and Weinstein 2008). Besides the ability to form biofilms (Mortensen and Skaar 2012), the rapid acquisition of multiple antibiotic resistance genes contribute to the virulent and persistent character of *Acinetobacter* spp. (Sandiumenge and Rello 2012). Moreover, the presence of genes encoding β-lactamases in the genome of e.g. *A. baumannii* confers resistance to several β-lactam antibiotics including cephalosporins and carbapenems (Bonomo and Szabo 2006; Peleg et al. 2007; Munoz-Price and Weinstein 2008; Towner 2009; Zarrilli et al. 2009; Kempf and Rolain 2012). Also, different types of efflux pumps can actively expel quinolones, tetracyclines, chloramphenicol, disinfectantia and tigecycline (Peleg et al. 2007).

Infections mostly caused by *Acinetobacter* spp. include VAP and bloodstream infections, both associated with a high degree (up to 35 %) of mortality and morbidity (Hartzell et al. 2007; Munoz-Price and Weinstein 2008; Celik et al. 2012; Sandiumenge and Rello 2012; Antunes et al. 2014). *Acinetobacter* spp. is already cultivated from ET biofilms, e.g. Inglis et al. (1995) and Feldman et al. (1999) isolated *Acinetobacter* spp. from ET biofilms. In addition, *A. baumanii* (Gil-Perotin et al. 2012; Liu et al. 2013) and *A. lwoffii* (Vandecandelaere et al. 2012) were cultivated from ET biofilms. No studies reported the presence of antibiotic resistant *Acinetobacter* spp. in ET biofilms.

9.2.1.4 *Enterobacteriaceae* Including *Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacter spp.*

Infections caused by *Enterobacteriaceae* (e.g. *E. coli* and *K. pneumoniae*) represent a serious threat for immunocompromised patients (Bodro et al. 2014; Pendleton et al. 2013; Trang et al. 2013; Ulett et al. 2013). For instance, members of the genus *Enterobacter* are known nosocomial pathogens, causing respiratory and bloodstream infections (Gallo et al. 2003; Grimont and Grimont 2006; Chang et al. 2009).

A major problem concerning *Enterobacteriaceae* is their high degree of resistance to β-lactam antibiotics (American Thoracic Society 2005; Nordmann 2013). The presence of extended-spectrum β-lactamases (ESBL) genes in the genome confers resistance to penicillins, cephalosporins and monobactams (Abdel-Hady et al. 2008; Abreu et al. 2011) and infections with ESBL-producing *Enterobacteriaceae* are often treated by carbapenems (Paterson 2000; Endimiani et al. 2005; Bassetti et al. 2007). Unfortunately, resistance to carbapenems conferred by carbapenemases is increasing (Wu et al. 2011; Liao et al. 2012; Khajuria et al. 2013). In addition, resistance to other non β-lactam antibiotics was already reported, including resistance to amikacin, ciprofloxacin and levofloxacin (Ronveaux et al. 1999; Krishnamurthy et al. 2013; Sisirak and Hukic 2013; Wellington et al. 2013).

Enterobacter spp. has been identified as the causative agent of VAP (Park 2005; Bahrani-Mougeot et al. 2007; Weber et al. 2007; Jones 2010; Joseph et al. 2010) and both *E. coli* and *K. pneumoniae* are frequently recognized to play an important role in the development of VAP (American Thoracic Society 2005; Park 2005; Bahrani-Mougeot et al. 2007; Charles et al. 2013; Ning et al. 2013).

E. coli and *K. pneumoniae* are the most frequently identified *Enterobacteriaceae* from ET biofilms (Inglis et al. 1989; Feldman et al. 1999; Perkins et al. 2010; Berra et al. 2012; Gibbs and Holzman 2012; Singhai et al. 2012; Vandecandelaere et al. 2012; Liu et al. 2013). *Enterobacter* spp., including *E. aerogenes* and *E. cloacae* are also often obtained from ET biofilms (Inglis et al. 1989; Feldman et al. 1999; Vandecandelaere et al. 2012; Liu et al. 2013). Other *Enterobacteriaceae* that were identified from ET biofilms include *Proteus mirabilis*, *Providencia stuartii*, *Raoultella ornithinolytica*, *Klebsiella oxytoca* and *Hafnia alvei* (Inglis et al. 1989; Vandecandelaere et al. 2012). In addition, pyrosequencing

of 16S rRNA genes showed the presence of *Serratia marcescens*, *Cronobacter muytjensii* and *Ewingella americana* (Vandecandelaere et al. 2012). Except *C. muytjensii*, all the other *Enterobacteriaceae* listed above are known to cause nosocomial infections in hospitalized patients (Da Costa et al. 2000; Endimiani et al. 2005; Rodriguez-Guardado et al. 2005; Liao et al. 2012; Ebringer and Rashid 2013; Khajuria et al. 2013; Mnif et al. 2013; Sisirak and Hukic 2013). Although, their role in the onset of VAP is not known.

Antibiotic resistance was observed among *Enterobacteriaceae* isolated from ET biofilms. Both *P. mirabilis* and *K. pneumoniae* are multidrug resistant with resistance to ofloxacin, gatifloxacin, ceftriaxone, cefixime, cefepime, cefoperazone, cefoperazone-sulbactam, amikacin, gentamicin, netilmicin, piperacillin-tazobactam and piperacillin reported (Singhai et al. 2012). Also, resistance of *E. coli*, isolated from ET biofilms, to a whole variety of antibiotics including tobramycin, ciprofloxacin and meropenem was observed (Vandecandelaere et al. 2013). As the ET biofilm represents a reservoir from which bacteria can infect the lungs, the presence of multidrug resistant *Enterobacteriaceae* in ET biofilms increases the risk for developing VAP.

9.2.1.5 *Staphylococcus epidermidis*

S. epidermidis is part of the normal human flora and represents the most frequently isolated species from human epithelia (Kloos and Musselwhite 1975; Ziebuhr et al. 2006; Otto 2009; Becker and von Eiff 2012). *S. epidermidis* belongs to the group of coagulase-negative staphylococci (CNS) and it is a commensal organism on human skin and mucous membranes (von Eiff et al. 2001; Otto 2009). Traditionally, CNS were regarded as non pathogenic organisms as the role of oropharyngeal commensals in infections is often difficult to interpret (Adair et al. 1999; American Thoracic Society 2005; Rogers et al. 2009). However, CNS have been increasingly recognized to be involved in the onset of nosocomial infections (Ziebuhr et al. 2006; Rogers et al. 2009; Becker and von Eiff 2012; Otto 2014) and the vast majority of CNS related infections are due to *S. epidermidis* (Otto 2009, 2012; Gomes et al. 2014). Also, *S. epidermidis* represents the most frequently isolated causative agent of infections involving indwelling medical devices (Otto 2012). Unlike *S. aureus*, *S. epidermidis* does not possess a whole arsenal of virulence factors (Otto 2012). The crucial virulence factor of *S. epidermidis* is its ability to form biofilms and in this way, to resist the host immune defense and antibiotic treatment (Cerca et al. 2005). It has also been suggested that *S. epidermidis* acts as a reservoir of antibiotic resistance genes which can be transferred to *S. aureus* (Bloemendaal et al. 2010; Otto 2013a).

More than 90 % of all nosocomial CNS (mainly *S. epidermidis* and *Staphylococcus haemolyticus*) are resistant to penicillins (Becker and von Eiff 2012) and up to 90 % of hospital isolates of *S. epidermidis* are resistant to methicillin (MRSE) (Otto 2009). In addition, *S. epidermidis* has acquired resistance to other antibiotics including rifamycin, fluoroquinolones, gentamicin, tetracycline, chloramphenicol, erythromycin, clindamycin and sulfonamides (Rogers et al. 2009).

The exact role of *S. epidermidis* in the development of VAP is not yet known (Park 2005). *S. epidermidis* was identified in ET biofilms by culture dependent (Zur et al. 2004; Singhai et al. 2012; Vandecandelaere et al. 2012; Liu et al. 2013) and culture independent techniques (Perkins et al. 2010; Vandecandelaere et al. 2012). In addition, several studies demonstrate the presence of other CNS in ET biofilms (Inglis et al. 1995; Perkins et al. 2010; Gil-Perotin et al. 2012; Vandecandelaere et al. 2012). For instance, *S. haemolyticus*, *Staphylococcus hominis*, *Staphylococcus saprophyticus* and *Staphylococcus xylosus* were already recovered from ET biofilms (Vandecandelaere et al. 2012). Although CNS were already identified from the lungs of VAP patients, the role of CNS in the development of VAP is not yet known (Park 2005; Weber et al. 2007; Kollef et al. 2008; Joseph et al. 2010). Antibiotic resistance was frequently observed among CNS isolated from ET biofilms and MRSE isolates were obtained from ET biofilms (Singhai et al. 2012; Vandecandelaere et al. 2013).

9.2.1.6 *Enterococcus faecium*

Enterococcus faecium has long been recognized as a harmless commensal of the mammalian gastrointestinal tract and the oral cavity (Willems and van Schaik 2009; Kouidhi et al. 2011). However, *E. faecium* has become an important source of nosocomial bacteraemia (Willems and van Schaik 2009). Infections caused by *E. faecium* include pneumonia (Sandiumenge and Rello 2012), urinary tract infections (Parameswarappa et al. 2013) and focal infections (Kouidhi et al. 2011). Acquired resistance against various antibiotics, including penicillin/ampicillin and aminoglycosides are reported in increasing numbers (Kouidhi et al. 2011) and especially, the emergence of vancomycin-resistant enterococci is a major problem (Wade 1997). *Enterococcus* spp. is not frequently identified as a causative agent of VAP e.g. Weber et al. (2007) reported that *Enterococcus* spp. is obtained from 1 % of VAP patients. In the guidelines of the American Thoracic Society, *Enterococcus* spp. is not present in the list of causative agents of VAP (American Thoracic Society 2005). Still, *Enterococcus* spp. isolates, including *E. faecalis* and *E. faecium* were cultured from ET biofilms from mechanically ventilated patients (Adair et al. 1999; Feldman et al. 1999; Zur et al. 2004; Perkins et al. 2010; Vandecandelaere et al. 2012; Wilson et al. 2012) and both organisms were also identified by culture independent techniques (Perkins et al. 2010; Vandecandelaere et al. 2012).

Thus far, only Adair et al. (1999) investigated the antibiotic resistance of *E. faecalis* in ET biofilms. MIC and MBIC for tobramycin, cefotaxime and cefuroxime were all above 1,024 µg/ml, showing resistance of *E. faecalis* to these three antibiotics (Adair et al. 1999).

9.2.2 Presence of the Normal Oral Flora in ET Biofilms

An overview of members of the normal oral flora that were identified in ET biofilms is shown in Table 9.1. The presence of typical oral bacteria in ET biofilms is not surprising as ET biofilms are inoculated by oral secretions (Inglis et al. 1995; Perkins et al. 2010). These bacteria belong to the lactic acid bacteria, *Bacteroidetes*, *Actinobacteria*, *Clostridia*, *Fusobacteria* and *Proteobacteria* (Adair et al. 1999; Perkins et al. 2010; Cairns et al. 2011; Gibbs and Holzman 2012; Gil-Perotin et al. 2012; Vandecandelaere et al. 2012; Liu et al. 2013). Members of the oral flora are commensal bacteria and are mostly harmless. However, several studies have described infections caused by members of the normal oral flora e.g. soft-tissue abscesses caused by *Actinomyces odontolyticus* (Sofianou et al. 2004), lower respiratory tract infections caused by *Corynebacterium striatum* (Diez-Aguilar et al. 2013) and endocarditis caused by *Rothia dentocariosa* (Ruben 1993). These infections are mostly caused by multiple organisms (Alcaraz et al. 2012) and interactions (both synergistic and antagonistic) play a major role in the onset of oral infection (Jenkinson 2011; Wang et al. 2013).

Literature data demonstrated that antibiotic resistance is also present among members of the normal oral flora. For instance, resistance of enterococci and viridans streptococci to penicillins, macrolides, tetracyclines cephalosporins and aminoglycosides was reported (Guiot et al. 1994; Ready et al. 2004; Eisenblatter et al. 2008; Ciric et al. 2011; Kouidhi et al. 2011; Pasquantonio et al. 2012; Warburton et al. 2013). In fact, it was suggested that oral viridans streptococci and *Veillonella* spp. (both abundant members of the typical oral flora) may serve as a reservoir of antibiotic resistance genes for nosocomial pathogens (Ready et al. 2006; Eisenblatter et al. 2008).

A considerable part of the oral flora is strictly anaerobic (Daniluk et al. 2006). For instance, members of the genera *Peptostreptococcus* (Downes and Wade 2006) and *Veillonella* (Gronow et al. 2010) are anaerobic bacteria. In addition, bacteria isolated from the oral cavity are often fastidious for growth conditions, e.g. members of the genera *Abiotrophia* and *Granulicatella* require the addition of pyridoxal to grow on blood agar (Ruoff 1991; Collins and Lawson 2000). Altogether, cultivation of oral cavity bacteria is not easy. In general, less than half of the oral bacteria can be cultured (Dewhirst et al. 2010; Sizova et al. 2012; Siqueira and Rocas

Table 9.1 Presence of typical oral bacteria in ET biofilms

Lactic acid bacteria	*Streptococcus*	Viridans streptococci: *S. mutans*[a], *S. parasanguinis*[b,c], *S. oralis*[b,c], *S. peroris*[b], *S. salivarius*[b], *S. pneumoniae*[b,c], *S. mutans*[b], *S. mitis*[b,c], *S. infantis*[b], *S. oralis*[b], *S. sanguinis*[b], *S. intermedius*[b], *S. constellatus*[b,c], *S. anginosus*[b], *S. viridans*[a]	Cairns et al. (2011), Gil-Perotin et al. (2012), Perkins et al. (2010), Gibbs and Holzman (2012), Liu et al. (2013)
		Group B streptococci: *S. agalactiae*[a]	
	Enterococcus	*E. faecalis*[a,b,c], *E. faecium*[b]	Adair et al. (1999), Vandecandelaere et al. (2012), Perkins et al. (2010)
	Lactococcus	*L. fermentum*[b]	Vandecandelaere et al. (2012)
	Gemella	*G. morbillorum*[c], *G. sanguinis*[c]	Vandecandelaere et al. (2012)
	Granulicatella	*G. adiacens*[b,c], *G.para-adiacens*[b]	Perkins et al. (2010), Vandecandelaere et al. (2012)
	Lactobacillus	*L. gasseri*[c], *L. salivarius*[c]	Vandecandelaere et al. (2012)
	Abiotrophia	*A. para-adiacens*[b]	Perkins et al. (2010)
Bacteroidetes	*Prevotella*	*P. histolica*[c], *P. melaninogenica*[b,c], *P. nigrescens*[b,c], *P. oris*[b,c], *P. salivae*[b,c], *P. denticola*[c], *P. pallens*[b,c], *P. ourolum*[c], *P. veroralis*[b,c], *P. intermedia*[b]	Vandecandelaere et al. (2012), Perkins et al. (2010)
Actinobacteria	*Mycoplasma*	*M. salivarium*[c]	Vandecandelaere et al. (2012)
	Atopbium	*A. parvulum*[c], *A. rimae*[c]	Vandecandelaere et al. (2012)
	Actinomyces	*A. odontolyticus*[c], *A. viscosus*[c]	Vandecandelaere et al. (2012)
	Corynebacterium	*C. striatum*[c], *C. jeikeium*[c], *C. propinquum*[c]	Vandecandelaere et al. (2012)
	Rothia	*R. mucilaginosa*[c], *R. dentocariosa*[c]	Vandecandelaere et al. (2012)
Clostridia	*Parvimonas*	*P. micra*[c]	Vandecandelaere et al. (2012)
	Peptostreptococcus	*P. stomatis*[c]	Vandecandelaere et al. (2012)
	Selenomonas	*S. sputigena*[c]	Vandecandelaere et al. (2012)
	Veillonella	*V. atypica*[c], *V. dispar*[c], *V. parvula*[c]	Vandecandelaere et al. (2012)
Fusobacteria	*Fusobacterium*	*F. nucleatum*[b,c]	Vandecandelaere et al. (2012), Perkins et al. (2010)
Proteobacteria	*Eikenella*	*E. corrodens*[c]	Vandecandelaere et al. (2012)
	Aggregatibacter	*A. segnis*[c]	Vandecandelaere et al. (2012)
	Haemophilus	*H. influenzae*[b,c], *H. parainfluenzae*[b], *H. haemolyticus*[b]	Vandecandelaere et al. (2012), Perkins et al. (2010)
	Neisseria	*N. sicca*[b], *N. mucosa*[b], *N. perflava*[b]	Perkins et al. (2010)

[a]Cultivation
[b]16S rRNA gene clone libraries
[c]Pyrosequencing

2013). Likewise, only a few studies identified typical oral bacteria in ET biofilms by cultivation (Adair et al. 1999; Gibbs and Holzman 2012; Gil-Perotin et al. 2012; Vandecandelaere et al. 2012; Liu et al. 2013). Other members of the oral flora (*Gemella* spp., *Granulicatella* spp., *Lactobacillus* spp., *Abiotrophia* spp., *Bacteroidetes*, *Actinobacteria*, *Clostridia*, *Fusobacteria* and *Proteobacteria*) were identified by culture independent methods including 16S rRNA gene clone libraries and 16S rRNA gene pyrosequencing (Perkins et al. 2010; Cairns et al. 2011; Vandecandelaere et al. 2012).

In contrast to nosocomial pathogens (see above), antibiotic resistance of members of the typical commensal oral flora is not so frequently studied. For instance, the antibiotic susceptibility of such isolates obtained from ET biofilms was rarely investigated (Gibbs and Holzman 2012; Gil-Perotin et al. 2012; Liu et al. 2013).

9.2.3 Presence of Yeasts in ET Biofilms

The main nosocomial fungal pathogens include *Candida* spp., *Aspergillus* spp. and *Fusarium* spp. (Perlroth et al. 2007; Branski et al. 2009; Alangaden 2011; Badiee et al. 2011). Nosocomial fungal pathogens cause different kinds of infections (e.g. in the airways or in the genitourinary tract) and are associated with high mortality rates (Poulain 2013; Ramage et al. 2014). Also, burn wounds can be infected by fungi (Branski et al. 2009). Biofilm formation plays an important role in the development of fungal infections (Poulain 2013) and indwelling medical devices are often colonized by fungi (Poulain 2013; Ramage et al. 2014). Also, treating *C. albicans* infections is hampered by the resistance of *C. albicans* to echinocandins (e.g. caspofungin) and azoles (e.g. fluconazole) (Pfaller 2012; Pierard et al. 2012; Soto 2013; Vandenbosch et al. 2013).

It was already suggested that *Candida* spp. is a causative agent of VAP (Park 2005; Joseph et al. 2010; Delisle et al. 2011; Charles et al. 2013). However, it is hard to differentiate between harmless colonization of *Candida* spp. in the airways and the development of an infection due to the presence of *Candida* spp. (el-Ebiary et al. 1997; American Thoracic Society 2005). Therefore, the exact role of *Candida* spp. in the development of VAP is not well understood (American Thoracic Society 2005).

Candida spp. is already cultivated from ET biofilms and most of them were identified as *C. albicans* (Inglis et al. 1995; Adair et al. 1999; Cairns et al. 2011; Berra et al. 2012; Gil-Perotin et al. 2012; Vandecandelaere et al. 2012; Liu et al. 2013). Thus far, only one study identified a fluconazole resistant *C. albicans* (Vandecandelaere et al. 2013).

9.3 ET Biofilms Are Polymicrobial

This overview shows that biofilms formed on ET have a polymicrobial nature. Based on literature data on biofilm formation in the oral cavity in function of time, we may suggest a model for the development of ET biofilms (Fig. 9.3).

As leakage occurs at the cuff of the ET, nasopharyngeal secretions (containing oral bacteria) ooze out into the trachea and accumulate at the distal end of the ET. Consequentially, the ET surface and the available nutrients in the secretions favor bacterial adhesion (Jones et al. 1997; Perkins et al. 2010; Liu et al. 2013). In fact, the ET represents a platform for the development of biofilms by bacteria present in nasopharyngeal secretions (Liu et al. 2013). The ET biofilm is predominantly composed of respiratory secretions and a water content of 90–95 % is observed (Inglis et al. 1995) (Fig. 9.3).

Bacteria, present in the respiratory secretions and initially sticking to the ET, are members of the typical oral flora. Primary colonizers represent e.g. *Streptococcus oralis*, *Streptococcus mitis* and *Streptococcus sanguis* (Kolenbrander et al. 2002). In fact, viridans streptococci coaggregate with a variety of oral bacteria (Kolenbrander et al. 2002) and this coaggregation enables them to start colonization and to initiate the formation of biofilms, thereby exploiting saliva as a nutrient source (Kolenbrander et al. 2002; Perkins et al. 2010; Kindblom et al. 2012). Other bacteria are attracted by the primary colonizers, e.g. *Veillonella atypica* and *Veillonella dispar* strongly coaggregate with *Streptococcus salivarius* (Hughes et al. 1988). Subsequently, oral streptococci and *Veillonella* spp. coaggregate with *Actinomyces* spp. (Shen et al. 2005; Kitada and Oho 2012; Yang et al. 2014). On their turn, *Veillonella* spp. attracts *Fusobacterium nucleatum*, which acts as a switch between early (e.g. viridans streptococci) and late colonizers (e.g. *Prevotella intermedia* and *Prevotella denticola*) (Kolenbrander et al. 2002; Bakaletz 2004; Shen et al. 2005). Finally, nosocomial pathogens adhere onto the biofilm formed by the oral bacteria (Fig. 9.3).

The interactions between oral bacteria are mediated by receptors and adhesins (Kolenbrander et al. 2002). In general, microbial interactions between commensal bacterial species may be important to determine which bacteria are going to persist (Bousbia et al. 2013). Also, co-aggregation of different microbial species can

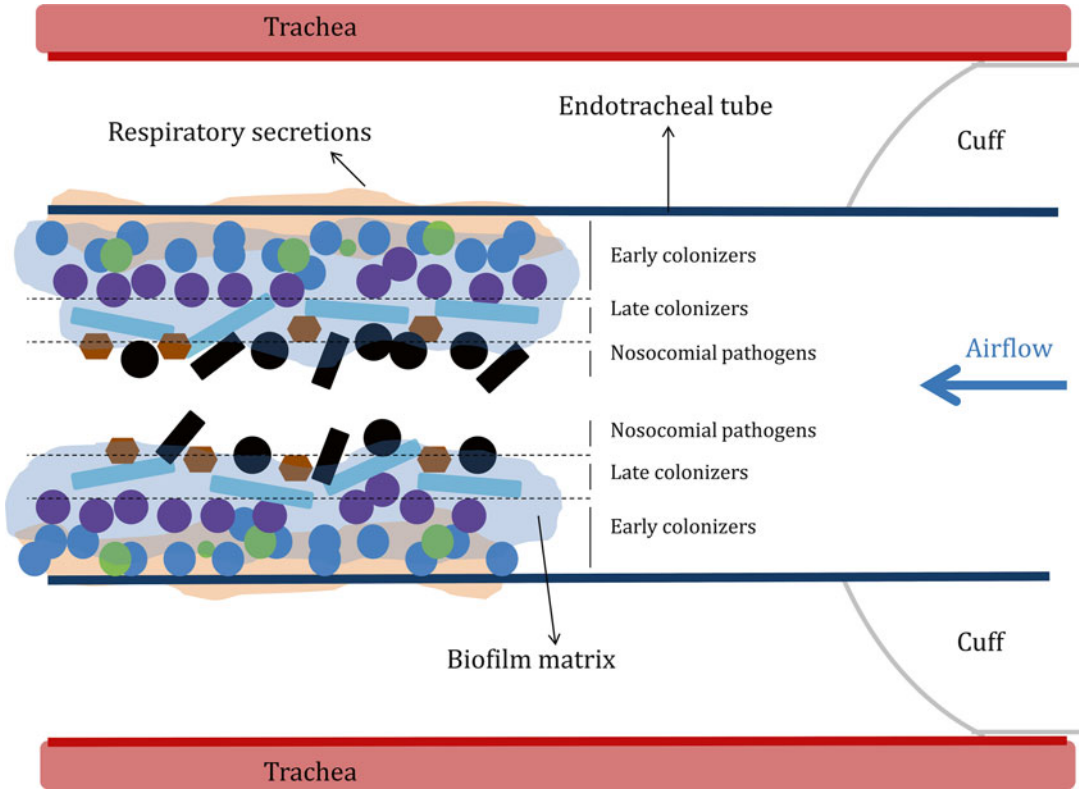

Fig. 9.3 Model of biofilm formation on the distal end of the ET. Examples of early colonizers are *Streptococcus oralis*, *Streptococcus mitis* and *Streptococcus sanguis* (*blue*, *green*, *purple*). *Fusobacterium nucleatum* acts as a switch between early and late colonizers (*light blue*) while *Prevotella intermedia* is a late colonizer (*brown*). Nosocomial pathogens are shown in *black*

enhance the virulent characteristics of certain bacteria as well increasing their tolerance to antimicrobials (Cairns et al. 2011). For instance, the synergistic interaction between *F. nucleatum* and *Prevotella* spp. increases their pathogenic potential (Jacinto et al. 2008). Also, interactions between nosocomial pathogens were already reported and these nosocomial pathogens may interact in a synergistic or in an antagonistic way. For instance, *P. aeruginosa* has an inhibitory effect on *S. epidermidis*, *S. aureus* and *C. albicans* in dual-species biofilms, resulting in the persistence and dominance of *P. aeruginosa* (Pihl et al. 2010; Park et al. 2012; Purschke et al. 2012). In contrast, it was already reported that *C. albicans* promotes *P. aeruginosa* pneumonia (Bousbia et al. 2013).

Overall, biofilm formation of oral microorganisms on ET represents a suitable environment that facilitates the adherence of potential respiratory pathogens such as *S. aureus* and *P. aeruginosa* (Cairns et al. 2011; Vandecandelaere et al. 2012). It is likely that oral bacteria initiate biofilm formation on the ET and are not directly involved in the onset of VAP.

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Biofilm-Based Central Line-Associated Bloodstream Infections

10

Ammar Yousif, Mohamed A. Jamal, and Issam Raad

Abstract

Different types of central venous catheters (CVCs) have been used in clinical practice to improve the quality of life of chronically and critically ill patients. Unfortunately, indwelling devices are usually associated with microbial biofilms and eventually lead to catheter-related bloodstream infections (CLABSIs).

An estimated 250,000–400,000 CLABSIs occur every year in the United States, at a rate of 1.5 per 1,000 CVC days and a mortality rate of 12–25 %. The annual cost of caring for patients with CLABSIs ranges from 296 million to 2.3 billion dollars.

Biofilm formation occurs on biotic and abiotic surfaces in the clinical setting. Extensive studies have been conducted to understand biofilm formation, including different biofilm developmental stages, biofilm matrix compositions, quorum-sensing regulated biofilm formation, biofilm dispersal (and its clinical implications), and multi-species biofilms that are relevant to polymicrobial infections.

When microbes form a matured biofilm within human hosts through medical devices such as CVCs, the infection becomes resistant to antibiotic treatment and can develop into a chronic condition. For that reason, many techniques have been used to prevent the formation of biofilm by targeting different stages of biofilm maturation. Other methods have been used to diagnose and treat established cases of CLABSI.

Catheter removal is the conventional management of catheter associated bacteremia; however, the procedure itself carries a relatively high risk

A. Yousif, MD • M.A. Jamal, PhD
I. Raad, MD, FACP, IDSA, FSHEA (✉)
Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas MD Anderson Cancer Center, Houston, TX, USA
e-mail: iraad@mdanderson.org

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of mechanical complications. Salvaging the catheter can help to minimize these complications.

In this article, we provide an overview of microbial biofilm formation; describe the involvement of various genetic determinants, adhesion proteins, organelles, mechanism(s) of biofilm formation, polymicrobial infections, and biofilm-associated infections on indwelling intravascular catheters; and describe the diagnosis, management, and prevention of catheter-related bloodstream infections.

10.1 Introduction

Central venous access is an essential medical procedure in the care of chronically and critically ill patients. An estimated 8 % of hospitalized patients require a central venous catheter (CVC) to be placed during the course of their hospitalization (Ruesch et al. 2002). Each year, more than five million CVCs are inserted in the United States (Ruesch et al. 2002; McGee and Gould 2003).

Different types of CVCs are widely used. We review the most commonly used types of CVCs below:

- **Non-tunneled CVCs**: These are the most common type of CVCs used for temporary access to the central circulation. Non-tunneled CVCs are available in different lengths (15–30 cm) and different materials (e.g., polyurethane, silicone). Non-tunneled CVCs can be single, double, or even triple lumen. CVCs for longer term infusion are supplied with valve mechanisms to decrease the backflow of the blood and thus possibly decrease the rate of infection and catheter thrombotic occlusion. The peripherally inserted central catheter (PICC) is another type of CVC that is widely used because of the relative ease of insertion; it is usually placed in the upper arm veins (cephalic or basilic veins) and has a lower rate of mechanical complications. However, the rate of thrombosis may increase with the increasing number of lumens.
- **Implanted CVCs**: This type of CVC provides long-term, semi-permanent central venous access. The removal of implanted CVCs is not recommended until there is no more need for the CVC or until complications occur. Two types of implanted CVCs are available, tunneled catheters and completely implantable venous access devices:
- **Tunneled CVCs**: contain a subcutaneous tunnel that crosses the catheterized vein and the skin exit site. By this mean the rate of biofilm formation and the infection are lower than the non-tunneled catheters (Dryden et al. 1991). The CVC may be round or flat. Sizes range from 2.7 to 12.5 F (e.g., Hickman, Broviac).
- **Subcutaneous port CVCs**: This type of CVC is a completely implanted device that provides long-term venous access through a CVC that is passed from the cannulated vein under the skin and connected to a subcutaneous infusion port or reservoir that is placed in a subcutaneous pocket. The port is accessed through the skin via a needle puncture of the port's septum. Subcutaneous ports are widely used to administer chemotherapy agents because of their low rates of extravasation and infection (Pegues et al. 1992); on the other hand, it is cosmetically more preferable as it is hidden beneath the skin. The only limiting infusion rate factor of this CVC is that the bore of the access needle is nearly always smaller than the internal diameter of the CVC attached to the port.

10.2 Epidemiology

Despite the utility of catheters, biofilm formation and bloodstream infections are major risks associated with catheter placement. More than five million catheters are inserted each year in the

United States (Ruesch et al. 2002), with 250,000–400,000 catheter-related bacteremia and fungaemias cases annually (if entire hospitals are assessed rather than ICUs only) (Maki et al. 2006), The National Healthcare Safety Network reports a rate of 1.5 central line-associated bloodstream infections (CLABSIs) per 1,000 CVC days in the United States (Edwards et al. 2009), with a mortality rate of 12–25 % (Centers for Disease and Prevention 2011; Pronovost et al. 2006). The attributable cost is estimated to be around $34,508–56,000 per infection (Rello et al. 2000; Soufir et al. 1999); the cumulative annual cost of caring for patients with CLABSIs ranges from $296 million to $2.3 billion (Mermel 2000; O'Grady et al. 2002).

CVCs are the source of 87 % of bloodstream infections that occur in intensive care units (Richards et al. 1999), with approximately 80,000 CLABSIs resulting in increased hospitalization stays of approximately 7–12 days (Pittet et al. 1994).

10.3 Adherence and Biofilm Formation

Biofilm is a microbially derived sessile community that is characterized by cells that are irreversibly attached to a substratum, interface, or to one another; they are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced and exhibit an altered phenotype with respect to growth rate and gene expression (Donlan and Costerton 2002). As many as 60 % of the microbial infections treated in developed nations are related to biofilm formation (Costerton et al. 1999); according to the NIH, biofilms are clinically important, accounting for over 80 % of microbial infections in the body (Lewis 2001).

Biofilm formation is a complex developmental process that involves distinct stages, including primary adherence and immobilization of planktonic microbes on a surface, cell-to-cell interaction, microcolony formation, the development of a matured three-dimensional biofilm structure, and detachment of cells from the matured biofilm to colonize new niches under desirable conditions (Fig. 10.1) (O'Toole et al. 2000). Microbes that constitute biofilm communities have extremely complex and heterogeneous physiological characteristics and are different from planktonic microbes.

The initial adhesion of microbes to biomaterial surfaces is influenced by physiochemical and electrostatic interactions between the microbial cell wall and the substrate; this is often conditioned by the fluids to which it is exposed (Dunne 2002), leading to reversible attachment that is strongly influenced by environmental factors, such as nutrient availability, pH, ionic strength, hydrodynamics, and temperature (Danese et al. 2000a). In addition, surface roughness, surface configuration, surface charge, and the hydrophobicity of material surfaces are key factors that influence microbial adherence to biomaterial surfaces (Donlan 2002; Katsikogianni and Missirlis 2004). The ability to perform this initial adherence is also linked to genetic factors, including genes encoding motility, quorum sensing, adhesins, and various metabolic activities (Costerton 1995; O'Toole et al. 2000). Host-specific adhesins that are located on the microbial cell surface or cellular components, such as pili and fimbriae, reinforce the reversible attachment between microbial cells and surfaces, resulting in the irreversible attachment of microbial cells to surfaces (Fig. 10.1).

Biofilm formation is initiated by the attachment of microbial cells to the surfaces of indwelling medical implants or devices such as catheters or infusion parts. The irreversible attachment phase of biofilm formation on a surface is likely to be involved with cell wall-associated proteins, such as microbial surface components that recognize adhesive matrix molecules (Patti et al. 1994). *S. aureus* produces multiple adhesive factors that can facilitate binding to host factors (Gotz 2002) and eventually mediate microbial attachment to implant surfaces covered by host plasma and other extracellular matrix components. Staphylococci produce several proteins that specifically bind to proteins of the extracellular matrix, including fibrinogen, fibronectin, vitronectin, and collagen (McDevitt et al. 1997; Menzies 2003; Ni Eidhin et al. 1998; Switalski

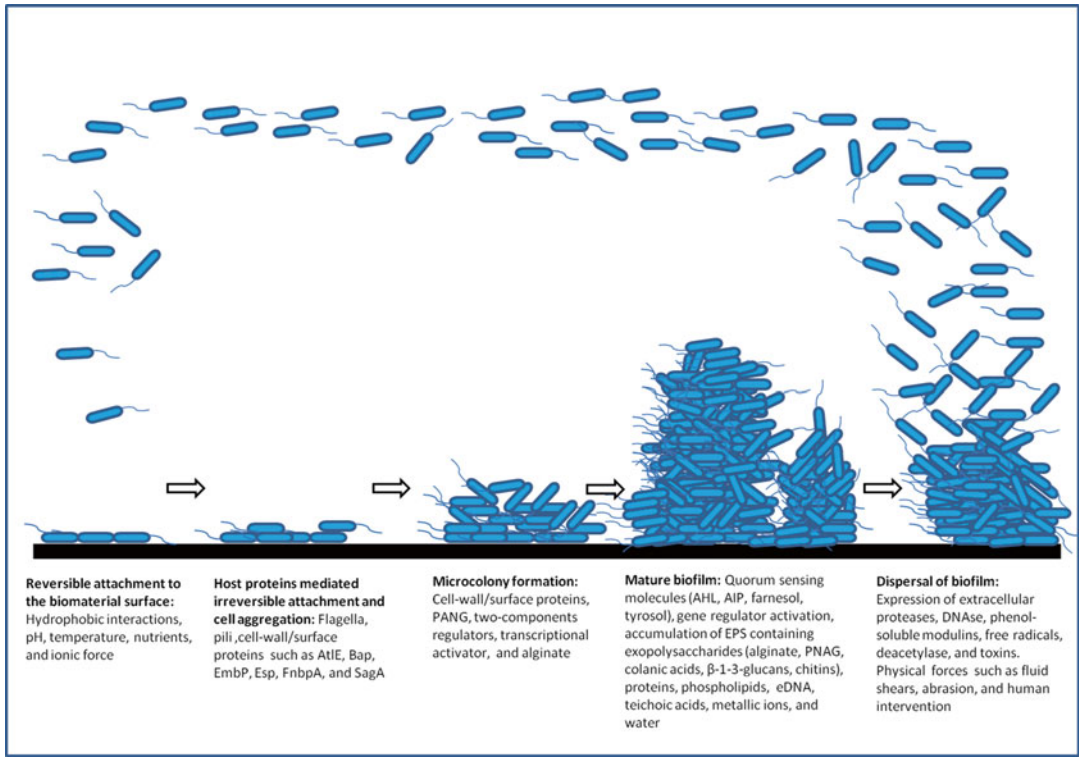

Fig. 10.1 Schematic representation of biofilm development and dispersion on a biomaterial surface

et al. 1993). These host components, including thrombin, platelets, and laminin, may deposit on catheters and foreign body material surfaces and provide specific ligands for microbial adhesins (Shenkman et al. 2002). Fibronectin and fibrinogen are known to influence the adherence of bacteria, especially staphylococci, to biomaterials (Dickinson et al. 1997; Herrmann et al. 1988). Thrombus proteins increase bacterial adherence on catheters and have been associated with catheter-related bloodstream infection; the formation of a fibrin sheath around the catheter greatly increases catheter colonization (Mehall et al. 2002). Furthermore, platelets have been shown to increase the adherence of *Staphylococcus aureus* in combination with thrombin. Activated platelets also bind to several soluble plasma proteins, including von Willebrand factor, fibronectin, fibrinogen, and thrombospondin, and consequently promoting microbial adhesion (Baumgartner and Cooper 1998). The implants are in direct contact with the bloodstream; thus, the surface becomes coated with blood components that act as a conditioning film to which microbes can attach by expressing specific adhesins.

Irreversible adherence is also performed using attachment organelles to the abiotic surface. Adherence is performed by the type I pili in *Escherichia coli* (Beloin et al. 2008; Thumbikat et al. 2009; Wellens et al. 2008). Curli, and antigen 43 have been reported to mediate bacterial interactions on biomaterial surfaces, in addition to primary attachment (Cegelski et al. 2009; Ulett et al. 2007). Curli also enables binding to extracellular matrix proteins, such as fibronectin and plasminogen (Cookson et al. 2002; Uhlich et al. 2006). Type IV pili-driven twitching motility has been reported in *Pseudomonas aeruginosa* that contacts the surface and maintains adherence (Klausen et al. 2003; O'Toole and Kolter 1998).

A plethora of primary attachment adhesins has been reported in gram-positive bacteria as well. A variety of Enterococcal adhesins such as

SagA, Ace, Esp, and enterococcal biofilm pili (Ebp) contribute to biofilm formation, endocarditis, and urinary tract infections (Kemp et al. 2007; Kline et al. 2010; Mohamed and Huang 2007; Mohamed et al. 2004, 2006; Nallapareddy et al. 2006). Staphylococcal proteins such as Bap, SasG, Aap, EmbP, FnbpA, and FnbpB are reported to be involved in bacterial adhesion and colonization on biomaterial surfaces (Geoghegan et al. 2010; O'Neill et al. 2008; Potter et al. 2009; Rohde et al. 2007).

Pathogenic microbes require various ion acquisition mechanisms to obtain ions from host tissues and establish infection in the form of biofilm in humans. Metallic cations such as iron, calcium (Ca2+), and magnesium (Mg2+) play roles in microbial adherence and biofilm formation. Numerous studies have shown that intracellular iron concentration plays a crucial role in biofilm formation and development. Iron regulates biofilm formation in many bacterial species, including *P. aeruginosa* and *E. coli* (Banin et al. 2005; Wu and Outten 2009). Lactoferrin, the iron chelator in human blood, restricted the maturation of *P. aeruginosa* biofilm (Singh et al. 2002). Apo-transferrin inhibits the adhesion of *S. aureus* and *Staphylococcus epidermidis* to polystyrene, polyurethane, and silicone surfaces (Ardehali et al. 2002), whereas catecholamine inotropes, which extract iron from plasma iron-binding proteins, stimulate biofilm formation by allowing *S. epidermidis* to adhere to solid surfaces (Lyte et al. 2003). Ca2+ has been reported as a key initiator that binds to extracellular DNA (eDNA) on bacterial surfaces and mediates bacterial aggregation and biofilm formation in both gram-positive and gram-negative bacteria (Das et al. 2014). Mg2+ is also known to be essential for biofilm formation. Mg2+ influenced attachment and subsequent biofilm formation and structure in *Pseudomonas* species (Song and Leff 2006). The increasing levels of Mg2+ enhanced biofilm production by *S. epidermidis* (Dunne 2002). Further, the increasing concentrations of potassium, the major intracellular cation, promoted biofilm formation in *S. aureus* (Beckingsale et al. 2011). Subsequently, flow conditions are considered a leading factor that strongly influences the number of adhered bacteria (Dickinson et al. 1995; Isberg and Barnes 2002) and the biofilm structure and performance (Klapper et al. 2002; Stoodley et al. 1999).

After the irreversible attachment, the multiplication of microbes on the surface lead cell-to-cell aggregations to form discrete EPS matrix-encased cell communities called microcolonies (Fig. 10.1); these microcolonies hold the cells together in a mass and firmly attach the microbial mass to the underlying surface. The continued growth of microbial cells on a surface leads to the development of mature biofilm that contains millions of tightly packed cells. These cells are assembled into a complex pillar- and mushroom-shaped structure, with towers interspersed with fluid-filled channels that facilitate nutrient supply (Hall-Stoodley et al. 2004). Thus, mature biofilm are complex, highly differentiated three-dimensional structures. Colonization and biofilm formation may occur within 3 days of catheterization (Fig. 10.2) (Anaissie et al. 1995). Raad et al. showed that catheters inserted for less than 10 days tend to have more extensive biofilm on the exterior surface of the catheter; in long-term catheters, biofilms were more extensive on the internal lumen (Fig. 10.3) (Raad 1998; Raad et al. 1993). At the final stage of biofilm development, microbes from the biofilm colony detach and disperse into new environmental sites to initiate another biofilm (Fig. 10.1). Dispersal of biofilms has been reported to be regulated by various environmental signals, signal transduction pathways, and effector molecules (Karatan and Watnick 2009). Such mechanisms could be linked to the cause of bacteremia and infections in new sites from implanted medical devices.

10.4 Extracellular Polymeric Substances

Depending on the species involved in the formation of the microcolonies (single or multiple species), biofilm is usually composed of 10–25 % cells and 75–90 % extracellular polymeric substances (EPS) (Costerton et al. 1987). EPS generally contain polysaccharides, proteins,

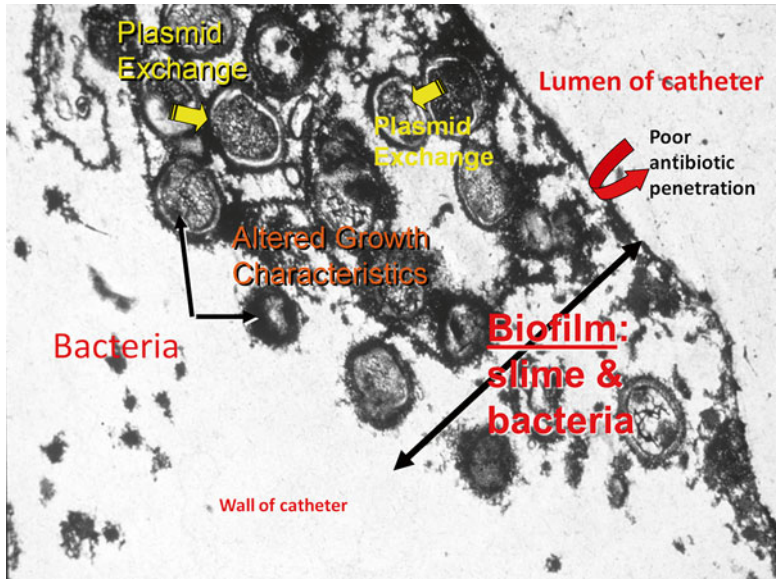

Fig. 10.2 Electron microscopic study showing biofilm formation on wall and lumen of the catheter

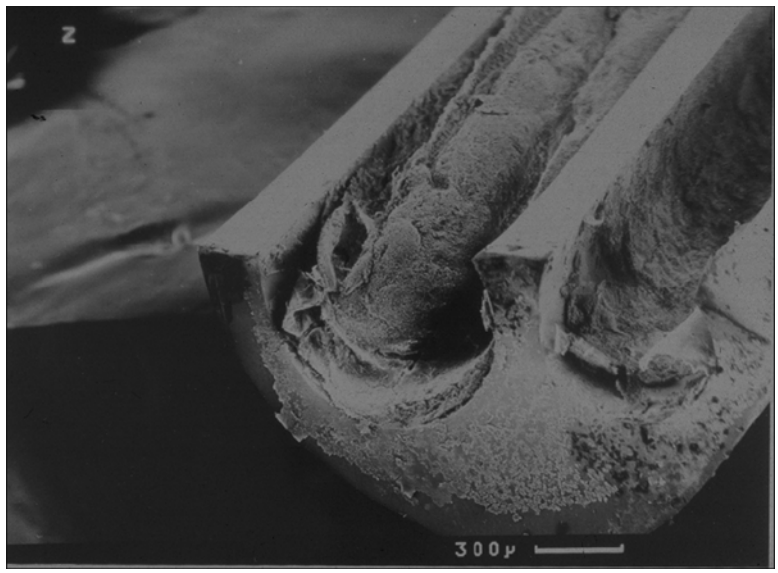

Fig. 10.3 Scanning electron microscopy showing accumulation of biofilm and biofilm matrix in lumen of the catheter

phospholipids, teichoic acids, eDNA, and other polymeric substances, hydrated to 85–95 % water (Costerton et al. 1981; Sutherland 1983). The biofilm matrix, in the form of EPS, contributes to the overall architecture, maintenance, and resistance phenotypes of biofilms (Branda et al. 2005; Sutherland 2001). Polysaccharides are a major component of the EPS matrix (Flemming and Wingender 2010). Exopolysaccharides are mostly heteropolysaccharides that consist of neutral and charged sugar residues. Many known exopolysaccharides, including alginate, xanthan, and colanic acid, are polyanionic in nature. In addition, polycationic exopolysaccharides, such

as polymer N-acetyl glucosamine (PNAG) (which is isolated from *S. aureus* and *S. epidermidis*), are reported to be responsible for colonizing medical implants, leading to biofilm-related infections (Gotz 2002).

EPS have been extensively studied in both gram-negative bacteria, gram-positive bacteria, and fungi. Polyglucosamine and colanic acid are the main components that contribute to the architecture of the biofilm in *E. coli* (Agladze et al. 2005; Danese et al. 2000b; Kostakioti et al. 2013; Wang et al. 2005). Three major exopolysaccharide components, Pel, Psl, and alginate were found to have increased attachment to mucin, airway epithelial cells and stabilizing the biofilm structure in *P. aeruginosa*. Interestingly, it was recently found that mucoid *P. aeruginosa* strains also depend on Psl to form biofilms (Ma et al. 2012; Yang et al. 2012). Another EPS component, alginate, is the exopolysaccharide that is expressed by *P. aeruginosa* clinical isolates from the lungs of CF patients (Govan and Deretic 1996); it plays an important role in structural stability and is associated with superior resistance to antibiotic treatment and host immune defenses during chronic infections (Hentzer et al. 2001; Leid et al. 2005). Furthermore, eDNA plays a critical role in cell-to-cell interactions and stabilization of *Pseudomonas* biofilm (Whitchurch et al. 2002; Yang et al. 2007). Extracellular proteins and several proteinaceous components are also considered to be matrix components, including type IV pili, flagella, and fimbriae. These components were found to mainly have secondary functions as adhesion factors and structural support in the biofilm formation of *P. aeruginosa* (Mann and Wozniak 2012).

The EPS of gram-positive bacteria such as staphylococci consist of different secreted polymers, exopolysaccharides, teichoic acids, and specific proteins, as well as eDNA. Majority of the strains of *S. aureus* utilize PNAG also referred as polysaccharide intercellular adhesin (PIA), to form biofilm (O'Gara 2007). The expression of icaADBC operon, which encodes enzymes that are required for the production of PIA on the surface of *S. aureus*, is critical to cell-to-cell adhesion and biofilm formation. The same study found that the *ica* locus is present both in *S. epidermidis*, *S. aureus*, and several other streptococcal species (Cramton et al. 1999). PIA plays a critical role in the adherence of *S. epidermidis* to biomaterials by providing favorable acid-base interactions with the surface (Olson et al. 2006).

EPS also harbor an adhesive protein, Bap, that is required for biofilm formation in *S. aureus* (Lasa and Penades 2006). Several surface proteins, including Aap and SasG, have also found to promote biofilm formation in *S. epidermidis* and *S. aureus* (Geoghegan et al. 2010; Rohde et al. 2007). Methicillin-resistant *S. aureus* (MRSA) biofilm is promoted by the fibronectin-binding proteins FnBpA, FnBpB, and Embp, as a component of a proteinaceous biofilm (Christner et al. 2010; O'Neill et al. 2008). Teichoic acids are another cell wall component that has been reported to take part in the structure of staphylococcal biofilms (Sadovskaya et al. 2004).

Besides PIA and proteins in the biofilm matrix, eDNA is a major component of biofilm that stimulates *S. epidermidis* biofilm formation (Qin et al. 2007). eDNA has also been shown to be indispensable for biofilm formation of *Streptococcus mutans*, *Streptococcus intermedius*, and *Enterococcus faecalis* (Thomas et al. 2008; Whitchurch et al. 2002).

Similarly, in bacterial biofilms, the extracellular matrix helps preserve the architectural integrity of fungal biofilms and contributes to antifungal tolerance (Flemming and Wingender 2010; Hawser and Douglas 1995). Andes's team identified soluble β-1,3-glucans as an important component of the biofilm matrix of *Candida albicans* in vivo and in vitro (Nett et al. 2007a, b). In addition, EPS of *C. albicans* biofilm contain chitins and eDNA (Al-Fattani and Douglas 2006; Ramage et al. 2009).

Polysaccharide molecules can interact with themselves or with heterologous molecules to yield gels, often with multivalent cations playing a substantial role in the process (Sutherland 2001). Among several ions, Ca2+ and Mg2+ bind to the majority of the most EPS components in a biofilm matrix (Decho 2010). Ca2+ may play a role in biofilm formation as an ionic cross-bridging matrix molecule in *P. aeruginosa*

(Sarkisova et al. 2005). The presence of Ca2+ affects the mechanical properties of biofilms and serves as ionic cross-bridging of the polysaccharide in the biofilm matrix (Kierek and Watnick 2003). As numerous matrix polymers are anionic in nature, they may also bind to cations and provide essential nutrients. The matrix itself can also act as a carbon and energy reserve (Sutherland 2001).

10.5 Quorum Sensing and Biofilm

Many microbial pathogens communicate through the production of and sensing of auto-induced signaling molecules known as auto-inducing peptides (AIPs) to control the expression of specific genes in response to population density; this is known as quorum sensing (Waters and Bassler 2005). In staphylococci, the quorum-sensing system is encoded by the *agr* (accessary gene regulator) locus, which consists of *agrA*, *agrC*, *agrD*, and *agrB* genes that are co-transcribed. Once AIP reach a threshold level, the bacteria respond by activating the expression of sequences of specific cell density-dependent gene. Most staphylococcal products are under the control of *agr*, which is activated during the transition from the exponential to the stationary growth phase. The *E. faecalis fsr* quorum-sensing system controls biofilm development (Hancock and Perego 2004). Like gram-positive bacteria, many gram-negative bacteria also use AIP, called N-acetyl homoserine lactone (AHL). *P. aeruginosa* has AHL-dependent QS systems (LasR/LasI, RhlR/RhlI, and PQS) that mediate biofilm architecture and the production of extracellular polymeric slime (Shih and Huang 2002; Yoon et al. 2002). The quorum-sensing molecules farnesol and tyrosol have also been identified in *C. albicans* (Chen et al. 2004; Ramage et al. 2009).

10.6 Biofilm Dispersal

Biofilm dispersal is the final stage of biofilm development, in which microbial cells from the matured biofilm detach and disperse into the milieu. This is an essential phase of the biofilm that contributes to biological dispersal, bacterial survival, and disease transmission (Kaplan 2010). Biofilm dispersal can be a complex and dynamic process, including multiple genetic determinants, environmental signals, signal transduction pathways, and effector molecules. Until recently, the mechanisms by which actual microbial dispersal from biofilms occur remained almost completely unexplored, and little was known about the functions or regulatory pathways involved in the release of microbes from biofilms. A better understanding of the mechanism of dispersion reveals the signals, which regulate the dispersal processes that leads to the development of clinically useful agents that inhibit biofilm formation or promote biofilm detachment on medical implants.

Biofilm dispersal or detachment can be split into different phases, such as detachment of microbes from the biofilm colony, translocation of the cells to the new site, and attachment of the cells to a substrate in the new site (Kaplan 2010). Both active and passive types of dispersal processes play a role in the initiation of biofilm detachment, such as nutrient availability (Gjermansen et al. 2005; Hunt et al. 2004; Sauer et al. 2004), enzyme-mediated breakdown of the biofilm matrix (O'Neill et al. 2007; Rohde et al. 2007; Whitchurch et al. 2002), free radical production (Barraud et al. 2006; Webb et al. 2003), surfactant production (Davey et al. 2003; Otto 2014; Periasamy et al. 2012; Wang et al. 2011), the control of quorum-sensing systems (Boles and Horswill 2008; Periasamy et al. 2012; Rice et al. 2005; Wang et al. 2011), and signaling molecules (Morgan et al. 2006).

Sauer and co-workers observed that spent medium from *P. aeruginosa* cultures induced dispersal of biofilm (Sauer et al. 2004). The RNA-binding protein CsrA (carbon storage regulator A) acts as an activator of biofilm dispersal in *E. coli* (Jackson et al. 2002). Depletion of oxygen was found to stimulate the production of a specific exopolysaccharide lyase, which degraded the matrix of *Pseudomonas fluorescens* biofilm and dispersed the bacteria (Allison et al. 1998). Increased production of alginate lyase digests

alginate in the biofilm matrix, promoting the detachment of *P. aeruginosa* biofilms (Boyd and Chakrabarty 1994); a separate study showed that digestion of alginate and eDNA with their respective enzymes enhanced the efficacy of tobramycin, amikacin, and gentamicin against *P. aeruginosa* biofilm (Alipour et al. 2009). Nitric oxide was reported to induce biofilm dispersal in *P. aeruginosa* biofilm (Barraud et al. 2006). *P. aeruginosa* produces extracellular surfactant rhamnolipids that can mediate biofilm dispersal (Boles and Horswill 2008). Cis-2 decanoic acid, an unsaturated fatty acid, is produced by *P. aeruginosa*, is capable of inducing the dispersion of established biofilm. The exogenous addition of this messenger molecule was also shown to induce the dispersion of biofilms formed by other pathogens, *E. coli*, *Klebsiella pneumonia*, *S. aureus*, and *C. albicans* (Davies and Marques 2009). Increased levels of cyclic-dimeric GMP, an intracellular signal, resulted in enhanced production of exopolysaccharides, while decreased levels of this molecule induced biofilm dispersal in different bacteria (Kaplan 2010).

It has been documented that activation of the *agr* quorum-sensing system leads to the upregulation of extracellular proteases (Aur and Spl) that contribute to *S. aureus* biofilm detachment (Boles and Horswill 2008). Another study showed that PNAG-degrading enzyme dispersin B was able to release preformed biofilm produced by *S. epidermidis* (Izano et al. 2008). Deoxyribonuclease has also been implicated in bacterial detachment in *S. aureus* biofilm (Mann et al. 2009).

In addition to proteases, other *agr*-regulated factors contribute to biofilm detachment. Surfactant-like molecules such as δ-toxin may exert dispersal effects on the biofilm of *S. aureus* (Kong et al. 2006). Otto's group recently identified a family of short staphylococcal peptides, the phenol-soluble modulins (PSMs), that are under the control of *agr*. The specific secreted surfactant PSMs promote biofilm structuring and detachment in *S. aureus* and *S. epidermidis* (Periasamy et al. 2012; Wang et al. 2011).

Fungi have been reported to be involved in biofilm dispersal as well. Yeast wall protein 1 was involved in the dispersal of *C. albicans* biofilm (Granger et al. 2005). NRG1 is a negative regulator that has been shown to be involved in biofilm detachment in *C. albicans* biofilm (Uppuluri et al. 2010). Very recently, it has been was reported that the histone deacetylase complex facilitates biofilm dispersal in *C. albicans* (Nobile et al. 2014).

10.7 Dissemination of Biofilm and Bacteremia

Microbial biofilm formation is the primary mode of growth in most natural and clinical settings. Dispersal plays a vital part in spreading pathogenic microbes from environmental reservoirs to human hosts and spreading infections within a host. Many biofilm-associated infections occur in the nosocomial setting as a result of the contamination of indwelling medical devices from the skin flora of patients or health care workers. The rate of biofilm dispersion is enhanced with increased biofilm thickness and external shear forces of the surrounding medium, such as urine, blood, saliva, and other body fluids. Bacterial biofilm on medical implants can cause bloodstream infections and systemic inflammation, in which biofilm bacteria detach and disperse during biofilm development (Otto 2014; Raad et al. 2008a); in these cases, the infected medical devices should be removed (Raad et al. 2008a).

PSMs contribute to the dissemination from a preformed biofilm to secondary infection sites. In a mouse model of biofilm-associated infections, PSMs of staphylococcal origin facilitated the dissemination of biofilm on CVCs to secondary infection sites (Periasamy et al. 2012; Wang et al. 2011). These studies provide evidence of the significance of such molecules in the dissemination of biofilm-associated infections, motivating us to identify potential therapeutic targets and thus prevent complications and the spread of infections. To achieve this goal as a primary step, Wang et al. generated antibodies against PSMβ1 of *S. epidermidis* peptides, which inhibit bacterial dissemination from the CVC (Wang et al. 2011).

Dissemination in patients with chronic infections is also mediated by biofilm dispersal, as it allows biofilm bacteria to spread throughout the infected organ or colonize other parts of the body. For instance, transient bacteremias have been diagnosed after dental procedures (Kinane et al. 2005). Nosocomial pneumonia was found to be caused by bacteria released from biofilms in a patient with an endotracheal tube (Adair et al. 1999); a kidney infection was caused by bacteria that detached from a biofilm in a patient's bladder (Mathoera et al. 2000); and suffering from other biofilm infections, such as endocarditis, (Parsek and Singh 2003). Remarkably, in cystic fibrosis (CF) patients, only *Burkholderia* species are able to cause systemic infections (Isles et al. 1984). Recently, biofilm dispersal of *Streptococcus pneumoniae* was documented to result in a significantly increased spread and cause infections to other sites such as the middle ear, lungs, and bloodstream in a mouse model (Marks et al. 2013).

10.8 Multi-species Biofilm and Its Clinical Relevance

Multi-species or mixed species of biofilm are certainly the dominant form in nature and are prominent in human host tissues and medical biomaterials, such as the oral cavity, the lungs of the CF patients, chronic wounds, and catheters. The extensive interaction between different species of microorganisms determines the structural and functional dynamics of multi-species biofilms. Coaggregation interactions are believed to contribute to multi-species biofilm formation in different environments (Rickard et al. 2003).

Several bacterial cell surface protein adhesins play important roles in coaggregation during multi-species biofilm formation. Five specific adhesins are expressed by *Streptococcus oralis* and aggregate with other species of oral bacteria in dental plaque (Yang et al. 2011). Since protein adhesins are commonly distributed among bacteria, adhesin-mediated coaggregation may be a major strategy for multi-species biofilm development. As protein adhesins are also found in fungi, these adhesins can mediate fungi-bacteria interactions (Li and Palecek 2008; Silverman et al. 2010). Bacterial pili, flagella, and their motilities are also essential for multi-species biofilm formation. *P. aeruginosa* type IV pili facilitate multi-species microcolony formation with *S. aureus* in multi-species biofilms (Yang et al. 2011). Further, pili promote multi-species biofilm of *E. coli* and *Citrobacter freundi* (Pereira et al. 2010). eDNA also widely exists among multi-species biofilms (Steinberger and Holden 2005). Recently, it was reported to enhance the mixed-species biofilm of *S. epidermidis* and *C. albicans* both in vitro and in vivo (Pammi et al. 2013). It has been documented that *C. albicans* interacts with 12 other species of *Candida* and bacteria, such as *P. aeruginosa* and *S. epidermidis*, in the form of multi-species biofilm in a polystyrene tube model (El-Azizi et al. 2004).

Some quorum-sensing molecules support interspecies communication in multi-species biofilm, enabling the microbes to sense the presence of other species (Waters and Bassler 2005). AHL autoinducers are the most common signaling molecules in bacteria and can influence a broad range of cross-species and cross-genus communications (Federle and Bassler 2003). *P. aeruginosa* and *Burkholderia cepacia*, which are sometimes found in the lungs of CF patients, can form mixed biofilms. *B. cepacia* is capable of perceiving the AHL signals produced by the CF pathogen *P. aeruginosa* (Riedel et al. 2001). Besides the involvement of signaling molecules in multi-species biofilm, the dispersion mechanism through such signaling molecules within multi-species biofilm can cause polymicrobial bloodstream infections and spread to other parts of the body.

One of the most alarming consequences of multi-species biofilm is that it is more resistant to antimicrobial agents and host immune responses than is mono-species biofilm. Many studies have compared the antibiotic resistance of multi-species and mono-species biofilms; in most cases, mixed-species biofilms were significantly more resistant to antimicrobial treatment. The CF pathogen *Stenotrophonomonas maltophilia* AHL

signaling molecule affects biofilm and polymyxin tolerance in *P. aeruginosa* (Ryan et al. 2008). The slime produced by *S. epidermidis* can inhibit the penetration of fluconazole, while *C. albicans* can protect slime-negative *S. epidermidis* against vancomycin in the mixed biofilms of *C. albicans* and *S. epidermidis* (Adam et al. 2002). In another study, *C. albicans* induced *S. aureus* vancomycin resistance in multi-species biofilm (Harriott and Noverr 2010).

Multi-species biofilms have been clinically linked to polymicrobial infections, which themselves are associated with significantly poorer clinical outcomes than are single microbial infections (Brogden et al. 2005; Sutter et al. 2008); they also account for roughly 15 % of infections in immunocompromised cancer patients. CRBSIs have also been reported in young and adults patients (Cairo et al. 2011; Downes et al. 2008). One of the significant risk factors for polymicrobial infections is the presence of indwelling vascular catheters that act as sites for mixed-species biofilm formation (Cairo et al. 2011; Downes et al. 2008). Many types of chronic infection are caused by biofilm-associated microbes; these are hard to eradicate because of the protective biofilm matrix, which may be further enforced if multiple species are present. Recently, it was demonstrated that in vivo wound healing was delayed when patients were coinfected with both *S. epidermidis* and *P. aeruginosa* (Pastar et al. 2013). Mixed-species *S. epidermidis* and *C. albicans* biofilms facilitate *S. epidermidis* infection and blood dissemination in the mouse subcutaneous CVC biofilm model; this study may explain the increased clinical mortality and morbidity in the polymicrobial environment.

Researchers have begun study multi-species biofilms to unravel the complexity of inter-species and inter-genus communications and their effect in clinical and environmental settings. The prevalence of mixed-species biofilms and their involvement in various infections highlights the need for a better understanding of the interactions and dynamics within mixed biofilm communities, which is necessary to successfully prevent or treat polymicrobial infections.

10.9 Diagnosis

Studies have shown that the formation of biofilm in venous lines is universal; quantitative electron microscopy has demonstrated that biofilm formation starts after insertion of the catheter, even with the absence of clinical manifestations of CLABSI (Raad et al. 1993). However, by exceeding certain threshold number of colony-forming units (CFUs) with dissemination, the clinical manifestation of bloodstream infections will be more apparent (Cleri et al. 1980; Sherertz et al. 1990).

The location of the biofilm depends on the type of the catheter (the duration of catheterization); for short-term catheters (<10 days), the external surface is the main site of biofilm formation, whereas for long-term catheters (>30 days), the internal surface of the lumen is the main site (Raad et al. 1993).

The roll-plate, semi-quantitative culture method of Maki et al. allows one to culture the external surface of the catheter (Cicalini et al. 2002). The catheter is rolled back and forth on a Columbia agar plate supplemented with 5 % sheep blood; the plate is then incubated for 3 days (72 h) at temperature of 35 °C with 5 % CO_2, and the CFUs of the microorganism are quantitated (Slobbe et al. 2009). This is a convenient method for culturing microorganisms in short-term catheter. However, the roll-plate technique can give high false-negative results in long-term catheters and fails to release biofilm-embedded organisms from the CVC surface (Sherertz et al. 1990). Other limitation of this method is that some catheters come out in an irregular shape, which makes it hard for the laboratory personnel to perform the procedure. This may also lead to false-negative results.

New quantitative methods have been developed to culture the external and internal surface of catheters. These include sonication and vortexing (Bjornson et al. 1982). The method includes placing the catheter in 5 ml of 0.9 % NaCl, sonicating it for 1 min, and vortexing the sonication fluid for 15 s. Subsequently, 50 µl of the sonication fluid is cultured on agar plates,

allowing for a detection limit of ≥100 CFU/catheter tip (Slobbe et al. 2009). Other quantitative diagnostic procedures include flushing the catheter with 2 ml of brain-heart infusion broth. After the fluid has been diluted to tenfold, 0.1 ml is streaked onto a blood agar; $\geq 10^3$ CFUs is considered significant colonization in the catheter lumen (Linares et al. 1985; Cleri et al. 1980).

Quantitative methods are considered superior to semi-quantitative methods, with the highest sensitivity (80–100 %) and specificity (more than 90 %) (Siegman-Igra et al. 1997). The most sensitive individual culture method is sonication of the tip or subcutaneous catheter segments. However, culturing both can increase the test sensitivity by 20 % (Sherertz et al. 1990; Kristinsson et al. 1989; Bjornson et al. 1982).

Studies have shown that using semi-quantitative methods prior to sonication of the catheter will decrease the sensitivity of the sonication; thus, it is not recommended to combine these two methods.

For all the quantitative and semi-quantitative methods described above, removal of the CVC is essential; unfortunately, the unnecessary removal of CVCs will make these methods clinically not useful.

For that reason, new approaches are designed to establish the diagnosis without removing the catheter, depending on the results of the clinical evaluation and confirmatory positive blood or catheter tip cultures.

The presence of positive blood cultures with no other apparent source of infection should raise suspicion for CLABSI, in addition to the clinical manifestations of systemic infection; although fever is the most sensitive indicator of a bloodstream infection, it is not specific. Other signs are chills, hypotension, and inflammation of the catheter insertion site, which is the most specific sign but has low sensitivity (Safdar and Maki 2002).

The Centers for Disease Control (CDC) has introduced a new term: laboratory-confirmed bloodstream infections; (LCBI) these must meet one of the following criteria (Horan et al. 2008):

1. One or more positive blood cultures with no apparent source of infection.
2. Clinical presentation of infection (fever, chills, and hypotension), two or more positive blood cultures collected on two different occasions, and no apparent source of infection or skin contaminant.
3. Patients <1 year of age must have at least one of the following signs or symptoms: fever, hypothermia, apnea, or bradycardia and a positive blood culture with no source of infection or skin contaminant.

A new device called a smart CVC (SCVC) which formed to detect biofilm formation (in vitro) through a biosensor. Information is sent through an antenna to an external base station to detect biofilm formation early; this provides more knowledge about colonization formation and leads to proper treatment. This device is still being researched, but is promising for enhancing the quality of patient care (Paredes et al. 2014).

10.10 Prevention of CLABSIs

CVC infection risk increases with the duration of use, yet routine changing of CVCs, unlike peripherally inserted catheters, is not recommended. Removing the CVC and inserting another in a different site carries high mechanical risks, and exchanging the CVC with a guidewire may increase the risk of bloodstream infection (Cobb et al. 1992). In addition, changing a CVC is not an easy procedure, as it is with a peripheral CVC. For that reason, different kinds of measurements are used to minimize and prevent the incidence of biofilm formation and bloodstream infections. The CDC recommends the following guidelines, which are considered as bundle of septic techniques to follow during CVC insertion (Casanova Vivas 2014):

1. Use gloves after washing hands with antiseptic-containing soap, alcohol gel, or foam.
2. Full sterile barrier precautions must be used during insertion of the catheter, including sterile gloves, a surgical gown, and a mask, with a large sterile sheet drape.
3. Apply skin disinfectant (2 % chlorhexidine) at the CVC insertion site and wait for it to fully air dry before proceeding with the insertion.

4. Remove the CVC once it is no longer needed.
5. Avoid sites with a high incidence of infection (e.g., femoral) (Goetz et al. 1998).

Described below are the most important and widely used modalities for preventing biofilm colonization. CVCs impregnated with antibacterial or antiseptic have been shown, in a huge number of in vitro, animal, and clinical studies, to have substantial efficacy at preventing biofilm colonization and eventually bloodstream infection (Raad and Hanna 2002; Darouiche et al. 1999; Hanna et al. 2004). Two types of impregnated CVCs are used widely in the United States: minocycline-rifampin (M/R) and chlorhexidine-silver sulfadiazine (CHX/SS).

CHX/SS-impregnated catheters, which are now considered the first-generation CVC, showed a twofold decrease in colonization and nearly fivefold decrease in the rate of bloodstream infection (Maki et al. 1997). A meta-analysis of 12 studies (Veenstra et al. 1999) showed that catheters impregnated with CHX/SS were effective at preventing colonization and bloodstream infections. However, in first-generation CHX/SS catheters, only the external surface of the catheter is coated, which compromises its efficacy over the long term (>3 weeks); in long-term catheters, the internal surface is the main source of biofilm formation (Mermel 2000; Raad et al. 1996; Bach et al. 1996). In addition, the antimicrobial durability of all CHX/SS CVCs (first and second generation) in plasma is limited to 1 week (Raad et al. 2012; Darouiche et al. 1999). On the other hand, reports have raised concerns about anaphylaxis shock associated with chlorhexidine. The risk of this complication is low but significant, and it could be genetically related as it was reported only in Japan (where it is prohibited for that cause) (Oda et al. 1997; Fujita et al. 1997).

Second-generation CHX/SS catheters have been developed that have threefold increases in the chlorhexidine concentration, in addition to coating the internal lumen, the hub, and extension lines. A study of 780 patients in intensive care units showed a significant decrease in colonization compared to non-coated catheters; however, there was a non-significant reduction in the rate of CRBSIs (Rupp et al. 2005).

Antibiotic-impregnated CVCs M/R resulted in significantly superior outcomes to those of CHX/SS catheters. In a large randomized, prospective, multi-center clinical trial (Darouiche et al. 1999), these CVCs led to a 12-fold decrease in the rate of bloodstream infection compared with first-generation CHX/SS catheters. The prolonged antimicrobial activity (around 50 days) (Darouiche et al. 2005) and established activity against multi-drug resistant (MDR) VRSA and gram-negative bacteria that are associated with CRBSIs (Raad et al. 2008b) were the reasons for its superior clinical efficacy.

Concerns have been raised regarding the emergence of resistant bacterial strains with the use of antibiotic-coated catheters; however, large clinical studies have failed to demonstrate resistance after prolonged use (up to 7 years) and more than 500,000 CVC days (Raad et al. 1997; Darouiche et al. 1999; Raad and Hanna 2002). In addition, these CVCs led to a decrease in the rate of nosocomial vancomycin-resistant enterococci (VRE)-related bacteremia in critically ill patients (Hanna et al. 2003).

The overuse of antiseptic skin preparations and sterile barrier precautions changed the epidemiological map of the pathogens that cause CLABSIs, shifting them toward resistant gram-negative bacteria, in which both CHX/SS and M/R CVCs have limited activity. For that reason, our team at The University of Texas MD Anderson Cancer Center has developed a new broad-spectrum catheter by adding chlorhexidine to M/R (CHX-M/R). In vitro results showed that it has excellent activity against multidrug-resistant, gram-negative *Acinetobacter baumannii*, *Enterobacter cloacae*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. maltophilia*, and is superior to M/R and CHX/SS catheters.

A new in vitro cross-contamination model of exchange study showed that exchanging uncoated CVCs over a guidewire in the presence of bacteremia using CHX-M/R catheters completely prevented cross-contamination by MRSA, *P. aeruginosa*, and *C. albicans* biofilm; exchanging them for CHX/SS CVCs reduced but did not prevent cross-contamination by MRSA. Furthermore, CHX-M/R CVCs showed

superior activity against *P. aeruginosa* and *C. albicans* to M/R catheters and were superior to CHX/SS CVCs against MRSA and *P. aeruginosa* (Jamal et al. 2014).

Recently, several studies have been conducted using locking solutions instead of the usual heparin lock. These solutions are mostly a combination of an antimicrobial and an anticoagulant (Campos et al. 2011).

Minocycline combined with EDTA (M-EDTA) showed high efficacy in preventing bloodstream infection in chronic hemodialysis patients (Campos et al. 2011; Raad et al. 2008a). This combination was highly active against *S. epidermidis*, *S. aureus*, and *C. albicans* that were embedded in biofilm (Raad et al. 2003). Randomized clinical trials show at least a threefold reduction in the occurrence of bacteremia (McIntyre et al. 2004; Dogra et al. 2002; Betjes and van Agteren 2004; Bleyer et al. 2005). On the other hand, a study by Bleyer et al. showed that minocycline/EDTA lock solution was significantly effective at preventing CLABSIs in patients with a history of recurrent bacteremia and a high risk of infection (Feely et al. 2007). Furthermore, this solution was used to prevent infection in an implantable port in children with cancer (Chatzinikolaou et al. 2003).

A new antimicrobial lock solution that was developed recently by Raad et al. contains 7 % citrate; this has been approved as an anticoagulant heparin-free catheter lock, 20 % ethanol; for its antimicrobial activity, plus 0.01 glyceryl trinitrate (GTN); which is well known for its intravenous use in treating hypertension. In vitro results show that these components rapidly and fully eradicate biofilms in all organisms tested (MRSA, methicillin-resistant S. *epidermidis*, *P. aeruginosa*, and *C. albicans*), in synergistic manner (Rosenblatt et al. 2013).

10.11 Management of Catheter Related Biofilm

The conventional treatment for all foreign body-associated biofilm infections is to remove the foreign body. However, removing the CVC and reinserting another in a different vascular access site carries a risk of iatrogenic mechanical complications; it is also time consuming and relatively expensive (Dimick et al. 2001). On the other hand, systemic antibiotics alone are not sufficient for treating biofilm bacteria because the extracellular materials in the biofilm, with their high concentrations of metal ions and low pH, cause metabolic inactivation of the antibiotics (Hoiby et al. 2010). These factors can contribute to the biofilm bacteria becoming 1,000 times more resistant than planktonic cells (Fig. 10.2) (Hoiby et al. 2010; Kostakioti et al. 2013). For that reason, many strategies have been designed to treat biofilm formation, either by killing the bacteria or dissolving the biofilm by targeting different developmental stages of biofilm formation.

Some biofilm disruption and treatment strategies are described below:

- **Chelating Agents**: Metal cations, such as calcium, iron, and magnesium, play an essential role in maintaining biofilm matrix integrity and inhibiting bacterial growth by affecting bacterial membrane stability (Patrauchan et al. 2005; Sarkisova et al. 2005; Raad et al. 2008a). Chelating agents can destabilize the biofilm matrix architecture, thus helping to dissolve it. Sodium citrate is one of the chelators that has an inhibitory effect on *Staphylococcus* species biofilm (Shanks et al. 2006). On the other hand, in vitro studies show high efficacy of tetra sodium-EDTA in eradicating biofilm (Kite et al. 2004; Percival et al. 2005). The combination of disodium-EDTA and antimicrobial agents such as tigecycline or gentamicin is effective at reducing biofilm formation in both staphylococcus species and *P. aeruginosa* (Bookstaver et al. 2009). Raad et al. showed a synergetic effect of Minocycline-EDTA (M-EDTA) in preventing colonization and biofilm formation (Raad et al. 2007). This combination was effective in organisms embedded in both fresh biofilm (in vitro) and mature biofilm (ex vivo) (Raad et al. 2003). To our knowledge, this is the only biofilm-disrupting and -dissolving treatment and technology with a large number of

successful clinical studies (Raad and Bodey 2011; Raad et al. 2002, 2007; Chatzinikolaou et al. 2003).

- **Phage Therapy**: An abundant, easily isolated, self-replicating, organism with a high mutation rate easily adapts to any given environment. One promising alternative to antibiotics is encoding phages with EPS-degrading enzymes (Hughes et al. 1998; Sutherland et al. 2004; Sillankorva et al. 2004), resulting in fast destruction of the bacterial cell wall. No clinical studies of this approach are available to confirm its efficacy and safety in humans.
- **Antimicrobial Peptides**: Cathelcidins are one of the most important antimicrobial peptide classes; as they show activity in activating the innate immune system response, they can be considered a possible strategy for treating biofilm formation (Pompilio et al. 2011). Pompilio et al. compared the activity of tobramycin, which is considered a first-choice treatment for *P. aeruginosa* in CF patients, with that of cathelcidins. The results show that cathelcidins peptides have faster kinetics and rapid bactericidal activity, while the overall extent of bacterial killing is greater with tobramycin. More precise and advanced studies are needed of the mechanisms involved before cathelcidins can be considered a treatment strategy (Pompilio et al. 2011; Kostakioti et al. 2013).
- **Polysaccharides**: Cell-to-surface and cell-to-cell interactions are mediated by exopolysaccharides, which is an essential step in biofilm formation. Mutations in polysaccharide synthesis cause instability in the biofilm structure, which makes it highly susceptible to antibiotics and immune defense (Rendueles et al. 2013). On the other hand, the results of new studies show that bacterial exopolysaccharides can interfere with the biofilm formation of other bacterial species, For example, *P. aeruginosa* exopolysaccharides can inhibit the biofilm formation of *Staphylococcus* species (Qin et al. 2009; Rendueles et al. 2013).
- **Signal Transduction Interference**: A promising new strategy involves targeting the bacterial signaling cascades. By inhibiting these signals, we can deprogram optimal gene expression without killing the bacteria or increasing the risk of bacterial resistance (Cegelski et al. 2008). An example of this model of treatment is targeting the QseBC two-component system that is common in biofilm-forming, gram-negative pathogens (Huang et al. 2006; Kostakioti et al. 2009; Khajanchi et al. 2012). Clinical data are required to demonstrate the clinical efficacy and safety of this approach.

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