Springer Series on Biofilms

Kendra P. Rumbaugh
Iqbal Ahmad *Editors*

# Antibiofilm Agents

## From Diagnosis to Treatment and Prevention

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Editors

# Antibiofilm Agents

## From Diagnosis to Treatment and Prevention

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# Preface

The editors would like to dedicate this text to the late Dr. Bill Costerton, who is regarded as "The Father of Biofilm." Bill spent the good part of his career working tirelessly to alert and convince the medical community about the existence and importance of biofilms. The fact that many medical specialties are now addressing the "biofilm problem" is in no small degree because of his contributions and those of the scientists he trained and mentored.

Biofilms comprise microbial microcolonies adhered to a surface and surrounded by a sticky exopolysaccharide matrix. Once adherent, microbes multiply and anchor themselves in quite intricate structures, which appear to allow for communication and transfer of nutrients, waste, and signaling compounds. Microbial biofilms constitute a major cause of chronic infections, especially in association with medical devices. Biofilms are extremely difficult to eradicate with conventional antibiotics and therefore represent an enormous healthcare burden.

While the "biofilm concept" has, for the most part, become accepted by the medical community, clinicians are left with the dilemma of how to diagnose and treat these infections. While there are a number of books highlighting research progress on understanding mechanisms of biofilm establishment and their roles in disease, there are currently no existing resources which provide a comprehensive review of the available antibiofilm options.

The purpose of this book is to provide a survey of the recent progress that has been made on the development of antibiofilm agents. Biofilm experts from across the globe have contributed and related their expertise on topics ranging from diagnosing and characterizing biofilm infections to treatment options and finally regulatory challenges to the commercial development of antibiofilm drugs. We intend for this book to serve as a valuable resource for medical professionals seeking to treat biofilm-related disease, academic and industry researchers interested in drug discovery, and instructors who teach microbial pathogenesis and medical microbiology.

Lubbock, TX — Kendra P. Rumbaugh
Aligarh, Uttar Pradesh, India — Iqbal Ahmad
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# About the Editors

**Iqbal Ahmad** is a senior faculty and Chairman of the Department of Agricultural Microbiology. Trained at the Central Drug Research Institute, Lucknow and The Himalaya Drug Company, India, Dr. Ahmad Joined AMU Aligarh as lecturer in 1995. His research works encompasses various disciplines such as drug resistance and virulence in *E. coli* and plasmid biology, microbial ecology, impact of wastewater on soil health and crop productivity, drug and metal resistance in microbes of clinical and environmental origin, and biological activities of Indian Medicinal plants. His present research interest is in the fields of molecular basis of drug resistance and virulence factors linkage in pathogenic bacteria, bacterial quorum sensing, and modulation of quorum sensing-linked bacterial traits and biofilms by natural products. Dr. Ahmad has guided many Ph.D. and several M.Sc. theses, completed four research projects, published seven Books. His research works have received fair citations by scientific community.

**Kendra Rumbaugh** is an Associate Professor of Surgery, with joint appointments in the departments of Immunology and Molecular Microbiology and Cell Biology and Biochemistry at the Texas Tech University Health Sciences Center in Lubbock, Texas. Dr. Rumbaugh's research focuses on understanding and treating wound infections, and she is especially interested in how biofilms, polymicrobial interactions and quorum sensing contribute to bacterial pathogenesis.

# Part I
# Medical Biofilms

# Biofilms in Disease

**Michael Otto**

**Abstract** Biofilms contribute to a majority of infectious diseases caused by bacterial and fungal pathogens. These range from chronic infections of indwelling medical devices and wounds to frequently fatal, serious infections like endocarditis. Biofilm research was initially focused on "environmental" biofilms, such as those present in wastewater tubing. More recently, "medical" biofilms as present during human infection have gained increased attention, and several animal models to mimic biofilm-associated infection in vivo have been established. Furthermore, biofilm research has shifted from the use of laboratory to clinical strains and is being complemented by the genetic analysis of isolates originating from biofilm infection. Often these investigations showed that in vitro results only have limited relevance for the in vivo situation, revealing the necessity of more intensive in vivo biofilm research. This introductory chapter will present an overview of biofilm infections, resistance, and the general model of biofilm development. It will also introduce important biofilm molecules and principles of regulation in premier biofilm-forming pathogens and finish with a general outline of possible routes of anti-biofilm drug development.

## 1 Introduction

According to the World Health Organization, infectious diseases are the second most frequent cause of death worldwide, responsible for more than 13 millions of deaths per year, which is second only to diseases of the heart. Many of these deaths are due to bacteria. Acute respiratory infections are the most frequent causes of deaths among infectious diseases; they are often directly due to, or exacerbated by,

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bacterial pathogens. In addition, bacteria cause a wide array of nonfatal but nevertheless severe infections, such as infections of the skin and soft tissues, the lung, the intestine, and the urinary tract, to name but a few.

Many bacterial infections occur in the hospital in patients with a weakened immune system, which is due to underlying genetic or infection-related immune deficiencies, or the generally weakened status of the patient. Widespread antibiotic resistance often makes these infections extremely difficult to treat.

Another important reason for the problems associated with treating bacterial infections is the formation of biofilms. The National Institutes of Health estimated that more than 60 % of microbial infections proceed with the involvement of biofilms. Biofilms are sticky agglomerations of bacteria or other microorganisms. They significantly decrease the efficacy of antibiotics and the patient's immune defenses.

In nature, bacteria commonly form biofilms. However, for more than a century, microbiological research was limited to growing bacteria under artificial conditions which we now know barely reflect their natural biofilm mode of growth. William J. Costerton, a pioneer of biofilm research, introduced the biofilm concept and the term "biofilms" to microbiology in the 1970s (Costerton et al. 1978). Initially focused on in vitro research and "environmental" bacteria, biofilm research over time increasingly included the investigation of "medical" biofilms formed by bacterial pathogens during infection.

Medical biofilm research comes with significant challenges that biofilm researchers are still struggling to cope with. This is due to the fact that in vitro biofilm models, despite the fact that they revealed many molecular determinants and principles of biofilm formation, barely reflect the situation that the bacteria encounter in the human host. The more recent focus on establishing animal models of biofilm infection and the capacity to directly investigate infectious isolates by modern genetic methods has taken biofilm research to a new level. Notably, concepts developed based on in vitro biofilm research often were not confirmed on the in vivo level, demonstrating the necessity to complement in vitro biofilm research by appropriate methods to ascertain their in vivo relevance (Joo and Otto 2012).

## 2 Biofilm Infections

Among the many types of infection in which biofilms are involved, a few have gained particular attention from researchers, owing to their frequency, severity, or potential model character for other biofilm-associated infections. Infections on indwelling medical devices, such as catheters or joint prostheses, are virtually always biofilm related. Owing to the high number of surgical interventions being performed nowadays, they are very common. By far the most important pathogens causing infections of indwelling medical devices are *Staphylococcus aureus* and coagulase-negative staphylococci, such as *Staphylococcus epidermidis* (Otto 2008). As these bacteria are commensals on the human skin and mucosal surfaces,

device-related infections commonly are caused by contamination of the devices during insertion, with the infectious isolates originating either from healthcare personnel or the patient. Of note, infected devices can be a source for life-threatening secondary infections, such as septicemia.

Biofilms on contact lenses are a common cause of keratitis (Elder et al. 1995). Similar to device-associated infections, they develop by contamination with commensal bacteria, often involving coagulase-negative staphylococci, corynebacteria, bacilli, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, or *Serratia marcescens*. The fungi *Candida albicans* and *Fusarium* ssp. also are frequent causes of biofilms on contact lenses.

Probably the most widespread biofilm infection is dental plaque, the source of several dental infections such as caries or periodontitis (Pihlstrom et al. 2005). In contrast to infections of indwelling medical devices, which are normally due to one single infectious isolate, dental plaque is a multi-species bacterial biofilm community (Hojo et al. 2009). Group B streptococci and lactobacilli are especially frequent among dental plaque-causing bacteria. We are only beginning to understand the many interactions between the members of the dental plaque biofilm community.

Urinary tract infections often involve biofilms. Most frequently the infecting bacterium is *Escherichia coli* (Marcus et al. 2008). Middle-ear infection (*Otitis media*) also is a common biofilm-associated disease, especially in children (Bakaletz 2007). The infecting bacteria include predominantly *S. pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. Moreover, biofilms may contribute to streptococcal pharyngitis ("Strep throat") (Murphy et al. 2009) and chronic wound infections (Percival et al. 2012). The latter often contain polymicrobial biofilms with skin-related and other bacteria, including anaerobes.

Unless complications occur, the biofilm-associated infections discussed so far are not life-threatening. However, there are also examples of extremely severe and frequently fatal diseases that involve biofilms. Infective endocarditis has a particularly high fatality rate and involves bacterial biofilms forming on the valves of the heart (Que and Moreillon 2011). *S. aureus*, Viridans group streptococci, and coagulase-negative staphylococci are the most common causes.

Cystic fibrosis (CF, mucoviscidosis) is an autosomal recessive genetic disorder, caused by a mutation in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR), which is involved in regulating sodium and chloride transfer across membranes (Riordan et al. 1989). Patients suffering from CF are particularly prone to chronic bacterial infection (Cohen and Prince 2012). *S. aureus* and *H. influenzae* dominate at early age, while *P. aeruginosa* is isolated in 80 % of cases from patients older than 18 years (Rajan and Saiman 2002). *P. aeruginosa* bacteria infecting the lungs of CF patients very likely grow in biofilms (Singh et al. 2000). Accordingly, *P. aeruginosa* CF isolates often show a characteristic "mucoid" phenotype associated with biofilm formation (May et al. 1991).

Owing to its involvement in CF infection as an especially severe form of biofilm-associated infection, in addition to the fact that molecular tools are more readily available for this bacterium compared to many other biofilm pathogens, biofilm formation in *P. aeruginosa* has been, and still is, the most intensely investigated

biofilm-forming bacterium. Much of what we know about biofilms and biofilm development stems from investigation using *P. aeruginosa*. However, we have also increasingly become aware of the fact that many mechanisms of biofilm formation discovered in *P. aeruginosa* have less of a model character than previously assumed, as the molecular mechanisms of biofilm formation may significantly differ between different biofilm-forming pathogens. Of note, an important problem associated with the model character of *P. aeruginosa* for biofilm infection is the fact that *P. aeruginosa* CF infections are difficult to mimic in animal infection models (Hoffmann 2007).

## 3 Biofilm Resistance

It has often been stressed that biofilms provide resistance to mechanisms of host defense, in particular, leukocyte phagocytosis. However, there have been conflicting results as to whether biofilm cells are inherently resistant to phagocytosis (Gunther et al. 2009). As investigation performed in staphylococcal biofilms suggests, protection is likely due mainly to the production of the extracellular biofilm matrix, which may inhibit the engulfment of biofilm cell clusters by phagocytes (Guenther et al. 2009; Vuong et al. 2004a). Furthermore, the matrix, which consists of polymers with low immunogenicity, shields biofilm cells from recognition of bacterial cell surface-exposed epitopes by the immune system (Thurlow et al. 2011).

Many antibiotics have significantly lower efficacy against biofilm as compared to planktonic (i.e., free-floating) cells (Stewart and Costerton 2001). The difference can reach factors of around 1,000 (Davies 2003). Biofilm resistance (or strictly speaking, tolerance, as opposed to specific mechanisms of resistance) is due to different reasons. The extracellular biofilm matrix provides a mechanical shield, preventing at least some antibiotics from reaching their target, often the bacterial peptidoglycan, the cytoplasmic membrane, or intracellular targets such as protein or DNA biosynthesis molecules. Furthermore, biofilm tolerance is due to the physiological status of biofilm cells, which is characterized by low activity of cell processes such as cell wall, protein, or DNA biosynthesis. Thus, the many antibiotics that target those processes are barely active against cells in biofilms (Davies 2003).

## 4 General Model of Biofilm Formation

Research initially performed in *P. aeruginosa*, but in the meantime also in many other bacteria, revealed a general model of how biofilms develop (O'Toole et al. 2000). For bacterial pathogens, the first step is attachment to tissue surfaces. Rarely, attachment may proceed directly on abiotic surfaces, such as on catheters,

but because human matrix proteins soon cover any foreign device in the human body, this form of attachment likely only plays a minor role even in device-associated biofilm infections. In the case of motile bacteria, such as *P. aeruginosa*, attachment may be preceded by active motion toward the surface, whereas nonmotile bacteria have to rely on passive modes of motion in that first step of biofilm development.

After attachment is accomplished, the bacteria proliferate and surround themselves with the characteristic biofilm matrix. This matrix is composed of many different molecules. Some are specific to the given bacterium, such as the exopolysaccharides and secreted proteins produced by many biofilm bacteria. Others may be produced by a large subset of bacteria, such as teichoic acids found in Gram-positive bacteria. As biofilms are in a stationary mode of growth, the biofilm matrix also comprises molecules that are released from dying cells. In particular, extracellular DNA (eDNA) was found to contribute to the biofilm matrix in many bacteria (Whitchurch et al. 2002). Electrostatic interactions between oppositely charged matrix polymers are believed to play a key role in matrix formation. It needs to be stressed that for some of these molecules, evidence for a participation in the biofilm matrix is only derived from in vitro investigation, such as in the case of eDNA. The environment in the human host contains factors, such as nucleases and proteases, which have the potential to interfere strongly with the composition of the biofilm matrix. Especially eDNA may be degraded by the efficient human serum DNaseI (Whitchurch et al. 2002). It may be because the human host cannot degrade them that biofilm bacteria produce specific biofilm exopolysaccharides, several of which have a proven function in in vivo biofilm formation (Rupp et al. 1999; Conway et al. 2004; Hoffmann et al. 2005).

Were it only for the biofilm matrix components, biofilms would be unstructured "clumps" of cells, and expansion of a biofilm would hardly be possible without leaving cells in deeper layers prone to death due to limited nutrient availability. However, we know from microscopic analysis that biofilms have a characteristic three-dimensional structure with cellular agglomerations in "mushroom" shape and channels that provide nutrients to those deeper layers. The molecular factors that facilitate channel formation have recently gained much attention. Several biofilm-forming bacteria were found to produce surfactant molecules to structure biofilms in that fashion (Otto 2013). Notably, the same forces that underlie channel formation are responsible for the detachment of cell clusters from a biofilm, a mechanism that leads to dissemination of the pathogenic bacteria to the bloodstream, and thus may cause second-site infections.

Biofilm formation is under the control of a series of regulatory systems, which often differ considerably between different biofilm-forming bacteria. However, there are also generally applicable concepts in biofilm regulation. In several bacteria, such as *E. coli*, sensory and regulatory systems trigger biofilm development upon contact with a surface (Otto and Silhavy 2002). Furthermore, the general switch from the planktonic to the biofilm mode of growth is often under control of the second messenger cyclic di-GMP (Romling et al. 2013). Finally, cell

density-dependent regulation ("quorum sensing," QS) controls biofilm differentiation in many microorganisms (Irie and Parsek 2008).

## 5 Biofilm Pathogens

While the general model of biofilm formation gives a good overall outline that is applicable to many biofilm-forming bacteria, most biofilm microorganisms produce highly specific biofilm factors. Some of those that were thoroughly investigated shall briefly be introduced in the following.

Biofilm formation in *P. aeruginosa* is best understood, at least in vitro. This species produces three main biofilm exopolysaccharides, the negatively charged alginate, the mannose-rich neutral "Psl," and the glucose-rich "Pel" exopolysaccharides (Ryder et al. 2007). Production of alginate in particular is associated with the "mucoid" phenotype of *P. aeruginosa* strains isolated from cystic fibrosis infection (May et al. 1991). The impact of QS on biofilms was first described in *P. aeruginosa*, where as in many other bacterial pathogens, it has a strong impact on the production of biofilm factors and biofilm development in general (Davies et al. 1998). QS regulation in *P. aeruginosa* involves at least three systems (Rhl, Las, and Qsc) forming a QS network (Jimenez et al. 2012). Early experiments performed in *P. aeruginosa* indicated that QS is a positive regulator of biofilm expansion (Davies et al. 1998), but we know now that the impact of QS on biofilm development is more complicated, affecting a series of factors involved in biofilm growth and structuring (Joo and Otto 2012). Rhamnolipids, for example, are QS-controlled surfactants that facilitate *P. aeruginosa* biofilm structuring (Boles et al. 2005). Furthermore, pili (or fimbriae) in *P. aeruginosa* provide motility and are not only important for reaching a surface, but also in QS-regulated detachment processes (Gibiansky et al. 2010), where cells regain pili-mediated motility starting in the center of biofilm "mushrooms" (Purevdorj-Gage et al. 2005).

*S. aureus* and coagulase-negative staphylococci contribute to a number of biofilm infections and dominate among pathogens causing infections of indwelling medical devices. Much of our knowledge on staphylococcal biofilm formation stems from research on the human commensal *S. epidermidis* (Otto 2009). *S. epidermidis*—as most other staphylococci—produces an exopolysacharide termed polysaccharide intercellular adhesin (PIA) or poly-*N*-acetyl glucosamine (PNAG). PIA/PNAG is a linear homopolymer of *N*-acetyl glucosamine with partial de-acetylation that introduces positive changes in the otherwise neutral molecule (Mack et al. 1996; Vuong et al. 2004b). It has a demonstrated significant function in in vitro and in vivo biofilm formation, although not all staphylococcal biofilm-forming strains (especially *S. aureus*) appear to rely on PIA/PNAG to form biofilms (Rohde et al. 2007). A large number of proteins also contribute to the formation of the staphylococcal biofilm matrix, such as the accumulation-associated protein Aap (Conrady et al. 2008). The biofilm-structuring surfactant phenol-soluble modulin (PSM) peptides of staphylococci are controlled by the accessory gene regulator

(Agr) QS system (Periasamy et al. 2012; Wang et al. 2007) and the exopolysaccharide PIA/PNAG by the LuxS QS system (Xu et al. 2006).

Group B Streptococci (GBS) such as *Streptococcus mutans* participate to a significant extent in dental plaque formation. *S. mutans* secretes glucosyl transferases and glucan binding proteins, which produce water-soluble and -insoluble glucans that facilitate biofilm formation (Banas and Vickerman 2003). Many GBS produce a polysaccharide capsule that contains moieties with similarity to host saccharides, which thus—in addition to their role in biofilm matrix formation—may provide protection from host defenses (Wyle et al. 1972). Biofilm formation in streptococci is regulated by a series of global regulators, including competence systems, which regulate the uptake of DNA (Suntharalingam and Cvitkovitch 2005). The competence/QS signal peptide CSP (competence-stimulating peptide) has a major role in controlling these phenotypes (Li et al. 2001).

In *E. coli*, a pathogen frequently involved in urinary tract infection, different forms of pili (type I fimbriae, curli fimbriae, and conjugative pili) participate in attachment and biofilm formation (Beloin et al. 2008). The Cpx system senses the surface and neighboring bacteria, affecting production of flagellae and biofilm maturation (Otto and Silhavy 2002). Interestingly, despite the fact that *E. coli* is not closely related to staphylococci, it produces the same matrix exopolysaccharide PIA/PNAG (called PGA in *E. coli*) (Wang et al. 2004), indicating that specific biofilm-related genes have been distributed far beyond species and genus barriers.

*Acinetobacter baumannii* is a biofilm-forming pathogen often involved with hospital-acquired pneumonia that has recently received much attention (Cerqueira and Peleg 2011). *A. baumannii* can form biofilms on abiotic surfaces that survive for several days, in which pili produced by the *csu* operon play a preeminent role (Tomaras et al. 2008). However, these pili are not important for attachment to mammalian cells (de Breij et al. 2009), exemplifying that in vitro results regarding biofilm factors may have limited relevance for the in vivo situation. *A. baumannii* produces two biofilm molecules that have previously been described in staphylococci: PIA/PNAG (Choi et al. 2009) and the biofilm-associated protein (Bap) (Loehfelm et al. 2008), again showing that key biofilm factors were distributed across genus barriers even between Gram-negative and Gram-positive bacteria.

*C. albicans* is the most frequent fungal human pathogen. While *C. albicans* biofilm development follows the same general model as do bacteria, the participating molecules are not related, owing to the fact that this pathogen is a eukaryotic organism (Cuellar-Cruz et al. 2012). Attachment occurs via cell wall proteins and is followed by the production of hyphae and a matrix that consists of several different polymers. Similar to bacteria, QS regulation has a strong impact on *Candida* biofilm development, with tyrosol and farnesol being the most important QS signals (Singh and Del Poeta 2011).

## 6 In Vitro and In Vivo Analysis of Biofilm Development

Analyzing biofilm formation in in vitro models ranges from simple microtiter plate assays to sophisticated flow reactors. Flow constantly provides fresh media to the biofilm cells and is often applied to mimic environmental biofilms, such as those formed in wastewater tubing. Which in vitro model best mimics "medical" biofilms as present during infection is debatable. Many observations and findings indicate that results achieved using in vitro biofilm models are difficult to transfer to the in vivo situation (Joo and Otto 2012). Nevertheless, modeling biofilm formation in vitro has the advantage that the biofilms can be analyzed using state-of-the-art microscopic techniques, such as confocal laser scanning microscopy (CLSM). By taking regular interval pictures of a biofilm forming in a flow cell, movies can be produced using CLSM that give detailed insight into biofilm development.

In addition to the genetic analysis of infectious isolates, the analysis of biofilms during infection relies primarily on animal models of biofilm-associated infection. Some biofilm infections, such as indwelling device-related infection, are easier to mimic in animal models than others, such as lung infection during cystic fibrosis or dental plaque formation. For that reason, we have a better understanding of in vivo biofilm factors in bacteria that cause device-related infections than many other biofilm-related diseases. Clearly, the development of better models of biofilm-associated infection is a premier task of current and future biofilm research.

## 7 Targeting Medical Biofilms

Biofilm formation is still a problem for drug development that has not been satisfactorily addressed. With the development of novel antibiotics almost having come to a halt (Cooper and Shlaes 2011), companies are often not focusing on biofilm-associated infections, as those are regarded as even more complicated to tackle. At least it is now common practice to monitor the efficacy of a drug in development against in vitro biofilms.

Generally, one can envision two different approaches to combat medical biofilms. First, novel antibiotics may be developed that have increased efficacy against biofilms. These should be antibiotics that penetrate the biofilm matrix and have a bactericidal rather than bacteriostatic mode of action. Second, drugs specifically inhibiting attachment, proliferation, or even biofilm structuring may target biofilm formation itself. It is also conceivable to develop drugs that promote biofilm dispersal, leaving biofilm cells more prone to attack by conventional antibiotics. However, biofilm molecules that are conserved in different biofilm pathogens are rare. This approach thus has the disadvantage of limited applicability and marketability. Some regulatory factors may be more widespread, but inhibiting regulators in antibacterial drug development requires much caution. Unfortunately, the outlook regarding the timeframe for the availability of drugs that are active against

biofilms is rather bleak, necessitating more extensive efforts both in general biofilm research and in the development of biofilm-active antibiotics.

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## References

Bakaletz LO (2007) Bacterial biofilms in otitis media: evidence and relevance. Pediatr Infect Dis J 26(10 Suppl):S17–S19

Banas JA, Vickerman MM (2003) Glucan-binding proteins of the oral streptococci. Crit Rev Oral Biol Med 14(2):89–99

Beloin C, Roux A, Ghigo JM (2008) *Escherichia coli* biofilms. Curr Top Microbiol Immunol 322:249–289

Boles BR, Thoendel M, Singh PK (2005) Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. Mol Microbiol 57(5):1210–1223

Cerqueira GM, Peleg AY (2011) Insights into *Acinetobacter baumannii* pathogenicity. IUBMB Life 63(12):1055–1060

Choi AH, Slamti L, Avci FY, Pier GB, Maira-Litran T (2009) The *pgaABCD* locus of *Acinetobacter baumannii* encodes the production of poly-beta-1-6-N-acetylglucosamine, which is critical for biofilm formation. J Bacteriol 191(19):5953–5963

Cohen TS, Prince A (2012) Cystic fibrosis: a mucosal immunodeficiency syndrome. Nat Med 18 (4):509–519

Conrady DG, Brescia CC, Horii K, Weiss AA, Hassett DJ, Herr AB (2008) A zinc-dependent adhesion module is responsible for intercellular adhesion in staphylococcal biofilms. Proc Natl Acad Sci USA 105(49):19456–19461

Conway BA, Chu KK, Bylund J, Altman E, Speert DP (2004) Production of exopolysaccharide by *Burkholderia cenocepacia* results in altered cell-surface interactions and altered bacterial clearance in mice. J Infect Dis 190(5):957–966

Cooper MA, Shlaes D (2011) Fix the antibiotics pipeline. Nature 472(7341):32

Costerton JW, Geesey GG, Cheng KJ (1978) How bacteria stick. Sci Am 238(1):86–95

Cuellar-Cruz M, Lopez-Romero E, Villagomez-Castro JC, Ruiz-Baca E (2012) Candida species: new insights into biofilm formation. Future Microbiol 7(6):755–771

Davies D (2003) Understanding biofilm resistance to antibacterial agents. Nat Rev Drug Discov 2 (2):114–122

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280 (5361):295–298

de Breij A, Gaddy J, van der Meer J, Koning R, Koster A, van den Broek P, Actis L, Nibbering P, Dijkshoorn L (2009) CsuA/BABCDE-dependent pili are not involved in the adherence of *Acinetobacter baumannii* ATCC19606(T) to human airway epithelial cells and their inflammatory response. Res Microbiol 160(3):213–218

Elder MJ, Stapleton F, Evans E, Dart JK (1995) Biofilm-related infections in ophthalmology. Eye (Lond) 9(Pt 1):102–109

Gibiansky ML, Conrad JC, Jin F, Gordon VD, Motto DA, Mathewson MA, Stopka WG, Zelasko DC, Shrout JD, Wong GC (2010) Bacteria use type IV pili to walk upright and detach from surfaces. Science 330(6001):197

Guenther F, Stroh P, Wagner C, Obst U, Hansch GM (2009) Phagocytosis of staphylococci biofilms by polymorphonuclear neutrophils: *S. aureus* and *S. epidermidis* differ with regard to their susceptibility towards the host defense. Int J Artif Organs 32(9):565–573

Gunther F, Wabnitz GH, Stroh P, Prior B, Obst U, Samstag Y, Wagner C, Hansch GM (2009) Host defence against *Staphylococcus aureus* biofilms infection: phagocytosis of biofilms by polymorphonuclear neutrophils (PMN). Mol Immunol 46(8–9):1805–1813

Hoffmann N (2007) Animal models of chronic *Pseudomonas aeruginosa* lung infection in cystic fibrosis. Drug Discov Today 4(3):99–104

Hoffmann N, Rasmussen TB, Jensen PO, Stub C, Hentzer M, Molin S, Ciofu O, Givskov M, Johansen HK, Hoiby N (2005) Novel mouse model of chronic *Pseudomonas aeruginosa* lung infection mimicking cystic fibrosis. Infect Immun 73(4):2504–2514

Hojo K, Nagaoka S, Ohshima T, Maeda N (2009) Bacterial interactions in dental biofilm development. J Dent Res 88(11):982–990

Irie Y, Parsek MR (2008) Quorum sensing and microbial biofilms. Curr Top Microbiol Immunol 322:67–84

Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ (2012) The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. Microbiol Mol Biol Rev 76(1):46–65

Joo HS, Otto M (2012) Molecular basis of in vivo biofilm formation by bacterial pathogens. Chem Biol 19(12):1503–1513

Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG (2001) Natural genetic transformation of *Streptococcus mutans* growing in biofilms. J Bacteriol 183(3):897–908

Loehfelm TW, Luke NR, Campagnari AA (2008) Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. J Bacteriol 190(3):1036–1044

Mack D, Fischer W, Krokotsch A, Leopold K, Hartmann R, Egge H, Laufs R (1996) The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. J Bacteriol 178 (1):175–183

Marcus RJ, Post JC, Stoodley P, Hall-Stoodley L, McGill RL, Sureshkumar KK, Gahlot V (2008) Biofilms in nephrology. Expert Opin Biol Ther 8(8):1159–1166

May TB, Shinabarger D, Maharaj R, Kato J, Chu L, DeVault JD, Roychoudhury S, Zielinski NA, Berry A, Rothmel RK et al (1991) Alginate synthesis by *Pseudomonas aeruginosa*: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. Clin Microbiol Rev 4(2):191–206

Murphy TF, Bakaletz LO, Smeesters PR (2009) Microbial interactions in the respiratory tract. Pediatr Infect Dis J 28(10 Suppl):S121–S126

O'Toole G, Kaplan HB, Kolter R (2000) Biofilm formation as microbial development. Annu Rev Microbiol 54:49–79

Otto M (2008) Staphylococcal biofilms. Curr Top Microbiol Immunol 322:207–228

Otto M (2009) *Staphylococcus epidermidis* – the 'accidental' pathogen. Nat Rev Microbiol 7:555–567

Otto M (2013) Staphylococcal infections: mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity. Annu Rev Med 64:175–188

Otto K, Silhavy TJ (2002) Surface sensing and adhesion of *Escherichia coli* controlled by the Cpx-signaling pathway. Proc Natl Acad Sci USA 99(4):2287–2292

Percival SL, Hill KE, Williams DW, Hooper SJ, Thomas DW, Costerton JW (2012) A review of the scientific evidence for biofilms in wounds. Wound Repair Regen 20(5):647–657

Periasamy S, Joo HS, Duong AC, Bach TH, Tan VY, Chatterjee SS, Cheung GY, Otto M (2012) How *Staphylococcus aureus* biofilms develop their characteristic structure. Proc Natl Acad Sci USA 109(4):1281–1286

Pihlstrom BL, Michalowicz BS, Johnson NW (2005) Periodontal diseases. Lancet 366 (9499):1809–1820

Purevdorj-Gage B, Costerton WJ, Stoodley P (2005) Phenotypic differentiation and seeding dispersal in non-mucoid and mucoid *Pseudomonas aeruginosa* biofilms. Microbiology 151 (Pt 5):1569–1576

Que YA, Moreillon P (2011) Infective endocarditis. Nat Rev Cardiol 8(6):322–336

Rajan S, Saiman L (2002) Pulmonary infections in patients with cystic fibrosis. Semin Respir Infect 17(1):47–56

Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL et al (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245(4922):1066–1073

Rohde H, Burandt EC, Siemssen N, Frommelt L, Burdelski C, Wurster S, Scherpe S, Davies AP, Harris LG, Horstkotte MA, Knobloch JK, Ragunath C, Kaplan JB, Mack D (2007) Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. Biomaterials 28(9):1711–1720

Romling U, Galperin MY, Gomelsky M (2013) Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. Microbiol Mol Biol Rev 77(1):1–52

Rupp ME, Ulphani JS, Fey PD, Bartscht K, Mack D (1999) Characterization of the importance of polysaccharide intercellular adhesin/hemagglutinin of *Staphylococcus epidermidis* in the pathogenesis of biomaterial-based infection in a mouse foreign body infection model. Infect Immun 67(5):2627–2632

Ryder C, Byrd M, Wozniak DJ (2007) Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. Curr Opin Microbiol 10(6):644–648

Singh A, Del Poeta M (2011) Lipid signalling in pathogenic fungi. Cell Microbiol 13(2):177–185

Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407(6805):762–764

Stewart PS, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. Lancet 358 (9276):135–138

Suntharalingam P, Cvitkovitch DG (2005) Quorum sensing in streptococcal biofilm formation. Trends Microbiol 13(1):3–6

Thurlow LR, Hanke ML, Fritz T, Angle A, Aldrich A, Williams SH, Engebretsen IL, Bayles KW, Horswill AR, Kielian T (2011) *Staphylococcus aureus* biofilms prevent macrophage phagocytosis and attenuate inflammation in vivo. J Immunol 186(11):6585–6596

Tomaras AP, Flagler MJ, Dorsey CW, Gaddy JA, Actis LA (2008) Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. Microbiology 154(Pt 11):3398–3409

Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR, Otto M (2004a) Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cell Microbiol 6(3):269–275

Vuong C, Kocianova S, Voyich JM, Yao Y, Fischer ER, DeLeo FR, Otto M (2004b) A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. J Biol Chem 279(52):54881–54886

Wang X, Preston JF 3rd, Romeo T (2004) The *pgaABCD* locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. J Bacteriol 186(9):2724–2734

Wang R, Braughton KR, Kretschmer D, Bach TH, Queck SY, Li M, Kennedy AD, Dorward DW, Klebanoff SJ, Peschel A, DeLeo FR, Otto M (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat Med 13(12):1510–1514

Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS (2002) Extracellular DNA required for bacterial biofilm formation. Science 295(5559):1487

Wyle FA, Artenstein MS, Brandt BL, Tramont EC, Kasper DL, Altieri PL, Berman SL, Lowenthal JP (1972) Immunologic response of man to group B meningococcal polysaccharide vaccines. J Infect Dis 126(5):514–521

Xu L, Li H, Vuong C, Vadyvaloo V, Wang J, Yao Y, Otto M, Gao Q (2006) Role of the *luxS* quorum-sensing system in biofilm formation and virulence of *Staphylococcus epidermidis*. Infect Immun 74(1):488–496

# The Use of DNA Methods to Characterize Biofilm Infection

**Randall Wolcott and Stephen B. Cox**

**Abstract** Because of biofilm's fundamental properties—its polymicrobial nature (genetic diversity) and "viable but not culturable" microbial constituents—clinical cultures are wholly unsuited for evaluating chronic infections associated with biofilm. DNA-based technologies (molecular methods) have a number of advantages for evaluating human infections. Real-time PCR and sequencing technologies are particularly robust for identifying microorganisms in human environments because of development of their methods by the human microbiome project. DNA methods enjoy much higher sensitivity and specificity than cultivation methods for identifying microorganisms regardless of their phenotype. Moreover, real-time PCR can be quantitative in an absolute sense, while sequencing methods yield accurate relative quantification of all constituents of the sampled infection. All methods for microbial identification have biases, yet molecular methods suffer the least from these biases. Although DNA-based identification of microorganisms has the limitation that sensitivities to antibiotics cannot be determined in a Petri dish and must be determined by identifying mobile genetic resistance elements within the microbes, molecular methods are a significant improvement in the identification of microorganisms for human infections and are currently the only reliable technology for diagnosing biofilm infection.

## 1 DNA-Based Testing

*In the land of the blind, the one-eyed man is king—Erasmus.*

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The human microbiome project (HMP) has forever changed how microorganisms will be identified (Chain et al. 2009). The HMP was established to identify and to quantitate bacteria living in normal human environments such as the gut, oral cavity, skin, urogenital, etc. Several challenges for the project were that the microbes in these host environments are polymicrobial, they are not quantifiable by cultivation methods, and they generally exist in a biofilm phenotype. In fact, the vast majority of the species known to inhabit normal host environments are not routinely culturable (Petrosino et al. 2009), which is characteristic of the biofilm phenotype (Fux et al. 2005). These facts led investigators to employ molecular methods.

Molecular methods are based on the idea of direct examination of the bacterial DNA existing in the sample to allow for identification of the bacteria that are present. There has been a very rapid and fluid progression of molecular technologies that can analyze microbial DNA. However, to get any of these molecular technologies to give a meaningful analysis, high-quality DNA first must be obtained. Therefore, one of the most important obstacles to using molecular methods for identifying and quantitating microorganisms in human infections is obtaining good microbial DNA from the sample (i.e., the process of DNA extraction). There are a number of excellent kits and laboratory methods for obtaining microbial DNA from mixed samples (samples that contain both microbial and human DNA). However, each method has different extraction efficiencies, and these efficiencies may vary for the different species within the sample. Yet even with these challenges, many extraction methods can approach 96 % efficiency (Fitzpatrick et al. 2010).

The process of DNA extraction, especially from samples that contain some of the host products, also can extract substances that inhibit later analysis of the microbial DNA. For example, polymerase chain reaction (PCR) is a common method used to amplify microbial DNA, yet the process can be inhibited by substances found in the sample. These PCR inhibitors include complex polysaccharides, bile salts, hemoglobin degradation products, polyphenolic compounds, heavy metals, and, most frequently, large amounts of human DNA (Stauffer et al. 2008). Many of the more common PCR inhibitors can be effectively mitigated, but if the inhibitors cannot be identified and controlled, resampling may be necessary. Once good DNA is obtained from the sample, most current molecular instrumentation can obtain reliable clinical results.

PCR is a widely used method of processing DNA that has a relatively long history of use in the clinic (Krishna and Cunnion 2012; Reddington et al. 2013). PCR utilizes primers that attach to complementary regions of bases in the microbial DNA and, through a polymerase reaction, create copies of this area. This copying process doubles the amount of target sequence with every cycle of the PCR. Real-time PCR has the ability to quantitate, in an absolute sense, how much microbial DNA is in the original sample. The number of cycles required before the real-time signal reaches a detection threshold (cycle threshold number or ct number) can be correlated to an absolute number of microbes present in the original sample. This is an extremely powerful feature of real-time PCR that can used to quantify “bacterial

load" (Verhoeven et al. 2012). However, PCR has several important limitations. Most limiting is the fact that real-time PCR requires a primer sequence to be developed for each species of microorganism present in the sample. With the literally thousands of different microorganisms that can be in human chronic infections, constructing thousands of primers for each analysis is inefficient, costly, and currently not feasible.

There also are a number of different parameters involved in the process of performing PCR, such as chemistries (e.g., Syber Green, TaqMan), platforms (e.g., Roche v. Abbott), factors in plate preparation, etc., that can impact results. Incomplete optimization of these parameters can lead to amplification inefficiencies, inconsistent reproducibility, random PCR products, and other problems. The optimization of chemistries, primers, and instrument variables is focused on improving sensitivity of the primer to the target microbe without sacrificing specificity for the organism (prevention of cross-reactivity with other species). Optimization must also take into account dynamic range so that minor species are detected and quantitated as accurately as the dominant species in the sample.

Diagnostic laboratories painstakingly optimize all of the PCR variables by choosing appropriate instruments, chemistries, and primers to mitigate the potential negative impacts of these variables. However, there are still limits to quantitative PCR methods. For example, even though the reported results will be extremely specific for the microbial species present, due to DNA extraction efficiencies for different species, different amplification efficiencies for different species, and other variables, quantification of the microbes in the sample remains mildly inconsistent. Calculating bacterial load by real-time PCR often yields up to an order of magnitude variation for known quantities (usually lower), yet this seems to be an acceptable level of variability for clinical decision-making.

Although real-time PCR can rapidly yield usable information on bacterial load and identify a limited number of microbial species, it is impractical for PCR to be used alone for the identification and quantification of microbes in most human infections. Investigators in the HMP encountered the same limitations and quickly turned to sequencing (Aagaard et al. 2012). One of the technologies used early on in the HMP was a whole metagenome survey of the microbes present. This methodology looks at all genes present in a sample, which is an excellent way to determine species of fungi, bacteria, and even viruses present in an infection. The problem with determining all the genes present was that it required a massive number of sequencing base pairs (bp-ATCG) for a sample, which only allowed a small number of samples to be evaluated per sequencing run. These surveys also lost some of their quantitative ability (Fodor et al. 2012).

An alternative methodology was developed in which a very specific gene, the ribosomal 16S rDNA gene for bacteria or 18S rDNA gene for fungi, can be amplified through a PCR step and then sequenced. The use of the 16S rDNA gene as an indicator of bacterial taxonomic relationships traces back to the pioneering work of Woese and Fox (1977). This method provides two important pieces of information. Once the 16S or 18S region has been sequenced, it can be compared to a database of known sequences, thus yielding the genus and species

with a high level of confidence. In addition, this method can allow for relative quantification of the microbes present within each sample. The number of "copies" of the gene for each species in the sample can be totaled, allowing for each species to be expressed as a percent of that total number. Although it does not provide for absolute quantification, this method does allow investigators to determine the dominant, major, and minor species within a sample (Rhoads et al. 2012a). Because this approach focused on sequencing only a single gene from each microbe, it allowed for several hundred samples to be analyzed on the same plate in a single run, greatly reducing the cost and increasing the speed of analysis. It was mainly through the development of sequencing technologies and methods that allowed investigators to elucidate fully the microorganisms present in the human microbiome (Morgan et al. 2013).

Sequencing is the molecular method for determining the exact order of nucleotides (i.e., adenine, thymine, cytosine, guanine) of a specific fragment of DNA or an entire genome. Sequencing instruments, such as the Roche 454, the PacBio (Pacific Biosciences), and Ion Torrent (Life Technologies), use different methods, but they all accurately determine the sequences of long segments of specific regions of microbial DNA, such as the 16S rDNA gene for bacteria and the 18S rDNA gene for fungi. These technologies can give a 99 % accurate code for the targeted gene, which is easily translated into taxonomic identification.

The microbial gene that codes for the 16S ribosomal subunit is conserved in all prokaryotic organisms except for a small subgroup of Archaea. The 16S ribosomal DNA has about 1,500 nucleotides, which contain nine hypervariable regions (v1–v9), and allows for the ability to identify bacteria at the species level. Fortunately, v1 can differentiate *Staphylococcus* to a genus level, and if the first three regions (v1–v3) can be sequenced, then the majority of other bacteria can be resolved to a genus level with a high degree of certainty. The 16S ribosomal DNA has been called the genomic fingerprint, and a 400+ nucleotide sequence of the 16S ribosomal DNA region is capable of reliably reading this genomic fingerprint.

Often, sequencing is carried out at multiple points along the 16S gene. It has been demonstrated that sequencing two fragments of the 16S gene consisting of 762 based pairs and 598 base pairs is more accurate in identifying bacteria than a single fragment of 1,343 base pairs (Jenkins et al. 2012). Therefore, sequencing methods often use primer sets consisting of two or more primers that cover different regions of the 16S gene. These primer sets can have some bias in how efficiently they sequence specific bacterial species.

Once sequencing has been completed, a data analysis pipeline is needed to begin processing the data. The data analysis process consists of two major stages: quality checking and diversity analysis. During the quality checking stage, denoising (Quince et al. 2009, 2011) and chimera checking (Haas et al. 2011) are performed on all the reads within the data. Each read is quality scanned and deficient reads are removed from the sample. The primary output of this stage is high-quality sequences. During the diversity analysis, sequences from each sample are run through an algorithm (typically involving a match to a database of known

sequences) to determine the taxonomic information for each sequence. Reference databases exist for sequences from the 16S, 18S, 23S, ITS, and/or SSU regions.

Bioinformatics, the post-analysis processing of the massive data, therefore becomes the overseer of the quality of the reported results to the clinician. It is very difficult for clinicians to abandon the visible, tangible, and familiar microorganism growing in a Petri dish for the very complex "black box" type of results produced by bioinformatics. However, current laboratory regulations requiring strict validation and reproducibility coupled with proficiency testing of unknown samples can allow the clinician to feel very comfortable with these new molecular methods. Also, a closer examination of clinical cultures demonstrates that clinicians may have placed their faith in an insufficient method all along.

## 2 Clinical Cultures: The Land of the Blind

Medical microbiology has clung to cultivation methods even while environmental microbiology migrated to DNA methods for microbial identification decades ago. This failure to take advantage of new technologies to improve microbial identification has left clinicians "blind" to the microbial reality of most infections. Many deficiencies in traditional cultivation methods make routine clinical cultures unacceptable for medical microbiology.

Only a handful of media, such as tryptic soy agar, blood agar, nutrient agar, brain–heart infusion agar, and a few others are used to plate routine samples and they are grown at only one temperature (usually 37 °C) for 24–48 h. These experimental conditions have been worked out to be adequate for *Staphylococcus* species, *Streptococcus* species, *Pseudomonas aeruginosa*, and several other bacteria that can grow under these limitations. However, the vast majority of bacterial and fungal species do not grow under these laboratory conditions. Therefore, hundreds to even thousands of specialty media have been developed along with various algorithms for microbes that require different atmospheres, nutrients, length of time, temperature, etc., to be grown. No other single fact could be more convincing for making the argument that routine clinical cultures are inadequate for diagnosing human infection.

Also, bacteria in the biofilm phenotype are notoriously difficult to grow in routine clinical cultures because they are "viable but not culturable." Biofilm infections also tend to be polymicrobial. Early investigators at the time of Koch found, "No matter how ingenious the machinery, how careful the researchers, they kept ending up with beakers of mixed bacteria. The inability to get anything but mixed cultures led many scientists to believe that the bacteria had to be in mixed groups in order to thrive, that they could never be separated. . ." (Hager 2006). To solve this problem, Koch developed the methodology of pure culture very similar to that of our current clinical culture.

Koch found on the semiliquid surface of agar infused with necessary nutrients that only one species of bacteria in his clinical sample would propagate and the rest

of the bacteria, "the contaminants," would not grow or would be outcompeted. What we now know is that the experimental design of the nutrient-enriched agar plate encourages planktonic phenotype propagation of the bacterial species in an exponential growth phase pattern. We also know that the experimental design has significant bias for the bacterial species that propagate well under the experimental conditions of temperature, nutrient, time, etc. This creates a huge selection bias to grow the microorganisms which the medical microbiologists have decided in advance are the pathogens. With molecular methods, we have discovered even more shortcomings of clinical cultures.

Many clinicians continue to hold Koch's view of one microorganism producing one clinical infection. While this generally may be true for acute infections that are commonly produced by bacteria in the planktonic phenotype, it does not hold true for biofilm infection. Chronic infections are associated with biofilm phenotype bacteria (Del Pozo and Patel 2007) and are often polymicrobial, which confounds the methods of clinical cultures. When molecular methods are compared with clinical culture to identify the microbes, we start to understand why clinical cultures provide little help in managing most chronic infections.

In pleural effusion samples, which tend to be culture negative even when the patient shows clear signs of infection, the use of universal 16S PCR, "bacterial load," demonstrated bacteria in 82 % of the clinically infected samples, whereas clinical cultures grew bacteria only 55 % of the time. Utilizing a single molecular test improved bacterial identification by 27 %. It should also be noted that this individual PCR test had only 0.9 % false positives whereas clinical cultures had a 2.6 % false positive rate (Insa et al. 2012).

Also, it has been found to be more advantageous to first identify the microorganisms utilizing molecular methods and then select media and growth conditions to cultivate the microorganisms present. Up to 20 different growth conditions were necessary to cultivate microorganisms in a single cystic fibrosis study (Sibley et al. 2011). This demonstrates that the "one size fits all" routine clinical culture is inadequate to handle the diversity of chronic infections.

A retrospective study that evaluated 168 chronic wounds with both clinical culture and molecular diagnostics (PCR and pyrosequencing) revealed the comprehensiveness of molecular methods (Rhoads et al. 2012a). Evaluating chronic wounds at a genus level for bacterial taxa only, cultures identified 17 different bacterial genera, whereas the DNA methods identified 338 bacterial taxa. Cultures underreported the diversity of the wound microbiota, but even more importantly, they failed to identify the most abundant bacteria in the wound over half the time (Rhoads et al. 2012b). Cultures obtained from polymicrobial biofilm infections fail to identify the diversity by a factor of 20-fold and fail to identify the cornerstone genus over half the time.

To improve on the design of the previous study, a prospective study was conducted in which 51 consecutive chronic wounds had a single sample taken from their surface (Rhoads et al. 2012b). The sample was homogenized and a portion was sent for clinical culture, a portion sent for PCR and pyrosequencing, and the remaining saved for further analysis if necessary. Once the clinical culture

was complete and all the sub-plates identified by phenotypic methods (biochemistries) the sub-plates were submitted for sequencing. The results showed that 5 wounds (10 %) were culture negative and 9 of the 46 remaining wounds (19 %) had discrepant results between the bacterial isolate identified by culture versus sequencing. For example, culturing methods identified *P. aeruginosa*, whereas sequencing evaluating the same sub-plate identified *Salmonella enterica*. Once again, culture failed to demonstrate the most abundant species over 50 % of the time (Rhoads et al. 2012a). It may be that one main reason clinicians struggle to manage chronic infections is because traditional culturing methods consistently report minor constituents of the infections rather than the dominant culprits.

Over 68 % of patients receive at least one course of antibiotics for the management of their chronic wounds (Howell-Jones et al. 2005). Unfortunately, multiple studies have demonstrated that treating wounds based on culture results does not improve the outcomes of the healing of the wound (Lipsky et al. 2004, 2011; Siami et al. 2001). This information has led some investigators to conclude that even though pathogens such as *P. aeruginosa* may be present in the wound, the pathogen is not doing any harm. That conclusion is made because when chronic infections are treated with anti-pseudomonal antibiotics specifically for *P. aeruginosa* identified by culture, there is no improvement in wound healing outcomes (Joseph 2013). The confusing results from clinical culture, which leads clinicians and scientists alike to conclude that pathogens may not behave pathogenically or that bacteria don't matter in certain chronic infections (O'Meara et al. 2010), may be due to the inadequacies of the cultivation methods.

Although routine clinical cultures are inadequate for evaluating chronic infections, we must first determine if the proposed replacement (i.e., molecular methods) is any better. That is, will adopting molecular methods improve clinical outcomes for chronic infections produced by biofilm phenotype microorganisms? After all, by growing bacteria, medical microbiologists can apply antibiotic discs and determine the "real-world" sensitivity of the isolated bacteria. Also, even though it has been demonstrated that DNA degrades quite quickly (2–3 days) once the bacteria dies within the host infection (Post et al. 1996), there is no clear determination that the microbial DNA identified by molecular methods is associated with a living bacterial cell. However, in a chronic wound infection model, when wound biofilm was comprehensibly diagnosed utilizing molecular methods and the microorganisms identified specifically treated, healing outcomes did improve (Dowd et al. 2011). Regardless, the primary tenant of medicine is for the clinician to fully diagnose the disease, and as demonstrated above, clinical cultures are mostly blind to the microbial reality of polymicrobial biofilm infection.

## 3 Advantages of DNA Diagnosis: The One Eye

An Oslerian (Sir William Osler) model of medicine mandates that the clinician diagnose a malady as fully as possible to formulate the most appropriate treatment available. Evidence-based medicine often requires not only diagnosis before the treatment regimen but also frequent intervals of reevaluation during the treatment to show efficacy. So, no matter the generation of the clinician or which model of medicine to which the clinician ascribes, diagnosis of the condition is fundamental. Diagnosis prior to treatment is especially important in the management of chronic infections.

However, most clinicians treating chronic infections have abandoned the fundamental principle of initial diagnosis. The problem seems to lie not in the clinicians but in the diagnostic tools available. Many different culturing methods have been tried, yet they do not improve outcomes in the treatment of chronic infections. The inadequacy of cultivation methods has led to a de facto management of chronic infections by an educated guess, trial and error method.

The transition toward adopting molecular methods for medical microbiology need not be difficult. For virology there are no other reliable methods other than nucleic acid-based analysis. Almost a decade ago it was established that not only was DNA-based testing more accurate and reliable than clinical culture, but it also had the advantage of reduced time to diagnosis and high throughput (Mothershed and Whitney 2006). New methods have also been developed to identify various different antibiotic resistance determinants while at the same time providing genetic surveillance for new and existing pathogens (Weile and Knabbe 2009). Indeed from 2001 to 2007, 215 novel bacterial species were identified in human infections by sequencing methods with 100 of these new species identified in four or more individual patients (Woo et al. 2008). Molecular methods offer faster and higher throughputs while staying true to the original purpose of identifying and quantifying microbes. Recent studies demonstrate that close to 100 % sensitivity and specificity can be achieved for evaluating clinical infections (Hansen et al. 2010). One issue is that molecular methods may be identifying too many microorganisms, leading the clinician to over treat a specific infection.

DGGE and imaging methods showed that there was much more diversity present in wounds than clinical cultures were reporting (Davies et al. 2004; James et al. 2008). Clinicians managing other chronic infections such as chronic rhinosinusitis (Stephenson et al. 2010), cystic fibrosis (Goddard et al. 2012), middle ear infections (Laufer et al. 2011), and burns utilized molecular methods to show similar findings. It has been generally agreed that these and other chronic infections are associated with bacteria propagating in biofilm phenotype (Del Pozo and Patel 2007). Although molecular methods can identify microbes regardless of their mode of growth, the same is not true for clinical cultures. Molecular technology provides the clinician a more robust understanding of the infection, but also forces the clinician to consider multiple microbial species. At the same time, molecular

methods do not provide any clear information on which species are producing the infection and which species are merely contaminants.

New methods are rapidly developing where microRNA (Martens-Uzunova et al. 2013) and messenger RNA (Mutz et al. 2013) can be sequenced and identified. This will provide critical information as to the inner workings of microbial cells which should provide insight as to strategies being used to cause infection. This may shed light on which microorganisms within the community are behaving as pathogens.

Before a bacterial species can be deemed a pathogen, or more importantly before that species can be dismissed as a contaminant, the clinician must take into account the synergies which arise within a polymicrobial infection. By including multiple bacterial and/or fungal species into a single community, the biofilm achieves numerous advantages such as passive resistance (Elias and Banin 2012), metabolic cooperation (Fischbach and Sonnenburg 2011), by-product influence (Elias and Banin 2012), quorum-sensing systems, an enlarged gene pool with more efficient DNA sharing (Madsen et al. 2012), and many other synergies that give the polymicrobial infection a competitive advantage. It is best to view a biofilm as a single entity possessing multiple genetic resources to allow it to adapt and thrive regardless of the stresses it encounters. In general, a more diverse population (i.e., greater the gene pool) will make the biofilm more robust in terms of its survivability (Tuttle et al. 2011).

Metabolic cross feeding has been well established between genetically distinct species. It has been shown that *Streptococcus gordonii* produces peroxide that can cause *Aggregatibacter actinomycetemcomitans* (Aa) to produce a factor H binding protein which limits the host's ability to kill Aa through a complement mediated lysis (Ramsey et al. 2011). This metabolic cooperation has been identified in numerous polymicrobial models (Dalton et al. 2011; Mikx and van der Hoeven 1975; Kuboniwa et al. 2006).

Waste products, molecules that bacteria produce that are end products and are of no benefit to the metabolizing member, are released into the local biofilm environment. Many of these metabolites such as ammonia, lactic acid, and carbon dioxide can have significant influence on the surrounding microorganisms (Elias and Banin 2012). Studies have demonstrated that *Fusobacterium nucleatum* and *Prevotella intermedia* generate ammonia which raises the pH suitable for *Porphyromonas gingivalis* (Takahashi 2003) and that *F. nucleatum* also provides an increased carbon dioxide environment which increases the pathogenicity of *P. gingivalis* (Diaz et al. 2002).

Passive resistance is when one of the members in the biofilm possesses a resistance factor that can protect other members of the biofilm which do not have the factor. There are numerous biofilm defenses which limit the effectiveness of antibiotics. For example, a beta-lactamase producing strain of *Haemophilus influenza* was cocultured with *Streptococcus pneumoniae* deficient in any resistance factors. *Haemophilus influenza* increased the MIC/MBC of *S. pneumoniae* by amoxicillin (Weimer et al. 2011).

The clinical concern relative to the synergies of polymicrobial biofilm is that the infection will be more severe and recalcitrant to treatment. There are many examples which show that this is indeed the case. Low levels of *P. aeruginosa* mixed with *Staphylococcus aureus* increased infection rates in a rat model (Hendricks et al. 2001). In the mouse model, *Prevotella* increases the pathogenicity of *S. aureus* (Mikamo et al. 1998). *Escherichia coli* produced marked increase in the size of abscess formation with *Bacteroides fragilis* in a diabetic mouse model (Mastropaolo et al. 2005). There also is clinical evidence to suggest that polymicrobial infections are more severe (Tuttle et al. 2011).

The synergies and general recalcitrance produced by polymicrobial infections argue for the full evaluation of every infection. This means not only identification of all species present but also their quantification. However, there is currently not enough information to give clear direction on which microorganisms are important to treat. Also, therapeutic tools for managing polymicrobial infections in conjunction with or separate from antibiotics are generally not available. If a clinician has no specific tools to address all the diversity of a polymicrobial infection then is it valuable to get the test in the first place?

## 4 The Clinical Use of Molecular Methods: Two Eyes

Identifying and quantitating the microorganisms present in an infection are only part of the diagnosis of an infection. Clinical findings play the major role in determining if the microorganisms present are harming the host. It is only through stereoscopic vision of laboratory results and clinical observation that we can clearly see the power of the detailed information provided by molecular methods. Just as when sophisticated imaging technologies emerged such as MRI, the full meaning and nuances of the images provided could not be appreciated until there was clinical application and experience.

Clinicians seem to be divided by the information provided by DNA-based testing. The unfamiliar microbes can both elucidate and complicate the diagnosis of chronic infections produced by biofilm. Through years of use of molecular methods in real-world chronic infections (mainly chronic wounds) several important principles have emerged. Uncommon bugs occur commonly in chronic wounds and many chronic infections. The clinical challenge of treating rare microbes is more difficult but doable. Literature searches usually will yield usable treatment options for the genera that are identified. Even though we like to know the species identification, most antibiotics, biocides, quorum-sensing inhibitors, and ancillary treatments work at the genus or even the family level for many microbes. That is, a treatment that would kill a rat would in general kill a mouse. Therefore, unfamiliar microbes for treatment purposes can be grouped with closely related microbes which are more familiar (e.g., *Raoultella planticola* and *Klebsiella* spp.) or categorized by common groupings such as gram negative, gram positive, anaerobic, etc. But all of the grouping and comparing of microbes to form a treatment plan

highlight the main inadequacy of molecular methods, which is the lack of antibiotic sensitivity data similar to that provided by culture methods.

There are several strategies for managing chronic biofilm infections with the lack of antibiotic sensitivity information. First, if the infection is accessible to topical treatment, high concentrations of antibiotics far in excess of resistance factors can overwhelm most mobile genetic element-induced antibiotic resistance. Second, if systemic antibiotics will be necessary then certain mobile genetic elements with limited diversity, such as mecA cassettes, van genes, and others can be identified by real-time PCR. Third, if sensitivity data is still critical, then molecular diagnosis is still very often the quickest and most cost-effective way to proceed because many microbes are not initially grown in routine clinical culture. By first identifying the microbes of interest by molecular methods, custom nutrients and methods can be used to cultivate microbes for sensitivity work or genomic study (Sibley et al. 2011). With the emerging massive increase in capacity per run, advances in bioinformatics and computing, along with steady decreases in costs, it is becoming feasible to evaluate all the genes in a sample which may allow molecular methods to eventually assess resistance directly in the near future.

Dealing with diversity is made easier by the data provided by DNA-based diagnostics, but caveats remain. Sequencing provides a relative abundance for each species identified in the sample; however, it yields no "absolute" quantification for how much microbial material is present. Real-time PCR has the ability to give reproducible estimates of the number of microbes per gram of tissue (such as $10^5$/g) which is termed the "microbial load" or "bacterial load." Several factors can fictitiously lower the value for "microbial load," such as inefficient extraction, decreased primer efficiency, and small variations throughout the analysis. As a result, a low "microbial load" should never be discounted as "not a significant infection." The diagnosis of infection is a clinical decision; therefore, chronic infection itself should always dictate treatment. To evaluate the progression or improvement of an infection it may be necessary to have the lab run the initial sample with subsequent samples in the same run to mitigate these variations, which allows for better comparison.

Quantification of microbes in the polymicrobial infections often encountered in biofilm infection is indispensable. For example, if a sample contains just 1 % MRSA but the bacterial load is $10^8$/g then there are still $10^6$ MRSA even though it is a minor component of the biofilm. So MRSA coverage would be reasonable. But 1 % MRSA with a bacterial load of $10^5$/g ($10^3$ MRSA) requires only observation which can greatly reduce the use of first-line MRSA antibiotics.

The diversity can be daunting at first, but it is amazing how the many disparate microbes resolve down to treatment groups that require only one or two treating agents. For example, a group of microbes in chronic wounds consisting of MRSA, *Streptococcus*, *Peptoniphilus*, *Anaerococcus*, *Bacteroides*, *Pseudomonas*, and *Serratia* can effectively be treated with the use of clindamycin and amikacin. By collapsing the gram positives and anaerobes into one treatment group covered by clindamycin and then covering the gram negatives with amikacin, only two

antibiotics are needed. In fact, high-dose (250 times MIC) amikacin can also provide double coverage for MRSA.

One study showed that by just adding the ability to assess chronic wounds with molecular methods (PCR and sequencing), the use of expensive first-line methicillin-resistant *S. aureus* (MRSA) treatments was greatly reduced (Wolcott et al. 2010). Molecular methods identified *S. aureus* along with the mecA cassette in a majority of the wounds evaluated, yet the quantification showed that MRSA was a minor population (less than 1 % of the bacteria present) and therefore was observed and not actively targeted by antibiotic therapy. Wound care outcomes were improved over standard of care with molecular diagnostics used in this manner. The study demonstrates that using currently available treatments directed by a better understanding of the microbial diversity in question improves outcomes.

Now that molecular tools are available to fully define an infection, it will be up to clinicians to develop appropriate solutions. For example, in the companion study to the one noted above, personalized gels to address what were considered the important species identified within the wound biofilm (usually greater than 1 %) were developed to treat each patient. Molecular diagnostics along with multivalent personalized treatment yielded much better healing outcomes (Dowd et al. 2011).

## 5 Conclusions

Dealing with the complexity of the results is just the beginning—DNA diagnostics face other barriers in routine clinical use. Clinicians must deal with accessibility, choosing the appropriate laboratory for the analysis, and, as always, cost. Yet the cost of DNA extraction, sequencing, bioinformatics, etc., currently rivals cultivation methods and will continue to drop rapidly. Accessibility is still a barrier.

Technologies now exist which very easily could move molecular diagnosis to the bedside in the next several years. Until then, reference laboratories currently offer the best choice of different DNA diagnostic tests utilizing multiple platforms. Nevertheless, the main barrier for general acceptance is the level of enthusiasm of the clinician for translating this technology into managing infections in individual patients. Not until clinicians embrace molecular methods for identifying and quantitating microbes will molecular methods revolutionize the management of chronic infections.

## References

Aagaard K, Petrosino J, Keitel W, Watson M, Katancik J, Garcia N, Patel S, Cutting M, Madden T, Hamilton H, Harris E, Gevers D, Simone G, McInnes P, Versalovic J (2012) The human microbiome project strategy for comprehensive sampling of the human microbiome and why it matters. FASEB J 27(3):1012–1022. doi:10.1096/fj.12-220806, fj.12-220806 [pii]

Chain PS, Grafham DV, Fulton RS, Fitzgerald MG, Hostetler J, Muzny D, Ali J, Birren B, Bruce DC, Buhay C, Cole JR, Ding Y, Dugan S, Field D, Garrity GM, Gibbs R, Graves T, Han CS, Harrison SH, Highlander S, Hugenholtz P, Khouri HM, Kodira CD, Kolker E, Kyrpides NC, Lang D, Lapidus A, Malfatti SA, Markowitz V, Metha T, Nelson KE, Parkhill J, Pitluck S, Qin X, Read TD, Schmutz J, Sozhamannan S, Sterk P, Strausberg RL, Sutton G, Thomson NR, Tiedje JM, Weinstock G, Wollam A, Detter JC (2009) Genomics. Genome project standards in a new era of sequencing. Science 326:236–237. doi:10.1126/science.1180614, 326/5950/236

Dalton T, Dowd SE, Wolcott RD, Sun Y, Watters C, Griswold JA, Rumbaugh KP (2011) An in vivo polymicrobial biofilm wound infection model to study interspecies interactions. PLoS One 6:e27317. doi:10.1371/journal.pone.0027317, PONE-D-11-09036 [pii]

Davies CE, Hill KE, Wilson MJ, Stephens P, Hill CM, Harding KG, Thomas DW (2004) Use of 16S ribosomal DNA PCR and denaturing gradient gel electrophoresis for analysis of the microfloras of healing and nonhealing chronic venous leg ulcers. J Clin Microbiol 42:3549–3557

Del Pozo JL, Patel R (2007) The challenge of treating biofilm-associated bacterial infections. Clin Pharmacol Ther 82:204–209. doi:10.1038/sj.clpt.6100247, 6100247 [pii]

Diaz PI, Zilm PS, Rogers AH (2002) Fusobacterium nucleatum supports the growth of Porphyromonas gingivalis in oxygenated and carbon-dioxide-depleted environments. Microbiology 148:467–472

Dowd SE, Wolcott RD, Kennedy J, Jones C, Cox SB (2011) Molecular diagnostics and personalised medicine in wound care: assessment of outcomes. J Wound Care 20:232, 234–232, 239

Elias S, Banin E (2012) Multi-species biofilms: living with friendly neighbors. FEMS Microbiol Rev. doi:10.1111/j.1574-6976.2012.00325.x

Fischbach MA, Sonnenburg JL (2011) Eating for two: how metabolism establishes interspecies interactions in the gut. Cell Host Microbe 10:336–347. doi:10.1016/j.chom.2011.10.002, S1931-3128(11)00295-2 [pii]

Fitzpatrick KA, Kersh GJ, Massung RF (2010) Practical method for extraction of PCR-quality DNA from environmental soil samples. Appl Environ Microbiol 76:4571–4573. doi:10.1128/AEM.02825-09, AEM.02825-09 [pii]

Fodor AA, DeSantis TZ, Wylie KM, Badger JH, Ye Y, Hepburn T, Hu P, Sodergren E, Liolios K, Huot-Creasy H, Birren BW, Earl AM (2012) The "most wanted" taxa from the human microbiome for whole genome sequencing. PLoS One 7:e41294. doi:10.1371/journal.pone.0041294, PONE-D-11-08927 [pii]

Fux CA, Costerton JW, Stewart PS, Stoodley P (2005) Survival strategies of infectious biofilms. Trends Microbiol 13:34–40

Goddard AF, Staudinger BJ, Dowd SE, Joshi-Datar A, Wolcott RD, Aitken ML, Fligner CL, Singh PK (2012) Direct sampling of cystic fibrosis lungs indicates that DNA-based analyses of upper-airway specimens can misrepresent lung microbiota. Proc Natl Acad Sci USA 109:13769–13774. doi:10.1073/pnas.1107435109, 1107435109 [pii]

Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, Ciulla D, Tabbaa D, Highlander SK, Sodergren E, Methe B, DeSantis TZ, Petrosino JF, Knight R, Birren BW (2011) Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res 21:494–504. doi:10.1101/gr.112730.110, gr.112730.110 [pii]

Hager T (2006) The demon under the microscope. Harmony Books, New York, NY, 46 p

Hansen WL, Beuving J, Bruggeman CA, Wolffs PF (2010) Molecular probes for diagnosis of clinically relevant bacterial infections in blood cultures. J Clin Microbiol 48:4432–4438. doi:10.1128/JCM.00562-10, JCM.00562-10 [pii]

Hendricks KJ, Burd TA, Anglen JO, Simpson AW, Christensen GD, Gainor BJ (2001) Synergy between Staphylococcus aureus and Pseudomonas aeruginosa in a rat model of complex orthopaedic wounds. J Bone Joint Surg Am 83-A:855–861