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# Biofilm Formation by Clinical Isolates and Its Relevance to Clinical Infections

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## 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.

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## 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

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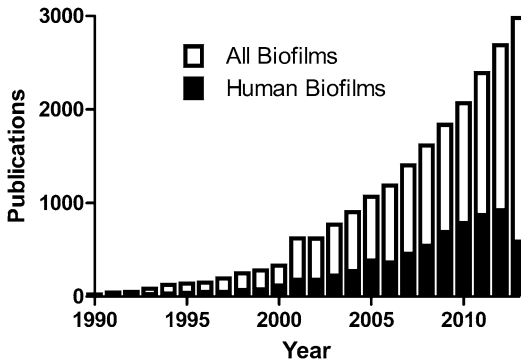
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**Fig. 1.1** PubMed-indexed literature citations for biofilms, 1990–2013. Annual study totals were obtained by searching the PubMed database using the term “biofilm” with and without application of the species record filter for “human”

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*, *setIA*, *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*, *hlyB*) 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 % of 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 *SCCmec* 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 (*SCCmec* 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 *SCCmec* 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 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 rima*, *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*, *Finigoldia*, *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<sup>2</sup> 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 on 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 *Finigoldia* spp., *B. fragilis*, *Clostridium* spp. and *Microsporium* 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 *Finigoldia* 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).

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## 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 “mucoïd” 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 mucoïd and non-mucoïd 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-mucoïd strains. However, a separate study documented similar increases in antimicrobial resistance in biofilms formed by both mucoïd and non-mucoïd 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-mucoïd 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 assessed by the FEV<sub>1</sub>. 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<sub>1</sub>, clinical symptom scores and weight gain were suggested in the group receiving garlic oil. The quorum sensing molecule 3-oxo-C<sub>12</sub>-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 multi-locus 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).

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## 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-sulfophenyl)-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. guilliermondii* 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

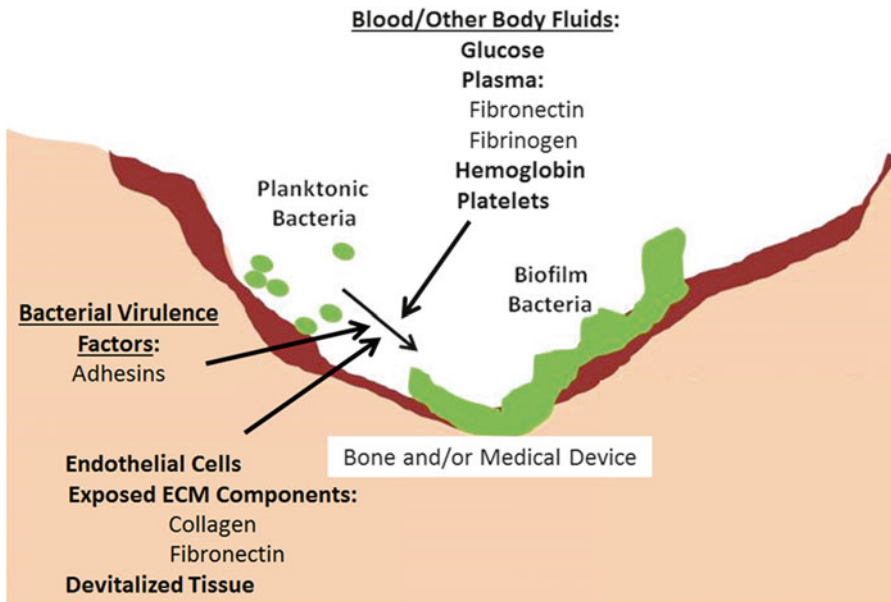
most compelling is the observation that treatment with an echinocandin, previously shown to disrupt *Candida* biofilms in vitro (Fiori et al. 2011), was associated with significantly reduced mortality and hospital length of stay. Thus *Candida* biofilms appear to represent a potent virulence factor for 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.

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## 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 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<sub>3</sub> or hemin resulted in the formation of more aggregates and microcolonies compared to cells grown in the presence of the synthetic iron chelator dipyriddy (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 flocs 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.

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## 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 <i>Staphylococcus</i> bacteremia      | Retrospective cohort  | 150 neonates                              | No difference in biofilm formation between infecting and colonizing isolates<br><br>Biofilm production more common in <i>S. epidermidis</i> and assoc. with 34 % reduction in C-reactive protein  | Klingenberg et al. (2005)  |
| <i>E. coli</i> bacteremia                                | Prospective cohort  | 177 patients                              | Biofilm formation not associated with outcomes<br><br>Predictors of <i>E. coli</i> bacteremia: neutropenia, catheter-assoc. infection, cirrhosis and pneumonia  | Martinez et al. (2006)     |
| 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 <sub>1</sub> , 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<br><br><i>H. influenzae</i> was associated with milder symptoms and faster resolution, whereas 10 patients with persistent disease had <i>S. aureus</i> biofilms requiring more clinic visits | Foreman and Wormald (2010) |
| 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<br><br>6-/12-month symptom and endoscopy scores worse among those demonstrating biofilms   | You et al. (2011)          |
| <i>Candida</i> 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<br><br>23 % excess mortality associated with biofilm-forming isolates<br><br>Echinocandin therapy reduced mortality  | Tumbarello et al. (2012)   |
| <i>Ureaplasma</i> spp./ bronchopulmonary dysplasia (BPD) | Retrospective cohort  | 43 neonates                               | 95 % of isolates formed biofilms<br>MBIC for macrolides was below planktonic MIC<br><br>67 % developed BPD including 4 who died   | Pandelidis et al. (2013)   |

(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<br>Trend toward increased biofilm formation among isolates from non-fluid sites<br>Isolates from relapsing infections displayed strong biofilm formation | Sanchez et al. (2013) |
| 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<br>Biofilm formation by <i>A. baumannii</i> associated with persistence<br>Multivariate model prevented by small sample size   | Akers et al. (2014)   |

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<sub>50</sub>) 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 experi-

ence 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.

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## 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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