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The Role of Biofilms in Device-Related Infections

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Preface

The Role of Biofilms on Indwelling Medical Devices is the third volume in the continuing Springer Series on Biofilms. In this volume, we explore biofilm infections of indwelling medical devices from the point of view of both the host and the infecting pathogen. We begin this volume with an analysis of the primary source of indwelling medical device fouling, namely the host microbial skin flora. Then, each major form of indwelling medical device infection, including orthopaedic implants, biofilms on ventilators, dental implant infections, urinary tract infections due to catheters, intravenous catheter fouling, and endophthalmitis infections, will be described. Not only will the general properties of the infection be discussed, but also the relationship and balance between the host and the infecting microbial species. The final two contributions take into account the biofilm phenotype to evaluate and describe both the specifics of the host response to biofilm infections and novel methods of diagnosis.

Device-related biofilm infections increase hospital stays and add over one billion dollars/year to U.S. hospitalization costs. As the use and the types of indwelling medical devices commonly used in modern healthcare are continuously expanding, especially with an aging population, the incidence of biofilm infections will also continue to rise. The central problem with microbial biofilm infections of foreign bodies is their propensity to resist clearance by the host immune system and all antimicrobial agents tested to date. In fact, compared to their free floating, planktonic counterparts, microbes within a biofilm are 50–500 times more resistant to antimicrobial agents. Therefore, achieving therapeutic and non-lethal dosing regimens within the human host is impossible. The end result is a conversion from an acute infection to one that is persistent, chronic, and recurrent, most often requiring device removal to eliminate the infection. This text will describe the major types of device-related infections, and will explain the host, the pathogen(s), and the unique properties of their interactions to gain a better understanding of these recalcitrant infections.

Chronic diseases such as AIDS, cancer, or diabetes are first understood as a collection of seemingly individual and anecdotal reports of symptoms. However, the link between the disparate signs and symptoms and the causal factor had to be made to make the first steps towards diagnosis, treatment, prevention and/or research towards a cure. While the causal factors of these maladies are recognized as a "disease",

such is not the case for biofilm diseases. The commonalities of chronic infection, tissue damage, recalcitrance to antimicrobial therapy, and resistance to clearance by the host immune system, are the symptoms of biofilm disease. It has been assumed that an understanding of the disease process can be obtained while ignoring the biofilm mode of growth from the host and microbiological standpoint. This approach has generally left us with two options: longterm, non-curative antibiotic therapy or surgical removal of the nidus of infection.

These chronic bacterial and fungal infections are not only difficult to treat, but in some cases impossible. In fact, 56% of all infections are due to biofilms, according to the Centers for Disease Control and that equates to approximately half a million deaths annually in the United States alone. The sheer number of infections and diseases related to the biofilm phenotype is clearly the number one cause of medical morbidity in the United States. Extrapolated to a global perspective, biofilm disease can be viewed as the major disease plaguing the human population. Until there is a greater realization of the role of biofilms in chronic diseases in humans and fitting directed research, the appropriate paradigm shift will not occur and in turn, the cost, treatment and morbidity and mortality associated with these diseases will not improve.

This book is meant to provide insight into the role of biofilms in implant-mediated infections. The authors and chapters have been focused on the most common types of implant infections, the most common pathogens associated with each infection, and in some cases, how the human host responds to the presence of these microbial communities. Although biofilm infections in humans are found outside the realm of medical devices, it is clear that the enormous increase in the clinical usage of medical devices presents a major challenge to health care not only in the United States, but across the globe. Hopefully this book and the chapters contained herein help to shed light on these issues and lead to further research that directly and positively impacts patient health.

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Microbial Ecology of Human Skin and Wounds

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Abstract Human skin is a complex organ that provides protection and regulates our interaction with the outside environment. The skin is composed of three layers, which include the epidermis, dermis, and hypodermis. Skin appendages include hair follicles, sebaceous glands, and sweat glands. These appendages are unevenly distributed on the skin. The stratum corneum is the outer protective layer of the epidermis and is composed of dead cells that are regularly shed from the surface. The outer layers of the epidermis are inhabited by microorganisms considered permanent skin residents as well as transient microorganisms that do not normally grow and multiply on the skin. The number and types of microorganisms inhabiting the skin are influenced by skin conditions, including the density and activity of sebaceous and sweat glands. The secretions of these glands provide nutrients and selective conditions that influence the composition of the resident microflora. This community is composed primarily of Gram-positive bacteria, including staphylococci, micrococci, and corynebacteria as well as lipophilic yeasts (*Malassezia*). This resident microflora is believed to help prevent skin colonization by pathogenic microorganisms. However, under certain conditions, skin disease can be caused by members of the resident flora. Skin infections are most often the result of injury to the skin. Cutaneous wounds enable access of microorganisms to normally sterile tissue and provide a much different niche for microbial growth than does intact skin. In the case of acute wounds, the healing process, including the immune response, is capable of stemming invasion by microbes and repairing the wound. However, in some cases wounds become chronic and fail to heal within a reasonable time frame. Most often chronic wounds afflict the ill and elderly with underlying disorders (e.g., diabetes) or weakened immune systems. Large bacterial populations in wounds have been correlated with delayed healing, and control of microbial infection is recognized as an important aspect of wound care. However, the role of specific microorganisms in preventing wound healing remains unclear. Similar types of microorganisms have been isolated from both acute and chronic wounds, although the latter tend to harbor more anaerobic bacteria. Growth of microorganisms as biofilms in wounds may also

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contribute to the delayed healing and poor response to treatment of chronic wounds. Overall, human skin and cutaneous wounds are complex ecosystems harboring diverse communities of microorganisms. A better understanding of these ecosystems may lead to improvements in human health.

1 Introduction

The skin is the largest organ of the human body and serves to regulate our interaction with the outside environment. It also provides a habitat for resident microorganisms that occupy the skin niche and help prevent colonization by pathogenic microorganisms. Regardless of the protective factors provided by skin and the resident microflora, pathogenic microorganisms can invade the skin niche and cause disease. Also, under certain conditions, members of the resident skin microflora that are usually benign can cause skin diseases. Although diseases can affect intact skin, infections are most often associated with skin trauma. Cutaneous wounds enable bacterial access to deeper tissues, which provide an environment much different than intact skin. This environment provides both nutrient-rich conditions for microbial growth and antagonistic effects of the immune system. Thus, wounds present a much different environment for microbial growth than does intact skin. Furthermore, chronic wounds involve other aspects that present a completely different niche for microbial growth than either intact skin or acute wounds. The following chapter provides an overview of skin structure and physiology, skin microbiology, wound repair, and wound microbiology.

2 Normal Human Skin

The skin acts as a barrier to microbial invasion. As well as providing protection, the skin contributes to thermoregulation, sensation, and secretion of various fluids. Although the basic structural components of skin are consistent, structural and physiological characteristics vary with body location and age. Skin characteristics also vary between individuals. Skin is inhabited by microorganisms, which include both resident microflora and transient organisms. The resident microflora is believed to be beneficial to human health when confined to the skin habitat.

2.1 Skin Structure and Physiology

The skin has been characterized into three layers, with the epidermis as the outermost layer, the dermis as the middle layer, and the hypodermis as the deepest layer (Fig. 1). The epidermis serves as a protective layer and is composed primarily of

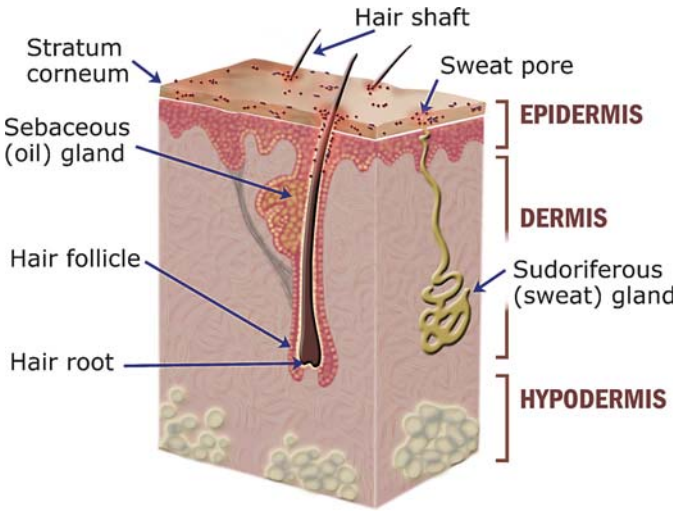

Fig. 1 Skin structure indicating sites for microflora attachment and growth. Bacteria, including cocci (*red*) and rods (*blue*), colonize the skin surface as both individual cells and small groups (microcolonies). The largest numbers of bacteria are associated with hair follicles as well as sebaceous and sudoriferous glands. Illustration by Peg Dirckx

keratinocytes. These cells produce the protein keratin, an important structural component of skin. Keratinocytes are involved in a variety of processes, including wound healing and modulating cutaneous immune responses. The outermost surface of the skin (stratum corneum) is composed of dead keratinocyte cells containing keratin and forms a tough water-repellant layer. The stratum corneum is regularly shed and varies in thickness depending on body location, thickest in certain areas such as the soles of the feet. Underlying the stratum corneum are living keratinocytes at decreasing stages of maturation with depth, as well as melanocytes, Langerhans cells, and Merkel cells. Melanocytes produce the pigment melanin, which contributes to skin color and provides protection from ultraviolet radiation. Langerhans cells are involved in the regulation of immune responses. Merkel cells are associated with nerve cells and are involved in the sensation of touch. Protruding through the epidermis are hairs and secretion ducts originating in the dermis and hypodermis. The epidermis is connected to the dermis by a basement membrane, which serves to anchor these two layers together.

The dermis is the structural layer of the skin that supports the epidermis and consists mainly of collagen and elastic fibers. A few cells such as fibroblasts, macrophages, and lymphocytes as well as blood vessels and nerves are present. The dermis contains two distinct regions, the uppermost of which is the papillary region characterized by thin collagen, elastic fibers, small blood vessels, and nerve endings. The deeper layer is the reticular region, where the collagen fibers are thick and the blood vessels and nerves are larger. Hair follicles and glands such as eccrine and sebaceous glands also reside in the reticular layer. Variations in the thickness of this

reticular region contribute to skin thickness in different areas of the body. The collagen and elastic fibers, which provide the dermis with strength and elasticity, are produced by the fibroblasts. Other cells in the dermis include immune cells such as macrophages, lymphocytes, and mast cells.

The deepest layer of skin, the subcutaneous tissue or hypodermis, attaches the skin to underlying tissues. The hypodermis is composed predominantly of adipose tissue, which provides thermal insulation, shock absorption, and energy storage. Large blood vessels in the hypodermis are essential for delivering blood to the dermis, and the main nerves that connect to the dermis are also located in the hypodermis. Some sweat glands and longer hair follicles of the scalp may extend down into the subcutaneous layer.

Appendages and glands in the skin include hair follicles, sebaceous glands, and types of sudoriferous or sweat glands. The density of these features varies with location; hair follicles and sebaceous glands are prevalent on the scalp, while eccrine glands, a type of sweat gland, are very numerous in the axillae. All of these skin features can influence the type and number of microbial flora present in certain areas of the skin. Sebaceous glands exist typically as lateral appendages of hair follicles. They secrete sebum, which is a mixture of lipids, including waxes and triglycerides, as well as protein, cholesterol, and salts. Most sebaceous glands secrete into the hair follicle, although some may secrete directly onto the surface in locations such as the lips and eyelids. They are nonexistent on the palms and soles and largest in size on the face, neck, and chest. Sebum provides nutrients for microbial growth, and bacteria on the skin are often found in microcolonies in or surrounding follicles (Fig. 1).

The main sweat glands in humans are eccrine glands, which are prevalent in areas such as the axillae, palms, and soles. The gland often extends from its opening on the epidermis down into the subcutaneous layer. The sweat secreted from these glands is an aqueous solution containing many solutes which influence growth of microorganisms. These include sodium, chloride, and potassium ions, urea, ammonia, glucose, lactic and ascorbic acids, as well as lysozyme. The pH of sweat can be neutral or slightly acidic, a factor that also influences microbial growth. Typically, increased densities of bacteria are found in areas of the skin containing a large number of eccrine glands.

Apocrine glands are sweat glands that have a thicker, milkier secretion than that of eccrine sweat glands. Located mainly on the axillae and around the genitalia, apocrine glands do not have a known purpose in humans. In other mammals, where these glands are much more developed, they serve as scent organs for sexual and territorial purposes.

As mentioned previously, composition and density of the microbial skin flora vary with body location. Factors that affect bacterial growth on the skin include, but may not be limited to, moisture, nutrients, pH, and presence of inhibitors. The moisture content of a certain area of the skin depends upon the density and activity of the eccrine glands in that area. These glands, as well as the sebaceous glands located in hair follicles, supply many different nutrients for microorganisms, and eccrine glands also help regulate pH for optimized growth conditions of some

species. Therefore, body locations that contain high numbers of these appendages are likely to house larger bacterial populations that can be quite diverse based on the variety of available nutrients.

In addition to variations in skin structure and physiology between individuals and among various body locations, skin also undergoes considerable changes with age, which influences the skin microflora (Somerville 1969). Although active at birth, because of the influence of maternal androgens, sebaceous glands are inactive in children and then become active again at puberty. Apocrine glands also become active at puberty. In general, adult glandular secretions (sebaceous, eccrine, and apocrine) decrease with age. The skin of adults also tends to have a higher water content than that of infants, children, or the elderly.

2.2 Skin Microflora

Research into the microbiology of human skin has been reviewed since 1965 in books authored or edited by Marples (1965), Sommerville and Noble (1973), and Noble (1981, 1993). The following section is covered in more detail by the most recent edition of *The Skin Microflora and Microbial Disease* edited by Noble (2004).

A huge variety of microorganisms have been isolated from human skin, many of which are transient from exposure of the skin to environmental sources such as soil and water and do not grow or multiply on the skin. Certain microorganisms are considered permanent inhabitants of human skin, while others may establish populations on the skin, temporarily, under certain conditions. Nobel (1981) established the terms *transient* for bacteria that do not multiply on the skin, *temporary residents* for those multiplying and persisting for a short period of time, and *residents* for those organisms believed to be permanent skin inhabitants.

As discussed earlier, the number of microorganisms on skin varies with body location. Areas such as the scalp, axillae, and groin, with numerous hair follicles, sebaceous glands, and eccrine glands, harbor the largest and most diverse populations of bacteria. There are also variations between individuals and individuals living in different environments with respect to the types of microorganisms present on the skin.

The resident microflora consists primarily of Gram-positive bacteria, including the genera *Staphylococcus*, *Micrococcus*, *Propionibacterium*, *Corynebacterium*, and others. In addition, yeasts of the genus *Malassezia* are also considered permanent residents of human skin. These microorganisms colonize the hair follicles and superficial layers of the epidermis and recolonize newly formed tissue as the stratum corneum is shed, utilizing secretions of the sebaceous, eccrine, and apocrine glands as nutrients (Fig. 1). The number and diversity of microorganisms are dependant on skin location, with the largest populations and most diverse communities in areas such as the axillae and groin, which have the most numerous glands. Although, all humans have a resident skin flora, the numbers and microbial species present vary widely between individuals. The resident microflora likely evolved

with humans and is well adapted to the skin environment. It is accepted that these microorganisms generally provide protection by occupying the skin niche and preventing colonization by pathogens, with minor consequences to the host (e.g., body odor). Resident microbes may also more directly inhibit invading microorganisms by the production of inhibitory compounds such as antibiotics, bacteriocins, and lytic enzymes (Allaker and Noble 2004).

The coagulase-negative staphylococci inhabiting human skin include *S. epidermidis*, *S. haemolyticus*, *S. hominis*, and others (Noble 2004). These bacteria are not considered skin pathogens, although they can cause disease in other niches of the human body, having been implicated in endocarditis and many device-related infections.

In contrast to the coagulase-negative staphylococci, the coagulase-positive species, *Staphylococcus aureus*, is considered a true pathogen (Lowy 1998). The primary human habitat of *S. aureus* is the nostrils, although they also are commonly members of the resident skin flora, particularly in the axillae, groin, and toe webs (Noble 2004).

Early studies of human skin flora used the term *diptheroids* to describe Gram-positive nonsporing rods; this group of bacteria was later referred to as *coryneform* bacteria because these pleomorphic bacteria often assumed club-shaped forms. The coryneform group is composed of numerous genera, including both aerobic and facultative species (Leyden and McGinley 2004). Many of the coryneform bacteria either depend on lipids for growth or show enhanced growth in the presence of lipids. Aerobic coryneforms considered residents of human skin include *Corynebacterium* species, such as *C. minutissimum*, *C. xerosis*, and *C. jeikeium* as well as *Brevibacterium epidermis*. Aerobic coryneforms have been linked to axillary odor (Leyden et al. 1981; Taylor et al. 2003). Anaerobic coryneforms constituting the resident flora are members of the genus *Propionibacterium*, with species including *P. acnes*, *P. granulosum*, and *P. avidum*. Of the propionibacteria, *P. acnes* is the most commonly isolated species of propionibacterium from human skin.

A variety of species of *Micrococcus* are skin residents, including *M. luteus* and *M. varians*. *Micrococcus* species are not considered skin pathogens and rarely cause infections in humans. However, they have been implicated in axillary malodor (Taylor et al. 2003).

Gram-negative bacteria are not generally considered part of the resident flora, with the exception of *Acinetobacter* species (Noble 2004). Species of this genus are found in locations such as the axillae, groin, and toe webs of some individuals. The toe webs may also be inhabited by other Gram-negative genera, including *Pseudomonas* and *Proteus*, although these genera are not considered resident flora (Noble 2004).

Yeasts of the genus *Malassezia* (*Pityrosporum*) are also considered part of the resident flora of human skin (Hay 2004). These yeasts are lipophilic and inhabit superficial layers of the stratum corneum near sebaceous glands, much like the lipophilic cutaneous bacteria.

Recent studies of skin microflora have utilized culture-independent techniques (PCR and cloning) to determine the presence of difficult-to-culture bacterial

species. These studies have served to further confirm the presence of species determined from the numerous culture-based studies described earlier. In addition, these studies also discovered bacteria not previously associated with human skin. These include such genera as *Acidovorax*, *Dietzia*, and *Methylophilus* found on human foreheads (Dekio et al. 2005). A study of human forearms revealed the presence of additional phyla not normally associated with human skin, including *Thermomicrobia*, *Cyanobacteria*, and *Deinococcus-Thermus* (Gao et al. 2007). In most instances, these new skin genera appear to be associated with a limited number of the volunteers sampled rather than on every individual. These studies serve to distinguish skin microflora on a subject-to-subject basis and provide further insight into the species diversity resident on normal human skin.

2.3 Microbial Skin Diseases

A vast number of microbially related skin diseases have been documented. As with the previous section on skin microflora, more detailed information on these diseases can be found in *The Skin Microflora and Microbial Disease* edited by Noble (2004). Some of these diseases are caused by invading microorganisms and viruses that are not considered part of the normal human skin flora. Such diseases include cellulitis and necrotizing fasciitis, caused by streptococcal species (Bisno and Stevens 1996). Other diseases can be caused by microorganisms considered normal resident skin flora that usually are benign. Such diseases include erythrasma, caused by *Corynebacterium minutissimum*, and pityriasis versicolor, caused by *Malassezia* yeasts. A common disease associated with a normally benign skin resident is acne vulgaris. In this case, increased sebum production and abnormal desquamation of follicular epithelium lead to proliferation of *Propionibacterium acnes* (Leyden and McGinley 2004). In most cases microbial skin diseases are associated with skin damage such as minor abrasions, burns, or other trauma. Infection of cutaneous wounds is discussed in more detail later. Diabetics and immunocompromised individuals are also predisposed to microbial skin infections caused by both members of the normal skin flora and invading microorganisms.

3 Skin Wounds

Skin wounds include superficial scrapes and cuts, bite wounds, traumatic wounds, surgical wounds, and pressure ulcers. As well as damaging the integrity of the skin, wounds also result in the introduction of cutaneous microflora and other microorganisms into the underlying tissues. In the case of acute wounds, the normal immune response and wound repair process are adequate to heal the wound in a relatively short time frame (days to weeks). This process can be augmented through the use of skin cleansers as well as topical and systemic antimicrobial agents.

In contrast to acute wounds, chronic wounds fail to heal within a reasonable time frame (months to years) and often remain in an inflammatory state. The microbiology of acute and chronic wounds is different, with the latter tending to harbor more diverse microbial communities that include anaerobic species. However, the role of microorganisms in preventing the healing of chronic wounds remains unclear.

3.1 Normal Wound Repair

Following injury to the skin, the inflammation phase begins, initiating the process of wound repair. Blood coagulation as well as platelet adhesion and aggregation form a blood clot, which serves to reestablish hemostasis and provide a provisional matrix for cell migration. This provisional matrix is rich in fibrin and also contains fibronectin. The platelets release chemotactic factors for blood leukocytes and growth factors. Blood clotting also leads to the generation of vasoactive agents and activation of the classical and alternative complement cascades, which further attract leukocytes to the wound. Concurrently with inflammation, epithelial cells migrate across the wound under the scab and proliferate, reestablishing the epithelial barrier.

Neutrophils are the first leukocytes to arrive at the wound site, drawn and activated by a variety of chemoattractants. The neutrophils engulf invading bacteria by phagocytosis and subsequently destroy them using enzymes and oxidative compounds. The length of the neutrophil infiltration phase depends on the amount of contamination in the wound. If a large number of bacteria and other foreign objects are present, neutrophil infiltration will persist along with further activation of the alternative complement cascade.

Following the initial neutrophil invasion of the wound, monocytes arrive on the scene, drawn by various chemoattractants, and are activated to become macrophages. The macrophages migrate along the provisional matrix phagocytosing bacteria, tissue debris, and exhausted neutrophils. Activated macrophages also produce chemoattractants and activators, recruiting additional inflammatory cells as well as fibroblasts. Cytokines produced by macrophages stimulate the formation of provisional extracellular matrix by fibroblasts as well as the formation of new blood vessels by endothelial cells.

The invasion of macrophages, fibroblasts, and endothelial cells into the wound bed, along with the formation of new blood vessels, gives the newly formed tissue a granular appearance, resulting in the term *granulation tissue*. Although fibroblasts initially produce primarily fibrin, synthesis of collagen eventually becomes predominant.

The final phase of wound healing is maturation, which involves matrix formation and remodeling. Fibroblasts form myofibroblasts, which contract the wound. The matrix matures, with the fibrin and hyaluronic acid of the provisional matrix replaced by collagen bundles of increasing thickness. As maturation progresses, collagen fibers become more organized, blood vessels are restored to normal, the scab is shed, and the epidermis is restored to normal thickness.

The wound healing process is guided by a large number of growth factors and enzymes. These compounds are released by various cells and regulate the proliferation, migration, and function of cells involved in the wound healing. Both interleukin-1 and tumor necrosis factor alpha are produced by keratinocytes in response to tissue damage. Release of these cytokines contributes to macrophage activation and induction of an inflammatory response. Macrophages also produce these cytokines along with interferon gamma. Important enzymes in the wound repair process include matrix metalloproteinases (MMPs) which facilitate cellular migration and tissue remodeling by specific cleavage of extracellular matrix protein. These enzymes are regulated by tissue inhibitors of metalloproteases (TIMPs), which bind and inactivate MMPs.

3.2 Chronic Wounds

Wounds that persist for more than about 2 months are considered chronic. These wounds often remain in an inflammatory state. Patients with chronic wounds are commonly also suffering from systemic disease, such as diabetes mellitus or peripheral vascular disease. A variety of factors may provide a barrier to wound healing. These factors include infection, poor blood perfusion, low oxygen pressure, malnutrition, and systemic disease.

Chronic wounds provide a much different environment than acute wounds. One of the key differences is the highly proteolytic condition (Yager and Nwomeh 1999). Typically, chronic wounds display a much higher ratio of MMPs to TIMPs than acute wounds do. This is at least partially due to a higher number of neutrophils present in chronic wounds (Yager and Nwomeh 1999). The proteolytic conditions in chronic wounds are also believed to be responsible for the degradation of growth factors and extracellular matrix proteins. However, the concentrations of some growth factors are elevated in chronic wounds. These include proinflammatory cytokines, such as interleukin-1, tumor necrosing factor alpha, and interferon gamma (Trengove et al. 1996a,b, 2000).

3.3 Wound Microbiology

Cutaneous wounds provide a much different environment for microbial growth than does intact skin. Depending on wound depth, tissues may be exposed, which provide a rich milieu for microbial growth. However, wounds also present a hostile environment for microorganisms because of the activity of the immune system. The microbial flora of wounds reflects these environmental differences, being quite different than the resident skin microflora. Although microbial infection of wounds is recognized as a barrier to healing, the role of microorganisms in chronic wounds remains unclear.

A myriad of microbial species have been isolated from wounds, including both aerobic and anaerobic bacteria. The predominant aerobic microorganisms isolated from clinical wound samples included *S. aureus*, coagulase-negative staphylococci, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella* species, *Streptococcus* species, *Enterococcus* species, and *Proteus* (Howell-Jones et al. 2005; Bowler et al. 2001). *S. aureus* is the most commonly isolated species from wounds (Bowler 1998) and is a recognized pathogen with a suite of virulence factors. However, it is often also isolated from chronic wounds showing no clinical signs of infection. In a comparative study of chronic and acute wounds, *S. aureus* predominated in infected acute wounds, while infected chronic wounds were primarily colonized by anaerobic bacteria (Bowler and Davies 1999a,b).

Genera of anaerobes cultured from wound samples include *Peptostreptococcus*, *Clostridium*, *Propioinacterium*, *Prevotella*, *Porphyromonas*, *Fusobacterium*, *Veillonella*, and *Bacteroides* (Howell-Jones et al. 2005;Wall et al. 2002; Bowler et al. 2001). Bowler and colleagues have stressed the potential role of anaerobic bacteria as a barrier to healing in chronic wounds (Bowler 1998; Bowler et al. 2001). *Peptostreptococcus* species, in particular, have been implicated in this regard (Wall et al. 2002). These authors suggested production of short-chain fatty acids and hydrolytic enzymes by *Peptostreptococcus* as potential factors in delaying wound healing. Anaerobes also appear to be responsible for malodor in wounds. Wounds that exhibit no odor were rarely colonized with anaerobic bacteria (Bowler et al. 1999). The lack of recognition of the importance of anaerobes in wound infections has been blamed on the difficulties of isolating and culturing anaerobes (Bowler 1998; Bowler et al. 2001). There appear to be instances of aerotolerance in some anaerobic species. The presence of *Clostridium tertium*, for example, was determined using 16S rDNA sequencing but has been mistaken as *Lactobacillus* or *Bacillus* using routine culture methodologies (Fujitani et al. 2007). Indeed, molecular-based studies have revealed that the microbial community of wounds is more diverse than revealed by culture (Davies et. al. 2001, 2004).

In polymicrobial communities, the growth of specific bacterial species may be aided by synergistic effects from growing in the presence of other bacterial species. For example, in a study of leg ulcers, *S. aureus* appeared to increase growth rates of Gram-negative anaerobes (Bowler and Davies 1999). Changes in virulence have been shown to occur in noncapsulate *Bacteroides fragilis*, which becomes capsulate (virulent) after passage with either *E. coli* or *S. pyogenes* (Brook 1988).

While the presence of bacteria does not necessarily indicate a wound infection, the concept of bacterial load appears to be an important determinant (Howell-Jones et al. 2005). As discussed by Bowler et al. (2001), quantitative culture of tissue biopsies or wound swab samples has been correlated with delayed wound healing. Generally, bacterial loads greater than 10^5 organisms are considered to indicate an infected wound (Robson et al. 1999). However, this guideline has been questioned (Bowler 2003). The use of rigorous and long incubation culture techniques has shown a positive correlation of increased bacterial diversity with nonhealing status of venous leg ulcers, instead of bacterial numbers alone (Davies et al. 2007).

3.4 Biofilms in Chronic Wounds

It has been speculated for several years that bacteria colonizing chronic wounds exist as biofilm communities (Serralta et al. 2001; Mertz 2003; Percival and Bowler 2004). Chronic wound infections share two important attributes with other biofilm diseases – persistent infection that is not cleared by the host immune system and resistance to systemic and topical antimicrobial agents (Costerton et al. 1999). Frequent debridement is one of the most clinically effective treatments to help heal chronic wounds (Steed et al. 1996). This may be an effective treatment because it removes the biofilm from the wound. This is similar to resolving infections from biofilm-colonized catheters; where antibiotic therapy is ineffective, the most effective approach is to remove the colonized catheter (Raad et al. 2002). However, direct evidence of biofilm involvement in chronic wound infections is scarce. One of the first published studies of biofilm formation in wounds was conducted using a porcine model (Serralta et al. 2001). The pigs were wounded, inoculated with *P. aeruginosa*, and a polyurethane dressing was applied. When the wounds were sampled, loosely adhered cells were removed with a saline flush and enumerated. Following flushing, the wounds were scrubbed to remove adhered bacteria and these too were enumerated. The scrub technique resulted in much higher bacterial counts than did the rinse technique, suggesting that the bacteria were tightly adhered to the wound as a biofilm. In this study plastic cover slips were also placed in some wounds for subsequent microscopic imaging. Clusters of bacterial cells surrounded by an extracellular polymer matrix were observed on the cover slips, which also indicated that biofilm formed in the wounds. These researchers also demonstrated that a *P. aeruginosa* strain isolated from a burn wound rapidly formed biofilms in vitro (Harrison-Balestra et al. 2003). Overall, preliminary evidence indicates that polymicrobial biofilm forms on chronic wounds, and clinical aspects of chronic wound infections resemble those of other biofilm infections. Recent research indicates that biofilms may be present in chronic wounds (James et al. 2008; Bjarnsholt et al. 2008). Further research is necessary to evaluate the role of biofilms in the prevention of wound healing.

4 Conclusion

Human skin is a complex organ that provides protection from the outside environment. The outermost layers of the skin are inhabited by microorganisms that are termed the resident microflora. In addition, a variety of other microorganisms that do not normally grow and multiply on the skin may be present on the skin. The resident microflora consists primarily of Gram-positive bacteria and yeasts of the genus *Malassezia*. Resident bacteria include staphylococci and micrococci as well as both aerobic and anaerobic coryneforms. These microorganisms grow and multiply on the skin using secretions from sebaceous glands as well as eccrine and apocrine sweat glands. The density of these glands and the associated microbial populations vary

with body location. The resident skin microflora is believed to serve a protective function by preventing colonization of the skin by pathogenic microorganisms, although in some cases microorganisms that are considered normal microflora can cause disease. Injuries to the skin result in the initiation of an immune response and cutaneous repair processes. Wound repair is complex and involves numerous cell types, growth factors, and enzymes. Infection with microorganisms can impair the healing process. Acute wounds heal within a few days or weeks, while chronic wounds persist for months or years. Chronic wounds usually have an underlying cause, such as systemic disease (e.g., diabetes) or immune dysfunction. A wide variety of bacteria have been isolated from wounds. The most common species of bacteria isolated from wounds is *S. aureus*. The role of specific bacteria in delayed healing of chronic wounds is unclear because many of the same bacterial species have been isolated from both acute and chronic wounds. Some studies have shown that anaerobic bacteria are more prevalent in chronic wounds than in acute wounds and have implicated them in delayed healing. Many studies may have overlooked anaerobic bacteria because they are difficult to grow in culture. It has been speculated that biofilms may play a role in the delayed healing of chronic wounds, but further research is necessary to evaluate this hypothesis. Overall, human skin and wounds are ecosystems often supporting complex microbial communities that can have both beneficial and detrimental influences on human health.

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Infections of Orthopaedic Implants and Devices

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Abstract Prosthetic implantation is rising in popularity in the United States, and use of prostheses will only continue to increase with the aging of the Baby Boomer generation. A concurrent rise in the number of infections of these implants is also being seen. Prosthetic implant infection (PII) can be caused by direct inoculation of bacteria to the implant or by seeding from the blood (hematogenous). Because the implant is quickly coated by host connective tissue upon implantation, bacteria such as the staphylococcal species *S. aureus* and *S. epidermidis* are able to readily gain a foothold in the host. *S. aureus* is able to quickly develop antibiotic resistance and methicillin-resistant strains (MRSA) are considered endemic in hospitals. This bacterium features a myriad of virulence factors that allow it to colonize and damage the host, as well as avoid the host immune response. These virulence factors are largely regulated through population-based quorum sensing via the *agr* system. *S. aureus* is also able to form biofilms, microbial communities encased in a polysaccharide matrix, which allows the bacteria within to persist in the face of antimicrobial therapies and the host response. Diagnosis of PII is difficult with many false results and confusion with aseptic loosening of the implant. Because of ineffective means of diagnosis, combined with S. aureus' high incidence of antibiotic resistance and its ability to evade both antibiotics and the host response through biofilm formation, treatment for PII is often inadequate and infection can become chronic. Therefore, PII has a high rate of morbidity and mortality for patients, as well as an extreme economic burden on the US healthcare system.

1 Introduction

Prosthetic implant infection (PII) is a medical problem that is rising in importance as usage of artificial joints, intramedullary rods, plates, and screws increases. The total number of hip and knee replacements alone is over 500,000

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in the USA, and this number is expected to rise. Though only occurring in a minority of patients, PII is distressing due to a high rate of mortality and the high cost of treatment associated with these infections (Trampuz and Zimmerli 2005). Treatment of PII costs as much as $50,000 per patient (Hebert et al. 1996), creating a large economic burden on the US healthcare system. In this chapter, we will first outline the ways in which PII can be initiated and the means of diagnosing and treating these infections. Next, we will detail aspects of the host that can contribute to the acquisition of PII, including some common risk factors. The most common etiologic agents of PII will be discussed, including the virulence factors that allow these bacteria to colonize, cause damage, and persist in the host. Finally, the host–pathogen interaction will be highlighted through outlining the normal host response to PII.

2 Routes of Infection

Prosthetic implant infection can originate from perioperative or postoperative exposure of the patient to the etiologic agent. Most infections that occur within 3 months of implantation are due to perioperative inoculation, where bacteria are introduced directly into the patient during, or soon after, surgery (Kaltsas 2004). This can occur due to the presence of commensal skin flora around the surgical site or from contamination from the healthcare provider. The usage of laminar flow in the operating room as well as antibiotic prophylaxis has decreased the incidence of microbial contamination of the surgical site during surgery (Hebert et al. 1996). The conditions of a surgical wound, including clotted blood and compromised soft tissues, also make it ideal for colonization. As well, infections can be caused by an associated bacteremia, where bacteria are seeded into the area of the implant, leading to an acute hematogenous infection. In these cases the prosthesis will function normally for some time before the onset of pain and swelling. Minor trauma likely predisposes the patient to infection by producing a small hematoma, vascular obstruction, and a subsequent bone necrosis that is susceptible to inoculation (Morrissy and Haynes 1989). Acute infection initially produces a local cellulitis that results in a destruction of leukocytes, increased bone pressure, decreased pH, and decreased oxygen tension. The collective effects of these physiologic factors further compromise the medullary circulation and enhance the spread of infection. Infection may proceed laterally through the Haversian and Volkmann canal system, perforate the bony cortex, and lift the periosteum from the surface of the bone. When this occurs in the presence of medullary extension, the periosteal and endosteal circulations are compromised; capillaries are lost, and large segments of cortical and cancellous bone die. If diagnosed more than 1 month after introduction into the body, the infection, whether caused peri- or postoperatively, is considered to be chronic and is often confused with aseptic loosening of the implant (Kaltsas 2004).

3 Diagnosis and Treatment of PII

Diagnosis of PII can be difficult. Because there is a lack of studies researching PII diagnosis and treatment, these often depend on the physician's personal knowledge, as well as tradition, and, ever increasingly, issues of liability (Hebert et al. 1996). Often, infection is only obvious if other indications are present, such as pain and fever. Aspiration of the suspected infected joint can aid in diagnosis, as can tests for PMN levels, C-reactive protein, and erythrocyte sedimentation rate (Bernard et al. 2004). However, sensitivities and specificities of these methods vary widely. PMN counts have a sensitivity that ranges from 20 to 83% and specificity from 80 to 100% (Bernard et al. 2004; Duff et al. 1996; Flivik et al. 1993; Lachiewicz et al. 1996; Spangehl et al. 1999). Measurement of C-reactive protein features sensitivity and specificity rates of 96–100% and 81–100%, respectively (Bernard et al. 2004; Sanzen and Carlsson 1989; Shih et al. 1987; Spangehl et al. 1999). The sensitivity and specificity of erythrocyte sedimentation rate varies from 29 to 100% and 0 to 100%, respectively (Bernard et al. 2004; Cuckler et al. 1991; Duff et al. 1996; Flivik et al. 1993; Lachiewicz et al. 1996; Levitsky et al. 1991; Roberts et al. 1992; Sanzen and Carlsson 1989; Shih et al. 1987; Spangehl et al. 1999; Teller et al. 2000; Thoren and Wigren 1991). Histopathological examination of the tissue surrounding the implant in order to gauge inflammation from infiltration of neutrophils can also aid in diagnosis and has a sensitivity of >80% and a specificity of >90% (Hebert et al. 1996).

Because aseptic loosening of the implant due to mechanical failure can closely resemble infection, it is of great importance to diagnose PII prior to corrective surgery (Bernard et al. 2004). There are several imaging methods that allow clinicians to envisage the region of possible infection. Conventional radiography is often used. However, radiographic changes to bone are often difficult to interpret, and can take up to 2 weeks following the onset of infection to reach a level that can be visualized because a 30–50% loss in bone density is often required (Butt 1973). Aseptic loosening can cause bone resorption, leading to false results as this will mimic infection radiographically (Bernard et al. 2004). Also, it can be difficult to determine the extent of infection. Ultrasound is another option, which is efficacious in showing fluid surrounding the artificial joint in suspected septic arthritis; however, this cannot differentiate between infection and joint effusion as a result of aseptic loosening (Bureau et al. 1999). Computed Tomography (CT) scans can be of better use due to their high level of detail. However, due to the problem of scatter in the presence of metal, this method is not always useful in the case of infected implants. It is also a very expensive procedure, which further limits its usefulness. Radionuclide scans are widely employed, and these help to identify areas of inflammation better than radiography alone. This method also has the benefit of being useful because there are no issues with scatter. One technique, technetium-99m methylene diphosphonate (Tc-99m MDP) scintigraphy, has proven to be effective and relies on pharmaceutical accumulation at areas of increased blood flow and bone repair. While sensitivity is high, specificity is lacking due to bone remodeling from the implantation of the

prosthetic device (Zimmerli et al. 2004), as well as new bone formation, fracture healing, heterotopic ossification, arthritis, and local minor trauma (Datz et al. 1984). Another radionuclide technique exploits indium-111 (In-111) labeled white blood cells, in which patient leukocytes are isolated, tagged with In-111, and injected back into the patient. This method works well in cases of suspected prosthetic implant infection when combined with bone marrow imaging with Tc-99m sulfur colloid marrow scintigraphy, since leukocyte uptake around prostheses may be caused by surgery. When an accumulation of leukocytes is seen, coupled with noncongruent bone marrow patterns and absent marrow uptake, an infection is likely. Tc-99m hexamethylpropylene amineoxime leukocytes (Tc-99m HMPAO WBC) are also employed to overcome the problems with In-111 WBC, such as the 24-h delay required for imaging, high levels of radiation in the spleen, and limited injection dose. Sensitivity of HMPAO WBC in diagnosis of PII is 63%, while specificity is 96% (Sonmezoglu et al. 2001). The combination of these two scans leads to a sensitivity of 100% and a specificity of 94% (Palestro et al. 1992). MRI is the final imaging technique that can be utilized for diagnosis. This method allows differentiation between bone and soft tissue infection. However, MRI also has the issue of scatter when metal implants are present.

The gold standard of diagnosis for PII is culture and identification of the infecting organism (Carek et al. 2001). Until a positive identification of the etiologic agent is made, the proper therapeutic treatment is delayed. Cultures cannot be taken from any superficial wounds or sinus tracts because they may feature contamination with skin flora (Hebert et al. 1996). Culture of the synovial fluid, in the case of infected joint replacements, can determine the etiologic agent 45–100% of the time (Hebert et al. 1996). Tissue biopsies taken via surgery are the most reliable, with accuracy ranging from 65 to 94% (Hebert et al. 1996). Once a positive diagnosis of infection has been made, treatment for PII often consists of antimicrobial therapy along with two-stage revision of the implant, where the infected implant is removed and replaced after antimicrobial therapy has ended and the infection is eradicated (usually >6 weeks) (Hebert et al. 1996; Zimmerli et al. 2004). If the infection is acute (less than 3 weeks duration), and the implant is stable, debridement and retention of the implant may be feasible (Hebert et al. 1996; Zimmerli et al. 2004). Debridement consists of the excision of all infected tissue, including bone, soft tissue, and sinus tracts (Zimmerli et al. 2004). If the soft tissue around the implant is relatively undamaged, one-stage revision, where the implant is replaced during the same surgery as the removal, is possible (Hebert et al. 1996). In patients who are severely immunocompromised, abuse IV drugs, or for whom no functional improvement after surgery is expected, the infected implant is often removed and not replaced. Alternatively, long-term antibiotic therapy can be commenced if the patient is inoperable, is on long-term bedrest, or is debilitated (Hebert et al. 1996). In all cases, a broad-spectrum antibiotic is given until culture results are obtained to cover the most common pathogens, and then specific therapy is begun once the infecting agent is identified.

Antibiotic treatment must be administered for at least 4–6 weeks, with 3 months being suggested for hip prosthesis infection and 6 months for infected knee

replacements (Zimmerli et al. 2004). When a two-stage revision is being performed, antibiotic therapy is administered for 6 weeks, with 2–3 weeks without antibiotics following the treatment before replacement of the device (Trampuz and Zimmerli 2005). For staphylococcal infections, the regimen of choice includes rifampin, often combined with quinolones. Because staphylococci are beginning to show increased resistance to quinolones, fusidic acid is an alternate option for combination with rifampin (Zimmerli and Ochsner 2003). For *Staphylococcus aureus* infections specifically, particularly methicillin-resistant *S. aureus* (MRSA), the antibiotic of choice is vancomycin, with teicoplanin, trimethoprim-sulfamethoxazole, or minocycline plus rifampin being alternatives (Carek et al. 2001). Beta-lactam antibiotics are most often administered for streptococcal infections, either alone or in conjunction with rifampicin (Everts et al. 2004).

4 Host Factors Involved in Prosthetic Implant Infection

These will be discussed in more detail in Chap. 12. In general, it is clear that the host immune system responds to both the implant and to the presence of colonized bacteria on the implant, yet this response is not effective at clearing the infection. This is most likely due to the fact that biofilms are inherently resistant to killing by factors of the host immune system. Avoidance of these host factors is discussed later.

4.1 Microbial Species Responsible for Prosthetic Joint Infection

Postoperative PII is generally monomicrobiotic in nature; that is, a single bacterial species is isolated from the infected region. Polymicrobial hematogenous PII is rare (Waldvogel et al. 1970a–c; . Staphylococci cause approximately 75% of infections, and almost all PII are caused by Gram-positive, aerobic cocci (Segawa et al. 1999). Coagulase-negative staphylococci are present in 30–43% of cases, while *S. aureus* is attributed to 12–23% (Zimmerli et al. 2004). *Staphylococcus spp.* are capable of causing PII in immunologically normal children and adults, as well as in immature and immunocompromised individuals. Incidences of *S. aureus* infection are becoming more worrisome with the emergence of multiple-antibiotic-resistant strains such as MRSA. Until recently, the only drug to which all *S. aureus* was susceptible was vancomycin, but vancomycin-resistant strains are beginning to be isolated as well (Pechous et al. 2004). *S. aureus* can cause infections when acquired in the community rather than in the hospital. Though these infections tend to be skin-related, community-acquired strains also acquire methicillin resistance and are becoming of greater concern, not only because they are beginning to become more virulent (Lindsay and Holden 2004), but also because these strains are able to infect hosts with no predisposing risk factors outside of the hospital setting. Community-

acquired strains are beginning to play a more prominent role in deep tissue infections. In one study, the community-acquired MRSA strain USA300 was shown to cause more than half of the PII seen in subjects (Kourbatova et al. 2005). *Staphylococcus epidermidis* causes the majority of PII, and the acquisition of biofilm-mediating and antibiotic-resistant genes contributes to its ability to do so. Though there are many strains of *S. epidermidis*, one study (Kozitskaya et al. 2005) showed that the majority of infections in a particular hospital were caused by one clone that was able to form biofilms and was resistant to beta-lactam antibiotics. This suggests that clinical isolates have an advantage when they contain these genes, which may be acquired by horizontal transfer (Kozitskaya et al. 2005). Overall, other pathogenic microorganisms associated with PII include *Enterococcus spp.* (3–7%)*, Streptococcus spp.* (9–10%), Gram-negative bacilli such as *Pseudomonas aeruginosa* and *Enterobacter spp.* (3–6%), *Mycobacterium spp.,* as well as anaerobic and mycoidal species (specifically *Candida spp.*) (Zimmerli et al. 2004). Each of these pathogenic species, individually, represents a small minority of infections. The immature or compromised immune status of the host is the primary cause of initial infection and development into a persistent and chronic PII infection by these other species.

4.1.1 The MRSA Epidemic

Once considered an aberrant, rare strain, MRSA is now the most commonly isolated nosocomial bacterial pathogen in most of the world (Grundmann et al. 2006). Approximately 40–60% of all nosocomially acquired *S. aureus* are methicillin-resistant, and these strains are now considered endemic in hospitals (Lindsay and Holden 2004). These infections can be devastating. A recent report of the Journal of the American Medical Association (JAMA) stated that approximately 94,000 individuals were diagnosed with invasive MRSA in 2005, leading to 19,000 deaths (Klevens et al. 2007). These numbers make deaths due to MRSA higher than those caused by HIV infection The infection rate of 31.8 per 100,000 for invasive MRSA is also higher than the rates for invasive pneumococcal disease, Group A streptococcus, meningococcal disease, and *Haemophilus influenzae* infection combined (Camargo and Gilmore 2008). This is an increasing burden on the healthcare system as MSSA (methicillin-susceptible strains) infections are not concurrently decreasing in number (Gould 2005). Colonization with MRSA does seem to be correlated with nosocomial infection, and patients who get infected while admitted often have a recurrence once discharged (Huang and Platt 2003) . As mentioned, MRSA is also rampant in the community; CA-MRSA is now also the most common cause of skin and soft tissue (SSTI) infections in the USA (Moran et al. 2006). CA-MRSA can cause significant mortality via invasive illness as well. CA-MRSA is all the more worrisome due to the fact that it is able to easily infect healthy young adults with no risk factors. This is exemplified in the rising incidence of CA-MRSA among young athletes (Centers for Disease Control 2003). As well, multidrug resistance also

seems to play a role in promoting biofilm formation (discussed later). In a study of 101 clinical isolates, 38% of MRSA strains could form biofilms, while only 14% of MSSA isolates could do the same (Kwon et al. 2008). This illustrates the idea that MRSA strains may be more apt to cause chronic infections such as PII than their MSSA counterparts. With the increasing age of the US population as the Baby Boomer generation nears retirement, the need for prosthetic implants will rise. This, combined with MRSA's increasing incidence in the community – both due to colonization acquired in the hospital and due to community-acquired disease – means that MRSA infections likely will continue to be a leading healthcare problem.

4.1.2 Virulence Factors of Staphylococci

S. aureus has a large number of virulence factors that allow it to cause a wide range of diseases. The *S. aureus* virulence factors are responsible for colonization and damage to the host, as well as avoidance of the immune response. These factors include regulatory systems that control virulence factor expression, biofilm formation, adherence proteins, toxins, and immuno-avoidance factors.

4.2 Regulation of Virulence Factors (Quorum Sensing)

Cell-to-cell communication between bacteria is a sophisticated process resulting from environmental cues, leading to the release of diffusible signal molecules that bind to a receptor, with the end result of modulated gene expression. This process, which is cell-density dependent (Miller and Bassler 2001), is termed quorum sensing, an expression first coined by Fuqua et al. (Fuqua et al. 1994). Quorum sensing depends upon the production and release of small molecules called autoinducers. For a molecule to be considered a cell-to-cell signal molecule (CCSM), it must meet several criteria, including the following: production during specific growth stages or environmental conditions; extracellular accumulation and the ability to bind to a receptor; and the generation of a concerted response when a critical concentration is reached, which extends beyond that required to simply metabolize or detoxify the molecule itself (Winzer et al. 2002).

There are currently four different identified autoinducer types utilized by bacteria for quorum sensing. AI-1, utilized by some Gram-negative species such as *P. aeruginosa*, is an *N*-acyl-homoserine lactone (AHL) (Pearson et al. 1994). AI-2, first discovered in *Vibrio harveyi*, is a furanosyl-borate-diester (Sun et al. 2004). As this molecule has been found to be produced by many bacteria (Sun et al. 2004), it is hypothesized that it may play a role in interspecies communication, as was shown in multispecies dental biofilms (McNab et al. 2003). This could have a significant implication in the formation of the multispecies biofilm infections common to contiguous focus osteomyelitis in the diabetic foot. Another autoinducer, AI-3, has been shown to modulate quorum sensing in Enterohemorrhagic *E. coli* (EHEC).

AI-3 has been shown to activate the transcription of the virulence genes of the LEE (Locus of Enterocyte Effacement) and can cross-talk with the human hormones epinephrine and norepinephrine to activate these genes (Sperandio et al. 2003). It is not currently understood whether norepinephrine triggers LEE expression in EHEC by directly substituting for AI-3, or whether it promotes endogenous AI-3 production or perhaps the synthesis of autoinducer(s) by the endogenous microflora (Vlisidou et al. 2004). As norepinephrine has been shown to increase adherence of EHEC to intestinal epithelium (Chen et al. 2003), perhaps AI-3 itself can also act in this same fashion to help increase colonization. Also, a study has found that incubation of *S. epidermidis* with norepenephrine significantly increased biofilm formation on polystyrene (Lyte et al. 2003). Norepinephrine also decreases bacterial clearance (Koch et al. 1996). The final autoinducer system is found in Gram-positive bacteria and involves a signal peptide. In this section, we will focus on the latter system in *Staphylococus spp.*

During early exponential growth, *S. aureus* has a low cell density and produces proteins involved in adherence and colonization (discussed later). However, in late exponential phase, the cell density increases, and *S. aureus* begins secreting proteins involved in acquiring nutrients, damaging the host, and disseminating to new areas (Shirtliff et al. 2002). Gram-positive bacteria utilize peptides typically structured as a 16-membered side chain to tail macrolytic peptide, with a short linear peptide attached to its amino-terminus (Miller and Bassler 2001). In *S. aureus*, quorum sensing is under partial or complete control of the staphylococcal accessory regulator (*sar*) and the accessory gene regulator (*agr*) systems. The *agr* locus, which is approximately 3 kb in size (Novick 2003), consists of two divergent transcription units, driven by promoters P2 and P3. The P2 operon contains four genes, *agrB*, *agrD*, *agrC*, and *agrA*, while the P3 operon codes for RNAIII (Novick et al. 1995). An octapeptide with a unique thioester ring structure (referred to as the *agr* autoinducing peptide (AIP)) is generated from its precursor, AgrD, and secreted out of the cell through the action of AgrB (Ji et al. 1997). AgrD is a propeptide that must be altered to become the active AIP with a unique thiolactone ring between a conserved central cysteine and the peptide's carboxy terminus (Novick 2003). This ring structure is vital for its function, as a replacement of the thiolactone by a lactone abrogates the activation properties of AIP (Novick 2003). Also, artificial compounds imitating this thiolactone structure were able to inhibit quorum sensing (Scott et al. 2003). It is believed that this processing is mediated by AgrB (Ji et al. 1997; Saenz et al. 2000; Zhang et al. 2002). AgrB is associated with the cytoplasmic membrane, with the N-terminus and C-terminus both being found within the cytoplasm, and six transmembrane segments in between (Zhang et al. 2002). Recently, AgrB was shown to be an endopeptidase (Qiu et al. 2005). It was also determined that two amino acids were required for this function – His^{77} and Cys^{84} (Qiu et al. 2005). Zhang et al. recently discovered that the AgrD propeptide is anchored into the cytoplasmic membrane via its amphipathic, α-helical N-terminal region, which stabilizes it for its interaction with AgrB (Zhang et al. 2004). AIP is sensed by the two-component signal regulatory system that is composed of AgrC and AgrA (Miller and Bassler 2001). As the concentration of AIP increases in the

extracellular microenvironment, the interaction between AIP and the histidine kinase receptor protein, AgrC, also increases. This interaction possibly acylates AgrC and enables it to phosphorylate and thereby activate an intracellular *agr*-encoded protein (AgrA) (See figure of agr system) (Mayville et al. 1999; Morfeldt et al. 1996). AgrA subsequently positively regulates transcription from P2 and activates transcription from P3 (Novick 2003) (Fig. 1).

The intergenic region between P2 and P3 likely houses the transcription factor binding sites necessary for activation of the operons. In fact, there is a 17-bp inverted repeat in this region that may be a bidirectional regulatory binding protein site (Bayer et al. 1996). However, AgrA does not contain a DNA binding domain. On the other hand, SarA, encoded by the regulatory locus *sar*, does contain a putative DNA-binding domain, and has been shown to bind a consensus motif within the *agr* promoter, leading to initiation of both RNAII (*agrBDCA*) and RNAIII transcription (Heinrichs et al. 1996). Recently, however, AgrA has been shown to bind to the P2-P3 promoter region via electrophoretic mobility shift assay (Koenig et al. 2004). SarA has also been shown to directly interact with the promoter regions of a number of other genes, including Protein A, fibronectin binding protein, and alpha-hemolysin (Chakrabarti and Misra 2000; Wolz et al. 2000). However, the interactions with this complex system are still being elucidated. Since *sar* and RNAIII homologs have been identified in a number of coagulase-negative *Staphylococcus spp*., including *S. lugdunensis* (Chan and Foster 1998), *S. epidermidis* (Lina et al. 1998; Van Wamel et al. 1998), *S. simulans*, and *S. warneri* (Van Wamel et al. 1998), this regulation system is also used by the other members of the staphylococcal genus. Therefore, it is

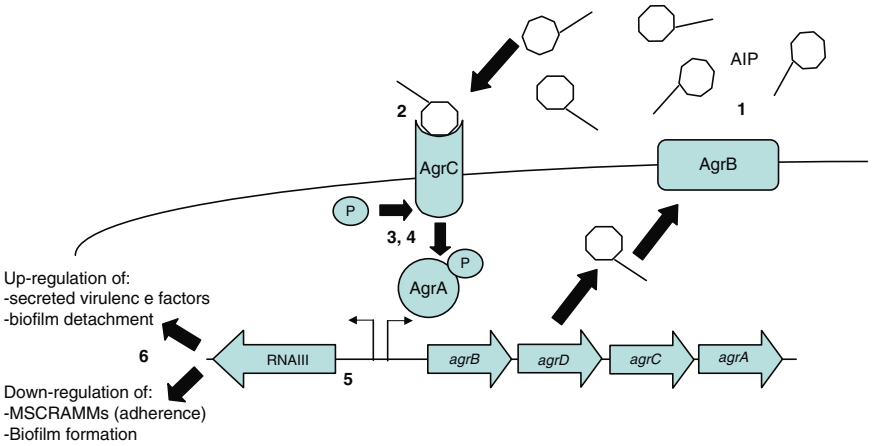

Fig. 1. The *agr* staphyloccal quorum sensing system. As the bacterial population grows, AIP molecules (produced from *agrD* and secreted via AgrB) accumulate in the extracellular millieu (1). These peptides then bind to AgrC (2), leading to the proteins autophosphorylation (3). This phosphate is then transferred to AgrA (4), which can bind to the intergenic region containing P2 and P3, leading to increased transcription of RNAIII (5). In this way the *agr* system is able to regulate the expression of genes for downstream effectors (6)

hypothesized that AgrA and SarA work cooperatively to bind within the *agr* promoter to initiate transcription. This is supported by a study in which RNAII and RNAIII levels decreased 2.6-fold in a *sar* mutant, suggesting that SarA is necessary for wild-type levels of transcription (Dunman et al. 2001).

During early logarithmic growth, a protein encoded by *rot* (repressor of toxins) inhibits the expression of *agr*-activated virulence factors (McNamara et al. 2000). Once activation of the *agr* and *sar* regulatory loci occurs during late exponential phase, there is increased transcription of an *agr* regulatory RNA molecule known as RNAIII (Balaban and Novick 1995). RNAIII, the product of the P3 promoter, is the real effector of the Agr response, in that it positively controls the expression of secreted proteins and negatively regulates cell surface-associated proteins (Zhang and Ji 2004). RNAIII immediately blocks the production of cell wall proteins that are upregulated in early exponential phase, such as coagulase, Protein A, and the fibronectin binding proteins (Chan et al. 2004), and, with a hypothesized timing signal, upregulates transcription of extracellular pathogenicity factors (such as exotoxins). The chief regulatory function of RNAIII is at the level of transcription by an unresolved mechanism, but may involve one or more regulatory proteins (Morfeldt et al. 1996). This RNA molecule is also capable of controlling production of at least two virulence factors, alpha-hemolysin (*hla*) and Protein A (*spa*), at the level of translation. Recently, a *hla* knockout was shown to be deficient in biofilm production at the level of microcolony formation, suggesting that this protein may be necessary for cell-to-cell communication (Caiazza and O'Toole 2003). At the beginning of exponential phase growth, the expression of alpha-hemolysin is normally inhibited through intramolecular base pairing that blocks the ribosomal binding site (Morfeldt et al. 1995; Morfeldt et al. 1996). Later in exponential phase, RNAIII is expressed and folds into a stable but inactive regulatory molecule. After a significant lag, the secondary structure of RNAIII changes through an unknown agent, and the 5′ region of RNAIII is then able to hybridize with a complementary 5′ untranslated region of *hla* mRNA, thereby making the transcripts accessible for translation initiation (Morfeldt et al. 1995). Conversely, the 3′ region of RNAIII contains sequences complementary to the leader sequence of *spa*, and hybridization is believed to inhibit translation of Protein A. In addition, SarA (the primary product of *sar*) has been shown to have an inhibitory effect on the expression of a number of genes, including *cna*, *sea*, *sar*, and the *agr* operon (Wolz et al. 2000). Therefore, *sarA* expression may be autoregulated, but the interactions with this complex system are still being elucidated. Another product of the *sar* locus, SarU, has been shown to be a positive regulator of RNAIII (Manna and Cheung 2003).

As the concentration of AIP rises in the extracellular environment, the level of RNAIII also increases. As mentioned, RNAIII is the intracellular effector of the *agr* system (Novick et al. 1993), enabling the growth phase-dependent reduction in adherence factor production and increase in extracellular pathogenicity factor production. AIP is not only capable of activating the *agr* regulon in self strains, but can also inhibit the *agr* activation of other S. *aureus* strains. Based on *agr* sequences and precise recognition between the AIP and AgrC, there are four identified groups of *S. aureus* (Jarraud et al. 2000). The groups are defined by the ability to mutually

inhibit one another's *agr* response, resulting in a unique regulation in which *agr* activity, but not growth, is inhibited (Ji et al. 1997). In one study, chimeric AgrB molecules were created in which sections of AgrB2 were swapped into AgrB1 and vice versa, and processing of AgrD1 and AgrD2 was examined. The first transmembrane α-helix and the extracellular loop 1 of AgrB1 were the most vital segments in determining the precise processing of AgrD1. On the other hand, the same segment in AgrB2 was not imperative in its group-specific contact with AgrD2. In its place, the two hydrophilic segments (methionine 67 to glycine 75 and alanine 126 to lysine 141) in AgrB2 were necessary for its specific processing of AgrD2 (Zhang and Ji 2004). In another study, AgrC chimeras were constructed and tested for activation or inhibition specificity against different AIPs. This work showed that AgrC/AIP binding is hydrophobic in nature, but activation or inhibition of *agr* via this binding is allele-specific (Wright et al. 2004). It has been shown that the AIP from group I (AIP1) is able to potently antagonize AgrC from groups II and III (Fig. 2) (Bhasin et al. 1998; Boles and Horswill 2008). This brought about the idea that AIPs from one group of *S. aureus* could be used therapeutically against infections by other groups. In a mouse protection test, skin abscesses were attenuated when Group II AIP was coadministered with Group I *S. aureus* in a subcutaneous infection (Mayville et al. 1999). In a more recent study, it was shown that *agr* antagonists work to prevent *agr* activation during the initial growth phase of the bacteria. In a mouse abscess model, this was able to abrogate virulence, suggesting that the *agr* quorum sensing that occurs in the early part of infection is critical for bacterial survival and abscess formation (Wright et al. 2005). While these AgrC antagonists may help to eradicate systemic *S. aureus* infections, they are unlikely to be successful against biofilm infections such as PII (discussed later). Reactive oxygen species released from phagocytes have been shown to interfere with the Group I autoinducer peptide, suggesting that these cells are able

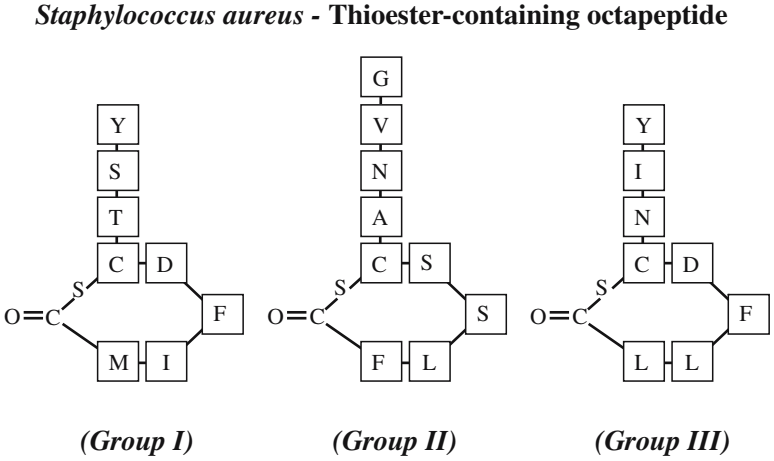

Fig. 2. Autoinducer peptide (AIP) structures. The three major groups of AIP molecules involved in quorum sensing are shown

to target quorum sensing in order to protect the host (Rothfork et al. 2004). This idea could also lead to novel therapeutic strategies in treating *S. aureus* infection.

The interaction of the quorum sensing system with certain host factors can also have a role in pathogenicity. Rothfork et al. showed that fibrinogen, a component of the inflammatory response that can cause staphylococcal clumping, was able to increase the virulence of wild-type *S. aureus* but not of an *agr* mutant. It was shown that fibrinogen's effect was on the delivery of AIP to the two-component AgrC-AgrA regulatory system that causes an increase in RNAIII transcription. The authors concluded that fibrinogen-induced clumping of *S. aureus* creates a microenvironment within the host that aids quorum sensing-dependent virulence, resulting in augmented bacterial burden and host morbidity and mortality (Rothfork et al. 2003).

Another two-component regulator, *saeRS*, has been shown to interact with Agr. This system represents the second two-component regulator involved in global virulence of *S. aureus* (Novick 2003). In a *sae-agr* double mutant, neither complementation with *agr* nor *sae* could restore production of secreted proteins (Novick 2003), suggesting that the two work together. Transcription of *sae* requires RNAIII, but *sae* is not required for *agr* activation, meaning that *sae* must be downstream of *agr* in the regulatory cascade (Novick and Jiang 2003). As varying environmental conditions, such as osmolarity and pH, seem to affect *sae* transcription, this locus may bridge the gap between cell density and environmental cues (Novick 2003).

4.3 Biofilm Formation

As mentioned previously, several bacteria responsible for PII are able to grow and persist chronically as a biofilm. Prosthetic implantation provides an ideal environment for biofilm infections to flourish, as the incision offers a route into the host, and the implant becomes an ideal attachment surface (Stoodley et al. 2005). A biofilm is defined as a microbially derived sessile community, typified by cells that are attached to a substratum, interface, or each other, are embedded in a matrix of extracellular polymeric substance, and exhibit an altered phenotype with regard to growth, gene expression, and protein production (Donlan and Costerton 2002). Biofilm depth can vary from a single cell layer to a thick community of cells surrounded by a substantial polymeric milieu. Structural analyses have shown that these thick biofilms possess a complex architecture in which microcolonies can exist in distinct pillar or mushroom-shaped structures (Costerton et al. 1995), through which an intricate channel network runs. These channels provide access to environmental nutrients even in the deepest areas of the biofilm.

Within this complex architecture, various miconiches of biofilm bacteria can exist. For example, DNA and protein production seems to be restricted to areas at the air/nutrient interface, and most of the biofilm's mass is metabolically inactive but viable (Rani et al. 2007). As well, biofilm-upregulated proteins have been shown via immunofluorescence confocal microscopy to be produced in some microcolonies but not others, or even within some individual cells within micro-

colonies (Brady et al. 2007). This indicates that protein production is heterogeneous, likely due to the varying levels of nutrient and oxygen availability within various areas of the biofilm.

By adopting this sessile mode of life, biofilm-embedded microbes benefit from a number of advantages over their planktonic counterparts. One advantage is the capability of the extracellular matrix to seize and concentrate a number of environmental nutrients, such as carbon, nitrogen, and phosphate (Beveridge et al. 1997). Another benefit to growing as a biofilm is the facilitation of resistance to a number of removal tactics, such as elimination by antimicrobial and antifouling agents, shear stress, host phagocytic clearance, and host oxygen radical and protease defenses. This innate resistance to antimicrobial factors is mediated through very low metabolic levels and radically downregulated rates of cell division (e.g., small colony variants) of the deeply entrenched microbes (Brown et al. 1988). One study even concluded that *P. aeruginosa* stationary phase cells and biofilm cells have comparable resistance to killing by antimicrobials (Spoering and Lewis 2001). While low metabolic rates may explain a great deal of the antimicrobial resistance properties of biofilms, other factors likely play a more major role. One such feature may be the capability of biofilms to act as a diffusion barrier to slow down the infiltration of some antimicrobial agents (Xu et al. 2000). For example, reactive chlorine species (such as hypochlorite, chloramines, or chlorine dioxide) in a number of antimicrobial/antifouling agents may be deactivated in the surface layers of the biofilm before they are able to disseminate into the lower layers (De Beer et al. 1994). In another study, alginate (a component of *P. aeruginosa* exopolysaccharide) was shown to be able to induce an α-helical conformation in antimicrobial peptides and likely entraps these peptides, preventing their diffusion into the biofilm (Chan et al. 2004). However, investigations of *S. epidermidis* biofilms with fluorescent molecules such as rhodamine demonstrated that these molecules were able to rapidly diffuse into the biofilm. Because the tracers used mimic the size of many antibiotics, at least in the case of *S. epidermidis*, prevention of antibiotic infiltration as a means of antimicrobial resistance is unlikely (Rani et al. 2005). As well, the nature of the biofilm allows for heterogeneous areas of oxygenation within the biofilm, with pockets of bacteria existing within an anaerobic environment (Stoodley et al. 2005). This can lead to the inactivation of antibiotics that are efficacious in physiologic conditions. Importantly, a recent study has shown that low-level exposure of *P. aeruginosa* to aminoglycoside antibiotics actually leads to the induction of biofilm formation (Hoffman et al. 2005). Resistance to the host response may include the prevention of host inflammatory molecules from entering the biofilm, although white blood cells are not limited in their ability to penetrate into staphlyococcal biofilms (Leid et al. 2002) As well, the host response can cause host cell lysis and subsequent damage to the host tissue. This can lead to the release of host cell components, which serve as nutrients for the bacteria (Stoodley et al. 2005).

The final benefit to the biofilm manner of growth is the potential for dispersion via detachment. As mentioned, microcolonies exist in discrete mushroom-shaped or tower structures. These microcolonies may detach under the direction of mechanical fluid shear or through a genetically programmed response that mediates

the detachment process (Boyd and Chakrabarty 1994). Under the direction of fluid flow, this microcolony travels to other regions of the host to attach and promote biofilm formation in previously uninfected areas. In *S. aureus*, this movement is via *tethered rolling* of biofilm flocs via viscoelastic attachments to the substratum (Rupp et al. 2005). In addition, detachment and seeding of virgin surfaces may be accomplished by the migration of single, motile cells from the cores of attached microcolonies (Sauer et al. 2002). Therefore, this advantage allows an enduring bacterial source population that is resilient against antimicrobial agents and the host immune response, while simultaneously enabling continuous shedding to encourage bacterial spread.

Patients with PII display many of the signs currently associated with biofilm-associated infections in other diseases, including negative culture results even in the face of signs of infection; chronicity of infection with periodic acute, systemic disease; and minimal responsiveness to antibiotics (Stoodley et al. 2005). However, because these symptoms can closely resemble those of aseptic loosening, it is of paramount importance to continue researching novel means of diagnosing these implant-associated biofilm infections (discussed earlier).

Staphylococcus spp. produce a multilayered biofilm embedded within a glycocalyx, or slime layer (Gristina et al. 1985). The glycocalyx develops on devitalized tissue and bone (such as the involucrum), or on medically implanted devices, to produce an infection (Akiyama et al. 1993). The presence of implants is a predisposing factor in the development of infection since they are coated in host proteins, including fibrinogen and fibronectin, soon after implantation, and this host protein coating provides an excellent source of attachment for any bacteria remaining after debridement surgery (Herrmann et al. 1988). Once attached, the bacteria can form the glycocalyx, which protects the bacteria from normal host defenses and systemic antibiotics (Oie et al. 1996), particularly those active against cell wall synthesis (Cerca et al. 2005). Though one study shows that biofilm formation is not necessary to cause persistent infections (Kristian et al. 2004), biofilms are difficult to eradicate and thus deserve special attention.

Early studies described the solid component of the glycocalyx as primarily composed of teichoic acids (80%) as well as staphylococcal and host proteins (Hussain et al. 1993). Host-derived proteins, such as fibrin, may result from the conversion of fibrinogen by the staphylococcal coagulase–prothrombin complex (Akiyama et al. 1997). In later studies, a specific polysaccharide antigen named polysaccharide intercellular antigen (PIA) was isolated. PIA is composed of β-1, 6-linked *N*-acetylglucosamine residues (80–85%) and an anionic fraction with a lower content of non-*N*-acetylated d-glucosaminyl residues that contains phosphate and ester-linked succinate (15–20%) (Mack et al. 1996). PIA is a polymer of approximately 130 residues, but other sizes of this β-1,6-linked *N*-acetylglucosamine have been identified, termed PNAG-I (the immunogenic 460-kDa compound), II (100 kDa), and III (21 kDa) (Maira-Litran et al. 2002). Depolymerization of PIA was recently shown to disrupt biofilms of *S. epidermidis*, supporting the role of this substance in biofilm formation (Itoh et al. 2005). PIA is produced in vitro from UDP-*N*-acetylglucosamine via products of the intercellular adhesion (*ica*) locus

(Cramton et al. 1999). The genes and products of the *ica* locus [*icaR* (regulatory) and *icaADBC* (biosynthetic)] have been demonstrated to be necessary for biofilm formation and virulence, and are upregulated in response to anaerobic growth, such as the conditions seen in the biofilm environment (Cramton et al. 2001). Though not found in all PII-causing clinical isolates of *S. epidermidis*, when the *ica* locus is present, it is always in its entirety, with every gene present (Arciola et al. 2005). This locus has been shown to be necessary for biofilm formation in *S. epidermidis* and for increased virulence in a rat model of infection (Li et al. 2005). Other studies, however, do not see a significant correlation between the presence of the *ica* locus and biofilm formation, but do find a relationship between the transcription of *icaA* and *icaD* and biofilm production (Cafiso et al. 2004). In another study, analysis of 112 *S. aureus* clinical isolates showed that, while all had the *ica* genes, none produced biofilms (Kim et al. 2008). Therefore, the relationship of the presence of *ica* and biofilm production is still being debated. In *S. aureus*, the *ica* locus seems to be dispensable, as the activation of the *ica* locus in four clinical isolates did not always lead to an increase in biofilm formation (Fitzpatrick et al. 2005a). This alludes to a possible *ica*-independent mechanism of biofilm formation. Deacetylation of PIA via IcaB has been shown to be important for biofilm formation (as an *icaB* mutant was unable to attach), resistance to phagocytosis by neutrophils, and colonization in a mouse model of infection (Vuong et al. 2004b). The regulation of *ica* in *S. epidermidis* is via reversible inactivation by insertion sequence (IS*256*) phase variation in 25–33% of variants (Conlon et al. 2002), and this has recently been observed in some *S. aureus* strains as well (Kiem et al. 2004). Recently, phase variation due to IS256 insertion was shown to be regulated via the stress-response sigma factor SigB (discussed later) (Valle et al. 2007). The protein GdpS has also been shown recently to be involved in biofilm formation through regulation of *ica*. While its means of *ica* regulation remains unclear, it has been demonstrated that GdpS, which is involved in c-di-GMP synthesis in other species of bacteria, is working in a c-di-GMP-independent manner in staphylococci (Holland et al. 2008). Regulation of PIA synthesis also seems linked to the TCA cycle, as when the TCA cycle was disrupted, PIA production was decreased (Vuong et al. 2005). Changes in the cycle may be utilized by the bacterial cells to detect alterations in the environment. Temperature may also play a role, as it has been shown that both elevated and decreased temperatures can induce *S. epidermidis* biofilm formation (Fitzpatrick et al. 2005b). Other levels of control in *S. epidermidis* are accomplished through IcaR-mediated transcriptional repression (relieved by ethanol stress) and the *sigB* operon product σ^B (regulated by operon genes *rsbU* and *rsbV*). Jefferson et al. (Jefferson et al. 2003) recently discovered that IcaR binds to a 42-bp region just upstream of *icaA*, and hypothesize that its role is to sterically hinder the binding of σ^B, thus preventing activation of the *ica* locus. Because σ^B regulates the expression of many genes involved in surviving in times of environmental stress, it has been hypothesized that PIA serves to protect *S. epidermidis* cells from these stresses (Jager et al. 2005). However, further studies are needed to evaluate this theory. Another component of *S. epidermidis* biofilms is poly-dl-glutamic acid (PGA), which is encoded by the *cap* locus. This locus is ubiquitous among *S. epidermidis* strains, unlike the *ica* locus, and was found to increase

resistance to high salt and the innate host immune response (Kocianova et al. 2005). Expression of PGA was also shown to be necessary for infection of a PII mouse model in this study.

In *S. aureus*, several virulence factors are σ^B-regulated genes, including clumping factor, fibronectin binding protein A, and coagulase (Nair et al. 2003; Nicholas et al. 1999), all of which are positively controlled, as well as alpha- and beta-hemolysin, enterotoxin B, SplA (a serine protease), cysteine protease (SplB), the metalloprotease Aur, staphopain, and leucotoxin D, all of which are negatively regulated (Kullik and Giachino 1997). Thus, the genes needed for attachment and biofilm formation are upregulated by σ^B. However, biofilm formation in vivo does not necessarily require σ^B, as a recent study showed that in a catheter infection model, both wild type and SigB-deficient mutants were able to form biofilms equally well (Lorenz et al. 2008). Also, IcaR is a strong negative regulator of the *ica* locus, as deletion of *icaR* augmented PIA production by nearly tenfold, and increased transcription of the *ica* locus approximately 100-fold (Jefferson et al. 2004). Another gene, *rbf*, has recently been identified by transposon mutagenesis. The Rbf protein was shown to be important in multicellular aggregation during biofilm formation, and also in the induction of biofilm formation by NaCl and glucose, but had no effect on *ica* transcription (Lim et al. 2004).

In addition to PIA, a number of other studies have elucidated vital genes and their products in the development of staphylococcal biofilms. There is recent evidence that attachment of bacterial cells to a polymer surface, a prerequisite for biofilm formation, may be promoted by an autolysin of *S. epidermidis* (Heilmann et al. 1997); the homologue in *S. aureus* (*atl*) may also function in this manner. In fact, a regulatory system termed WalK/WalR has been shown to upregulate AtlA and biofilm formation in *S. aureus* (Dubrac et al. 2007). Recently, an ica-independent biofilm phenotype was determined to be due to FnBPA and B, two proteins involved in initial ligand binding. This biofilm growth was limited to MRSA strains and could be activated through increased glucose concentrations in vitro (Huang and Platt 2003). A two-component regulatory gene locus that mediates adhesion and influences biofilm formation in *S. aureus* has also been studied recently. This locus is a system encoded by *arlRS*, a member of the OmpR-PhoB family of response regulators, that is regulated by the *agr* and *sarA* loci (Fournier and Hooper 2000; Fournier et al. 2001). When upregulated, the product of *arlS* prevents biofilm formation and may mediate attachment to polymer surfaces by affecting peptidoglycan hydrolase activity. Recent work determined that *arlRS* negatively regulates genes involved in the early attachment of *S. aureus* to surfaces, and that its repression is in effect under both static and flow growth conditions. In an *arlRS* mutant strain, biofilm thickness is significantly greater than wild type, but this increased density is not due to increased PIA production, *agr* activity, or autolysin (Toledo-Arana et al. 2005). Extracellular teichoic acids have been shown to be crucial in the development of *S. epidermidis* biofilms (Sadovskaya et al. 2005), and their structure is also crucial. Specifically, the addition of D-alanine esters to teichoic acids via *dltA* may be an important factor in imparting the proper charge balance on the Gram-positive cell surface, enabling initial attachment and subsequent biofilm formation. The accumulation associated protein (Aap) of *S. epidermidis* is implicated in biofilm

formation in strains that lack the *ica* locus. In particular, Aap seems to be processed to a truncated form by host proteases, and this smaller form is able to induce biofilm formation, thus aiding the bacterium in avoiding the host response (Rohde et al. 2005). Monoclonal antibodies generated against Aap are able to block biofilm formation by *S. epidermidis*, supporting the protein's importance (Sun et al. 2005). A similar protein in *S. aureus*, SasG, has been implicated in nasal colonization in vivo (Corrigan et al. 2007). Another *S. aureus* gene, biofilm associated protein (Bap), which was required for biofilm formation on inert surfaces, was discovered via transposon mutagenesis. This protein is found on the cell surface and its gene expression is positively regulated by the SarA protein (Trotonda et al. 2005). Found in mastitis-causing *S. aureus* strains, biofilm production has been shown to be inhibited through the binding of calcium ions to this protein, illustrating the idea that mastitis may be exacerbated by a lack of biofilm formation (Arrizubieta et al. 2004). This gene is encoded on the pathogenicity island SaPIbov2, and evidence of horizontal gene transfer of this island between staphylococcal species is beginning to be uncovered (Tormo et al. 2005a). However, testing of 262 clinical and animal isolates showed that none carried the *bap* gene; thus, the in vivo significance of this protein may be doubtful (Vautor et al. 2008). Lipoteichoic acid (LTA) also seems important, as an LTA mutant completely lost its ability to form biofilms, likely due to changes in the surface hydrophobicity of the cells. Because prosthetic implants are often hydrophobic in nature, this could have important implications for potential biofilm therapies (Fedtke et al. 2007).

Besides polysaccharide and proteins, extracellular DNA (eDNA) has also been shown to be important for biofilm formation. Rice et al. recently showed that eDNA is important for *S. aureus* adherence and biofilm formation and that its release is due, at least in part, to the *cidA* murein hydrolase regulator (Rice et al. 2007). It is thought that the CidA protein is a holin that allows for bacterial cell lysis. The autolysin AtlE in *S. epidermidis* has also been implicated in this process for the release of chromosomal DNA and early attachment (Seol et al. 1997). The *cidA* gene's expression has been shown to be upregulated by CcpA, a protein involved in catabolite repression that augments biofilm production in the presence of glucose. CcpA has also been shown to upregulate *ica* (Seidl et al. 2008). Though also present in *S. epidermidis* biofilms, eDNA seems to play a more vital role in *S. aureus* biofilms, as DNAse I was able to prevent biofilm formation by this species, as well as promote detachment of preformed biofilms and render *S. aureus* biofilms sensitive to detergents, while *S. epidermidis* biofilms were less affected (Izano et al. 2008).

In another study, the differential gene expression in planktonic (shaken) vs. biofilm (static) *S. aureus* cultures was evaluated, and five genes whose expression was increased in biofilms were identified (Becker et al. 2001). These included the gene encoding threonyl-tRNA synthetase (upregulated by amino acid starvation), three oxygen starvation response genes, and the ATPase ClpC. This protein has been shown to be involved in regulating the TCA cycle and entry into death phase, as well as the response to environmental stresses (Chatterjee et al. 2005; Frees et al. 2004). Another study employing microarrays to study differential gene expression between these conditions found 48 genes that were enhanced at least twofold in the biofilm

compared to planktonic conditions (Beenken et al. 2004). Resch and colleagues recently discovered that, besides the genes mentioned earlier, other genes upregulated under biofilm conditions include *sdrC*, which is involved in binding to bone sialoprotein; staphylococcal secretory antigen A; and staphyloxanthin, a pigment involved in protection from UV radiation (Resch et al. 2005). Yao et al. performed analogous studies on *S. epidermidis*, and found that, like in *S. aureus*, many metabolic and secreted virulence genes are downregulated, while expression of genes involved in salt protection, stress response, and resistance is increased under biofilm conditions (Yao et al. 2005). As mentioned earlier, research has shown that biofilm formation is upregulated by anaerobic, osmotic, and ethanol stress due to the stress-induced alternative sigma factor, σ^B (Rachid et al. 2000). The overriding theme is that proteins involved in cell wall synthesis and protection from environmental stresses are upregulated in biofilm conditions, while virulence factors and secreted toxins are increased during planktonic growth.

4.4 Adherence to Host Tissues/Implants

The ability of *S. aureus* and *S. epidermidis* to adhere to host cells and implanted biomaterials is crucial in initiation of infection. This adherence is what allows *Staphylococcus spp.* to subsequently colonize in the form of a biofilm. *Staphyloccus spp.* have several proteins, termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that can bind to a variety of host proteins that comprise the extracellular matrix, including host proteins such as fibrinogen, fibronectin, collagen, and elastin (Williams et al. 2002). Soon after implantation, foreign material will be coated with these host proteins. Because *S. aureus* and *S. epidermidis* have adhesins that specifically bind to these host factors, the implant becomes an ideal environment for bacterial attachment and subsequent infection. The MSCRAMM proteins include the fibrinogen binding proteins Fib, CflA, and FbpA; the fibronectin binding proteins FnbA and FnbB; the collagen receptor Cna; and EbpS, which is important in binding elastin. All of these adherence proteins are alike in that they possess a LPXTG motif in their C-terminal domains, which is cleaved by sortase so that the N-terminal ligand-binding domain is attached to the cell wall surface (Harris et al. 2002). Besides initial adherence, these proteins can also be important for other functions. For example, fibronectin binding protein has been show to be necessary for invasion of host cells by *S. aureus* (Dziewanowska et al. 1999). This protein mediates invasion via the $\alpha5\beta1$ integrin (Sinha et al. 1999). Eap, an extracellular protein that can bind to a variety of host proteins, has also been shown to be important for eukaryotic cell invasion (Haggar et al. 2003).

Because these proteins are vital for infection, several therapeutic strategies are currently under development to target the MSCRAMMs, particularly ClfA. Humanized monoclonal antibodies against ClfA have been shown to have efficacy in binding fibrinogen and in preventing MRSA infection in animal models of sepsis and endocarditis (Patti 2004; Vernachio et al. 2003). A mouse monoclonal antibody

against ClfA has also demonstrated an ability to block *S. aureus* adhesion, to displace previously adherent *S. aureus*, and to prevent infection in a murine sepsis model (Hall et al. 2003). In another study, monoclonal antibodies raised against a peptide fragment of Cna were shown to be able to block binding of collagen by the receptor, and to also block collagen binding to intact *S. aureus* cells and attachment of *S. aureus* to a collagen substrate (Visai et al. 2000).

4.5 Avoidance of the Host Immune Response

S. aureus has several tools to prevent clearance by the immune system. Capsular polysaccharides prevent the bacterium from being phagocytosed by immune cells such as macrophages and PMNs. Though complement proteins and antibodies can be deposited on the bacterial surface, the capsule prevents the interaction of these proteins with their receptors on the host's phagocytic cells (O'Riordan and Lee 2004). Most *S. aureus* strains are encapsulated. There are currently 11 capsular serotypes, with serotypes 5 and 8 being most prevalent. These two polysaccharides are processed by proteins of the *cap5* and *cap8* loci, respectively. Strains that express the type 5 capsule are more virulent than acapsulated strains in animal models of infection (Tzianabos et al. 2001), and phagocytosis of capsulated strains is lessened due to the need for anticapsule antibodies and not simply complement binding (Bhasin et al. 1998; Cunnion et al. 2001; Tzianabos et al. 2001). The virulence of strains expressing these two capsules has been shown to differ, with *S. aureus* that expresses type 5 capsules being more virulent than strains expressing type 8 (Tzianabos et al. 2001). Isogenic mutants lacking type 5, type 8, or both capsules supported the idea that the difference in virulence is due to the difference in capsule type, and that cells expressing type 5 capsule are more resistant to in vitro killing by whole blood and human PMNs than are cells expressing type 8 capsules (Watts et al. 2005). Adherence and capsule production seem to be inversely regulated, with adherence being maximal at times when capsule production is lessened. This could be expected since a thick capsule would effectively mask adherence proteins on the cellular surface (O'Riordan and Lee 2004). Capsules have also been correlated with abscess formation in rat models of *S. aureus* infection (Tzianabos et al. 2001).

Protein A (Spa) is another mechanism employed by *S. aureus* to avoid the host immune response. This 47-kDa, cell wall-associated protein binds the Fc portion of host immunoglobulins, thus rendering them ineffective by pointing the Fab fragments (antigen binding domain) out, away from the bacterium, consequently preventing opsonization (Gao and Stewart 2004). Spa is expressed maximally during exponential growth, unlike most staphylococcal exotoxins, and seems to be negatively regulated by the *agr* system (Recsei et al. 1986). During active infections, Spa can be released from the bacterial surface (Silverman et al. 2005). Spa has been shown to be important in contributing to staphylococcal sepsis through binding to the TNFα receptor and mimicking TNFα (Gomez et al. 2004).

This leads to a strong proinflammatory response in the host and subsequent damage to host tissues (Fournier and Philpott 2005). Spa has also been shown to target marginal zone (MZ) B cells, which are then apoptotically deleted (Goodyear and Silverman 2004). This function has given Spa an identity as a sort of B cell *superantigen* (superantigens discussed later) (Silverman et al. 2005). Thus, Spa may not only function in preventing opsonization via the adaptive immune response, but also stop the B cells before they can even produce antistaphylococcal antibodies, as well as the more predetermined response of *innate-like* B-1 B cells (Silverman et al. 2005).

Another means of avoiding the host immune response is via invasion of host cells. In general, *Staphylococcus spp*. are considered noninvasive, extracellular organisms, but in recent years it has been demonstrated that these bacteria can enter eukaryotic cells (Alexander and Hudson 2001), including fibroblasts (Murai et al. 1992), epithelial cells (Bayles et al. 1998), and osteoblasts (Bost et al. 1999; Jevon et al. 1999). Adherence to cells is a prerequisite for invasion, and this adherence is mediated through the MSCRAMM proteins (discussed earlier). In particular, fibronectin binding protein (FnBP) seems to be the most important for invasion (Alexander and Hudson 2001). It is proposed that fibronectin serves as a sort of bridge, binding to FnBP on *S. aureus* and to β1 integrins on the host cell surface (Ruoslahti 1996), leading to receptor-mediated endocytosis (Alexander and Hudson 2001). Once inside the cell, *S. aureus* is able to avoid the humoral immune response as well as clearance by antibiotic treatment. The *agr* and *sar* quorum sensing systems seem to be implicated in causing apoptosis of invaded host cells (Wesson et al. 1998), and may provide a means of escape for the bacteria. Osteoblasts that have been invaded by *S. aureus* produce heightened levels of the proinflammatory cytokines IL-6 and IL-12 (Bost et al. 1999), which could aid to further destroy infected bone tissue during PII or osteomyelitis. Often, the invading *S. aureus* are *small colony variants*, which feature a defect in the electron transport chain (McNamara and Proctor 2000), leading to a slower metabolic rate. Small colony variants have also been implicated in chronic infection and show superior resistance to vancomycin and oxacillin (Chuard et al. 1997).

One of the key strategies of the innate response is production of nitric oxide (NO), which is able to modify numerous cellular targets such as lipids and DNA (Radi et al. 1991) (Wink et al. 1991). Though there is evidence that NO is found at high concentrations in areas where staphylococcal colonization occurs (Andersson et al. 2002) *S. aureus* is able to resist this compound. It has been shown that this resistance is due to S. aureus' ability to adapt to growth in a NO-rich environment via increased lactate dehydrogenase activity (Richardson et al. 2008). This gene is encoded by the hmp-ldh1 cassette, and mutation of ldh1 leads to decreased virulence and a competitive disadvantage in a murine model. Therefore, *S. aureus* is uniquely suited to thrive in the face of the innate NO response by converting its metabolism to homolactic fermentation during nitric stress (Richardson et al. 2008).

Other immunoavoidance factors or strategies are the gamma-hemolysin and Panton-Valentine leukocidin. These exotoxins work to elicit an inflammatory

response by affecting neutrophils and macrophages (Dinges et al. 2000). Gamma-hemolysin is also able to lyse many types of mammalian erythrocytes (Dinges et al. 2000). These toxins work to lyse immune cells by inserting into the cell membrane and creating a pore. Each toxin has two components, S and F, which alone are inactive but together have lytic activity. Gamma-hemolysin has two genes encoding S components (*hlgA* and *hlgC*) while PV leukocidin only has one S component gene (*lukS*-PV). Each toxin only has one F component gene (Dinges et al. 2000). Because any of the S components can combine with any of the F components from either toxin, there are six possible forms of gamma-hemolysin/PV leukocidin. Gamma-hemolysin has been shown to be an important virulence factor when expressed in conjunction with alpha-hemolysin (discussed later) in a model of septic arthritis (Nilsson et al. 1999). PV leukocidin is able to cause granule secretion in PMNs and release of inflammatory mediators (Siqueira et al. 1997). Finally, biofilm formation (discussed earlier) is crucial for immune evasion as well.

4.6 Damage to the Host

S. aureus has a myriad of toxins and secreted enzymes with which it can damage the host. Several of these are enterotoxins, which cause food poisoning. Another toxin, toxic shock syndrome toxin (TSST), causes toxic shock syndrome. These toxins are superantigens, which bind simultaneously and nonspecifically to MHC class II and T-cell receptors, constitutively activating the T cells and causing massive T-cell proliferation (Baker and Acharya 2004) and a TH_1 response (Krakauer 1995). This also leads to a large increase in cytokine production and inflammation, eliciting damage that weakens the host. There are 17 staphylococcal superantigens, including enterotoxins A, B, C, D, E, and G–Q, as well as TSST (Baker and Acharya 2004).

Superantigens consist of N- and C-terminal domains with a long, solvent-accessible α-helix in the center (Baker and Acharya 2004). A flexible disulfide loop is present in several enteroxins, and it is believed that this loop is responsible for the emetic properties of staphylococcal enterotoxins that are characteristic of food poisoning (Acharya et al. 1994). The main targets of superantigens are the CD4 T cells (Bavari and Ulrich 1995). Superantigens are not processed by antigen-presenting cells (APCs). These proteins instead can bind directly to MHC class II molecules on the APC surface. This binding is mediated, in the case of many enterotoxins, through the binding of a zinc ion to a zinc binding site on the toxin (Baker and Acharya 2004). Most superantigens bind to the Vβ region on the T-cell receptor (TCR), and up to 10% of resting T cells are stimulated to produce a TH_1 response (Baker and Acharya 2004). This response leads to the production of proinflammatory cytokines, including IL-2, IFNγ, and TNFα (Herrmann et al. 1992; Litton et al. 1994; Miethke et al. 1992), which leads to massive T-cell proliferation and the onset of shock (Johnson et al. 1992).

Another class, the exotoxins, includes four hemolysins (α, β, γ, and δ) as well as Panton-Valentine leukocidin. Alpha-hemolysin inserts into the eukaryotic cell membrane, forming a pore and lysing the cell. It is specifically known to lyse erythrocytes (Dinges et al. 2000), and also has cytolytic, dermonecrotic, and lethal properties. It is this exotoxin that bestows upon *S. aureus* its beta-hemolytic property (Bhakdi and Tranum-Jensen 1991). Alpha-hemolysin is encoded by the *hla* gene, and the structure of the protein is mainly composed of beta sheets (Dinges et al. 2000). Expression of *hla* is under the control of the *agr* locus, leading to expression of alpha-hemolysin during late exponential phase. Though produced by many strains, it has been demonstrated that TSS isolates often contain *hla* but do not produce alpha-hemolysin due to mutations that prevent translation (Dinges et al. 2000). Alpha-hemolysin monomers insert into eukaryotic cell membranes, where they form a heptamer and a small pore (Belmonte et al. 1987). This pore then allows for rapid efflux of K^+ and influx of Na^+, Ca^{2+}, and other small solutes. Osmotic swelling leads to lysis of the cell (Dinges et al. 2000).

Beta-hemolysin's function in disease is still being elucidated, but this toxin is found mostly in animal strains and shows specificity for sphingomyelin, which has high levels in macrophages (Dinges et al. 2000). It has been demonstrated that beta-hemolysin has phosphorylase C activity (Doery et al. 1963). Delta-hemolysin is encoded by *hld*, which is translated from RNAIII, the effector molecule of the *agr* quorum sensing system (Yarwood et al. 2004). It is capable of lysing macrophages and neutrophils, but, like beta-hemolysin, its role in disease is unclear (Dinges et al. 2000). The gamma-hemolysin and Panton-Valentine leukocidin, as mentioned earlier, work to elicit an inflammatory response by affecting neutrophils and macrophages (Dinges et al. 2000).

S. epidermidis has another means by which it is able to elicit host damage. A cell wall-associated PAMP (pathogen-associated molecular pattern) called phenol soluble modulin (PSM) (Mehlin et al. 1999) has been shown to be proinflammatory and chemoattractive, stimulating innate immune cells to cause higher cytokine production, degranulation, enhanced respiratory burst, and inhibition of apoptosis in neutrophils (Liles et al. 2001). There are three PSM molecules (α, β, and γ) (Mehlin et al. 1999) and while these peptides show similarity to other staphylococcal proteins, their biological roles are still being determined. Otto and colleagues have shown that PSM production is not essential for infection, as approximately 22% of clinical isolates do not produce the peptides (Vuong et al. 2004a, b). As well, PSMs seem to inhibit biofilm formation, and the genes encoding these peptides are the most significantly downregulated under biofilm growth conditions when compared with planktonic; concentrations of these peptides within biofilm cultures were also significantly less than in planktonic cultures(Yao et al. 2005). The researchers also determined that PSM production is controlled by the agr system; indeed, PSMγ is encoded by RNAIII (Vuong et al. 2004a, b). Agr mutant strains showed markedly downregulated production of PSM peptides (Batzilla et al. 2006). This regulation leads to a delayed onset of PSM production until the bacterial population has reached a threshold and may help to keep the bacteria *hidden* until they are at a level to resist the proinflammatory response.

The reduced production of these peptides in a biofilm state illustrates the less inflammatory, chronic nature of these types of infections compared with acute, planktonic-associated disease.

4.7 Quorum Sensing and Staphylococcal Biofilms

One of the most exciting areas of current research involves determining what influence quorum sensing has on the growth, development, and pathogenesis of staphylococcal biofilms. There is growing evidence that the *agr* phenotype and expression patterns may impact several features of biofilm behavior, including attachment of cells to substrates, biofilm detachment and dispersal, and even the chronic nature of many biofilm-associated infections (Yarwood and Schlievert 2003). However, the relatively few studies that have been done on the relationship between quorum sensing and biofilms seem to give conflicting results. Pratten et al. showed that there was little difference in biofilm formation between wild type and an *agr* mutant of *S. aureus*, and that *agr* and *sar* were expressed most highly in the deepest areas of the biofilm, which the authors contest would be expected with genes that are expressed in a cell-density-dependent manner (Pratten et al. 2001). Shenkman et al. showed that expression of RNAIII increases the binding of *S. aureus* to fibronectin, but decreases its binding to fibrinogen (Shenkman et al. 2002). Another study looked at the effect of mutating *sarA*, and found that this mutant had a decreased ability to form biofilms, which could be due to decreased binding to substrates such as fibronectin (Beenken et al. 2003). An additional study in which *sarA* was mutated found a subsequent decrease in transcription of the *ica* locus, which is responsible for production of polysaccharide intracellular adhesion (PIA), a necessary component of biofilm formation (Valle et al. 2003). This was recently shown to be the case in *S. epidermidis* as well (Tormo et al. 2005a, b). However, a different report in which *ica* was mutated showed that this mutant was still able to form a biofilm (Beenken et al. 2004), which means that lack of PIA, or *sarA*'s effect on the *ica* locus, must not explain why a *sarA* mutant is attenuated in biofilm formation. It is suspected that the expression of the *ica* genes occurs early in biofilm formation and is needed for initial colonization, rather than persistence (Vandecasteele et al. 2003). A recent study of biofilm growth in iron-limited conditions contends that Agr and SarA are both required for the expression of two adhesive factors that are required for low-iron biofilm growth – Eap and Emp – thus making quorum sensing a requirement for biofilms under iron stress (Johnson et al. 2008).

Agr-mediated quorum sensing activates the transcription of secreted proteins in late exponential phase, while subsequently downregulating expression of adhesion proteins. Vuong et al. (Vuong et al. 2000) studied the difference in biofilm formation between *agr*-positive and *agr*-negative strains. Out of 105 *S. aureus* strains, 78% of those that were *agr*-negative could form biofilms, while only 6% of the *agr*-positive strains did so. These results were found to be independent of PIA levels. In another

study, Vuong and colleagues (Vuong et al. 2004c) showed that an *agr* mutant in *S. epidermidis* was able to form a significantly thicker biofilm in a static in vitro biofilm model than could the wild type. AtlE production was increased in this mutant, which promotes attachment (Vuong et al. 2003). These results were confirmed by another study in which an *agrD* deletion mutant of *S. aureus* was employed under static conditions (Yarwood et al. 2004). Vuong et al. also showed that, in the wild type, *agr* expression was limited to the outer areas of the biofilm (Vuong et al. 2003), unlike the aforementioned study by Pratten et al., where *agr* was expressed most highly inside the biofilm (Pratten et al. 2001). As planktonic bacteria in this study also showed *agr* expression, the researchers concluded that *agr* may be involved in promoting biofilm detachment, rather than its formation (Vuong et al. 2004c). Along these same lines, a very recent study indicated that a peptide that mimics the *agr* peptide AIP-I is able to augment biofilm formation (Fowler et al. 2008). This phenotype is consistent with blockade of the AIP-I receptor AgrC-I (Vuong et al. 2000). The globlal regulator protein CodY has also been implicated in repressing *agr* during biofilm growth (Majerczyk et al. 2008). Therefore, targeting *agr* for treatment of biofilms may actually exacerbate the biofilm. As well, clinical isolates of *S. epidermidis* obtained from prosthetic implant infections were found to have significantly less *agr* function than isolates obtained from skin, further illustrating the idea that *agr*-targeted therapies would not be effective against biofilm infections (Vuong et al. 2004c). It has been hypothesized that delta-hemolysin, which is encoded within RNAIII and has surfactant properties, may contribute to the detachment of cells from both *S. aureus* and *S. epidermidis* biofilms (Vuong et al. 2003; Vuong et al. 2000). This was supported recently when exogenous purified AIP-I was added to biofilms and caused detachment of the cells via activation of the agr locus. This effect could also be induced through reduction in available glucose in the growth media, illustrating how environmental cues can upregulate Agr and lead to detachment (Boles and Horswill 2008). If this is in fact true, it has significant clinical implications. Cells that are expressing *agr* and actively detaching from a biofilm may initiate additional infection sites while also playing a role in the toxemia linked to acute staphylococcal infections. It is probable that these cells would express secreted virulence factors, such as exotoxins and superantigens. Meanwhile, as there is a high frequency of strains that are naturally *agr*-null (Shirtliff et al. 2002) or have defective *agr* system (Cafiso et al. 2007), cells that do not express *agr* and remain part of the biofilm may be a factor in chronic, low-level infections (Yarwood and Schlievert 2003).

Recently a study emerged that may tie together the varying opinions of the role of *agr* in biofilms. Yarwood et al. showed that there are virulence factor variants within a biofilm. The population that dominated the biofilm was non-hemolytic and thus, Agr deficient, which could be due to the repression of *sarU*. However, the Agr-positive population does remain, albeit at lower levels, and likely reflects the population that is able to detach and cause an acute illness (Yarwood et al. 2007).

Recently, biofilm formation by *S. aureus* was shown to be augmented in the presence of heparin, a normal component of the eukaryotic cell membrane and a commonly administered anticoagulant drug (Shanks et al. 2005). It was hypothesized

that this mechanism may be via cell–cell communication, possibly by inhibiting *agr*, since, as discussed earlier, *agr* mutants also exhibit robust biofilm growth. As well, because staphylococcal species have been shown to contain a heparin-binding protein (Fallgren et al. 2001), it was also hypothesized that *S. aureus* bacterial cells may utilize heparin to cross-bridge in order to better adhere to each other (Shanks et al. 2005). The notion that heparin can increase biofilm formation is particularly troubling in light of the fact that heparin is regularly given in the case of catheterization, where bacterial biofilms are often found (Gorman et al. 1993).

Another facet of quorum sensing and its relation to biofilms could lie in antibiotic resistance. It has been shown that while wild-type *S. aureus* shows appreciable resistance to both oxacillin and rifampin, an *agr* mutant is sensitive to rifampin (Yarwood et al. 2004). Because rifampin is often used with other antibiotics to treat staphylococcal biofilm infections, this finding could have clinical relevance; if *agr* could be targeted therapeutically, rifampin may be more effective. Targeting quorum sensing could be a way to treat antibiotic-resistant biofilm infections. The quorum-sensing inhibitor RNAIII-inhibiting peptide (RIP), a heptapeptide (YSPWTNF-NH2), is able to abrogate toxin production and biofilm formation (Balaban et al. 1998) by both *S. aureus* and *S. epidermidis* by preventing adhesion to epithelial cells and plastic polymers (Balaban et al. 2003). When rats were implanted with RIP-soaked grafts or injected with RIP and subsequently infected with *S. aureus* or *S. epidermidis*, the rats showed no infection compared with controls (Dell'Acqua et al. 2004). A more recent study further illustrated this idea by showing that soaking a graft with RIP and a variety of other antimicrobial compounds and then implanting it into a rat completely prevented the onset of biofilm infection on the graft by several *Staphylococcus* species (Balaban et al. 2005). Another study using a rat model of urinary catheterization showed that coating of the stents abrogated biofilm formation, particularly when combined with teicoplanin therapy (Simonetti et al. 2008). This implies that RIP may be successful if used therapeutically, perhaps in combination with antibiotics, but more studies will be needed to elucidate this.

4.8 Case Example of Normal Immune Responses in S. Aureus PII

During acute PII, the innate immune system reacts to the peptidoglycan wall (via *N*-formyl methionine proteins and teichoic acids) of *S. aureus* to create proinflammatory cytokines (such as IL-1, IL-6, and TNFα) and C-reactive protein. These factors allow the host to build up a protective inflammatory response that controls this pathogen and frequently clears the infection. However, when the infection is not resolved by the host's innate immune response, *S. aureus* is able to persist via a number of virulence factors and strategies, including its ability to invade and survive in mammalian cells, the production of a biofilm, or encasing itself within a thick, antiphagocytic capsule. As well, the cell-mediated (TH_1) and humoral (TH_2) adaptive immune responses are often inadequate. In a study by Yoon et al., using a murine model of

acute hematogenous osteomyelitis, the major cytokines of cell-mediated immunity (IL-2 and IFNγ) seemed to rise only transiently while inflammatory cytokines remained at elevated levels in infected bone (Yoon et al. 1999). This cytokine profile led to an early expansion and activation of T-cell subsets followed by apoptosis. Thus, *S. aureus* seemed to hinder the normal immune response by downregulating both T-cell immunity and the production of cytokines important in the adaptive response. While a staphylococcal infection typically pushes the immune system toward a TH_1 response, due to the low oxygen partial pressures of infected bone where immune cell function is inhibited, the efficacy of this response is debatable. Also, a study in mice showed that a high level of IFNγ (a TH_1 cytokine) plays a detrimental role in the eradication of staphylococcal infection, and the TH_2 response (evidenced through high levels of IL-4 and IL-10) is involved in host resistance to infection through regulation of gamma interferon (Sasaki et al. 2000). However, the requirement of the TH_2 response to clear *S. aureus* infection was questioned in a study using IL-4-deficient mice (Hultgren et al. 1999). It seems that a TH_2 response is only required for clearance of *S. aureus* infection in certain mice depending upon their genetic background. In addition, it has been determined that when IFNγ was administered to mice infected with *S. epidermidis* well after the initial inflammatory response, the animal was able to reduce the level of biomaterial-associated infection (Boelens et al. 2000). Also, while intracellular persistence occurred in untreated mice, those animals that received IFNγ did not demonstrate any Gram-positive intracellular invasion. Therefore, while an increase in TH_1 cytokines during the initial inflammatory response may often result in host tissue damage, pathogen eradication may occur when these cytokines are provided to the infected host after this early phase. This may be an important method to trigger a correct and appropriately timed TH_1 immune response.

In summary, upon infection with *S. aureus*, a strong native immune response, cytokine release, and high T-cell activation are elicited. This pathogen is able to use a number of immunoavoidance strategies during this time (discussed earlier), while the host's immune system concurrently causes damage to *self* tissues and blood vessels in the area of infection. This damage may lead to local circulatory and immune compromise. High levels of T-cell activation ultimately result in apoptosis and a helpless immune system, allowing *S. aureus* to persist. By artificially pushing the host immune system toward an effective TH_1 response (via administration of IFNγ) after the preliminary inflammatory response, this persistent pathogen may be cleared more easily by the host.

5 Special Case: Ilizarov Fixator

Though it used to be believed that adult bone was unable to grow, it is now known that such bones are able to be lengthened. Transosseous osteosynthesis, a method developed by G. Ilizarov, is now commonly used to treat many conditions involving bone nonunions (Ilizarov 1989a, b). A section of bone is removed, and a fracture is created above the empty space. An external metal frame, the Ilizarov fixator, is

applied to the limb, and screws are inserted from the frame to the bone. This holds the two bone segments apart, providing stabilization. The fixator is adjusted periodically to *stretch* the bone such that new bone will form in the fracture. This causes the bone segment to lengthen, and eventually the two segments will meet, thus filling the empty space.

The Ilizarov fixator is indicated in several clinical cases, often when sections of bone need to be removed. This includes compound fractures (Dagher and Roukoz 1991), osteomyelitis (Parsons and Strauss 2004; Pearson and Perry 1989), PII with subsequent prosthesis removal and debridement (Manzotti et al. 2001; Manzotti et al. 2002), and bone defects (Song et al. 1998). The device can also be useful in limb lengthening in children (Birch and Samchukov 2004), such as in the case of osteogenesis imperfecta (Saldanha et al. 2004). In cases of infection, the infected bone is debrided, leaving behind a dead space that can be large, depending on the extent of infection. The Ilizarov technique has been shown to be efficacous in lengthening bone and filling this space, with 100% union and >76% resolution of infection in one study (Parsons and Strauss 2004). However, because the device must be adjusted daily, it requires a good deal of labor and patient compliance to be effective.

Complications with the Ilizarov fixator can occur, and one such complication is the risk of pin site infection (Clasper and Phillips 2005; Naudie et al. 1998). Because the fixation device is external and must be attached to bone through the skin and soft tissues, the integrity of these tissues is compromised. This allows relatively easy access of bacteria into the soft tissues and bone. As well, the presence of the pin itself can prevent healing (Davies et al. 2005). Superficial pin site infections are quite common (Manzotti et al. 2001; Naudie et al. 1998; Tomak et al. 2005). Those infections that move deeper can be persistent and form sequestra and draining sinus tracts (Ring et al. 1996), necessitating removal of the pin (Tomak et al. 2005). In particular, those pins that are placed near joints are at a higher risk for causing sepsis, due to the amount of movement present at those sites (Hutson and Zych 1998). Infection is defined as the presence of tenderness, erythema, heat, and discharge (Hedin and Larsson 2004; Mahan et al. 1991). Severity of the infection is classified according to the Checketts-Otterburns scale, with a 1 being considered a minor infection and a 6 being a very severe infection of the pin tract that requires debridement (Dahl and Toksvig-Larsen 2004). Often these severe infections occur after removal of the device. Minor infections (1, 2, or 3 on the Checketts-Otterburns scale) can be managed with oral antibiotics and pin site care, and perhaps resiting of the pins; external fixation can continue. More severe infections involve multiple pin sites, severe soft tissue infection, or the spreading of the infection to the bone (Dahl and Toksvig-Larsen 2004). The most commonly found etiologic agents in pin site infections are *S. epidermidis* and *S. aureus* (Mahan et al. 1991). Because *S. epidermidis* is a skin bacterium, it is not surprising that it would be found in the pin tract, and may or may not cause infection. Proper care of the pin site can help to prevent these infections and also keep superficial infections from spreading to the deeper tissues or becoming systemic (Davies et al. 2005). However, the best course of treatment of pin sites is debatable, with some

advocating a nihilistic approach with simple daily showers for cleansing, and others suggesting a more aggressive treatment with antiseptics and bandaging (Hedin and Larsson 2004). When antiseptics were used, chlorhexidine proved to be more effective at preventing infection than saline solution. Chlorhexidine has antistaphylococcal properties, and using this solution for cleansing can help to lessen the need for prophylactic antibiotic treatment (Dahl and Toksvig-Larsen 2004; Hutson and Zych 1998).

6 Conclusion

Prosthetic implantation is an ever-increasing medical practice. With a high level of mortality and the cost of treatment being approximately $50,000 per patient (Hebert et al. 1996), PII is a distressing public health concern. The most common etiologic agents of PII are *Staphylococcus spp*., including *S. aureus* and *S. epidermidis*, which often harbor resistance genes allowing them to persist in the face of commonly used antibiotics. Staphylococci possess a number of virulence factors that allow them to colonize and damage the host, all the while avoiding the host's immune response. Quorum sensing allows for tight regulation of virulence factor expression. Perhaps the most important staphylococcal virulence factor is its ability to form a biofilm. This mode of growth allows the bacteria to persist in the presence of antimicrobials and the host response, leading to a chronic state of infection that can only be eradicated through removal of the infected device. With the increased usage of intramedullary rods, plates, and screws, as well as prosthetic joints, the number of prosthetic implant infections (PII) will only rise as well unless significant research is dedicated to developing better ways of treating and preventing these infections. Because *Staphylococcus spp*. rely on biofilm formation to cause persistent infections and on quorum sensing to trigger the expression of virulence factors, therapeutics aimed at these phenomena could prove to be promising candidates for the treatment of PII.

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Biofilms and Aseptic Loosening

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Abstract Orthopaedic implants play a remarkable role in maintaining health and mobility. Unfortunately, implanted orthopaedic devices sometimes fail owing to a variety of factors, one of the most important of which is bacterial infection. However, in a significant proportion of cases, failure can not be clearly attributed to infection based primarily on the inability to isolate an offending bacterium. Such failures are often referred to as "aseptic loosening". There is an ongoing debate as to whether at least some of these cases may in fact have an infectious etiology. If so, then there must be some explanation for why current diagnostic methods fail to detect the underlying infection. This is a critical debate in that the failure to detect infection necessarily compromises the ability to utilize the most appropriate antimicrobial therapy. This chapter summarizes the clinical issues surrounding aseptic loosening and discusses the possible reasons why conventional culture techniques may fail to detect infection.

1 Introduction

The medical significance of total joint arthoplasty is evident in the growing number of arthroplastic procedures. For instance, the National Center for Health Statistics reported a total of 165,000 total hip replacements (THR) and 326,000 total knee replacements (TKR) in the United States in 2001. According to the National Hospital Discharge Survey, these numbers had risen to 220,000 and 418,000, respectively, by 2003. It can be anticipated that this trend will continue as medical advances lead to longer life spans and the need to maintain mobility in an aging population. Indeed, projections made by the American Academy of Orthopaedic Surgeons indicate that the numbers of THR and TKR will increase to ~274,000 and 474,000, respectively, by 2030.

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Although the reported failure rate varies considerably, some studies suggest that ~8% of both THR and TKR fail to the point that surgical revision is required (Herberts and Malchau 2000; Robertsson et al. 2001). Based on the 2003 statistics cited earlier, this would represent 17,600 THR revisions and 33,400 TKR revisions in the United States alone. Based on average hospitalization costs of $45,000 for THR revision and $42,000 for TKR revision, this would represent a total cost of more than $2 billion per year. Although infection is an important factor contributing to implant failure, in most cases there is no clear infectious etiology (McDowell and Patrick 2005), and such cases are generally referred to as "aseptic loosening." Indeed, Wooley and Schwarz (2004) recently concluded from their review of the literature that more than 25% of all prosthetic implants will ultimately show signs of aseptic loosening, and the American Academy of Orthopaedic Surgeons (as cited by Greenfield et al. 2005) has estimated that this accounts for more than 40,000 total joint revisions per year in the USA alone.

There are a number of recent reviews that address possible mechanisms that contribute to aseptic loosening (Callaghan et al. 2004; Sundfeldt et al. 2006; Wooley and Schwarz 2004). It is clear that implant failure occurs in the absence of any infectious process, but there is also evidence to suggest that a significant number of cases may be initiated by bacteria or their products. Greenfield et al. (2005) concluded that bacterial lipopolysaccharide (LPS) may play a particularly important role by inducing cytokine production, osteoclast differentiation, and osteolysis. Indeed, many investigators have demonstrated that LPS adheres to polyethylene wear particles (Akisue et al. 2002; Bi et al. 2001; Cho et al. 2002), and Xing et al. (2006) recently demonstrated using a rat model that such particles decrease bone attachment to implants and that this effect is exacerbated by the presence of adherent LPS. The staphylococci are clearly the most prominent musculoskeletal pathogens, and although they do not produce the classic LPS endotoxins, they do produce components with endotoxin-like properties, including peptidoglycan fragments and various forms of techoic acid. Presumably, such products would remain present and are capable of inducing changes leading to loosening even in patients in whom the infection has been eradicated by host defenses or antimicrobial therapy, or both. Because samples from such patients would be culture-negative, the ultimate diagnosis would be aseptic loosening despite the fact that the precipitating events had an infectious etiology. Moreover, Xing et al. (2006) suggested that the presence of polyethylene wear particles around implants can promote the accumulation of LPS from exogenous sources and that this can induce changes that ultimately result in implant failure. This implies that failure may occur as a result of bacterial processes irrespective of whether the implant itself is, or ever was, infected. However, the issue here, and the focus of this chapter, is whether some cases of aseptic loosening do in fact arise as a result of ongoing but undetected infection of the implant and, if so, why clinical and microbiological evaluations fail to detect the presence of the causative bacteria. This is a critical issue because it has a direct impact on the need for antimicrobial therapy and the ability to implement the most appropriate form of therapy. Moreover, the failure to include adequate antimicrobial therapy in such cases would ultimately lead to failed revision

and a high rate of recurrence, and there is little doubt that recurrent cases become progressively more problematic to manage. There are several possibilities that may account for the failure to detect infection (McDowell and Patrick 2005; Nelson et al. 2005), and this chapter is devoted to a discussion of these possibilities and their potential impact on the diagnosis and treatment of failed orthopedic implants.

2 Is Aseptic Loosening Really Aseptic?

Many patients with infected orthopedic implants present clinically with few if any symptoms other than minor tenderness and pain in the affected joint at the extremes of motion. Indeed, a later chapter will discuss how various physiological parameters (e.g., erythrocyte sedimentation rate) and imaging modalities (e.g., radionuclide scans) can be diagnostically useful but are rarely definitive. It will also be appropriately pointed out that the utility of many imaging modalities is compromised by the presence of the implant itself. This accounts for why culture and identification of the offending bacterium remains the gold standard for the diagnosis of infected prosthetic devices. However, even this gold standard is far from absolute, and by definition it is inadequate in cases of aseptic loosening. For instance, Mikkelsen et al. (2006) used various culture techniques to examine 120 surgical biopsies from 118 patients undergoing knee arthroplasty. All patients were placed into the clinical categories of infected, aseptic loosening, or mechanical failure, based on preoperative data and operative observations. Of 58 patients diagnosed with aseptic loosening, none showed any degree of bacterial growth on culture. Such studies support the hypothesis that implant failure in these patients is in fact aseptic. However, of 26 patients judged to be infected, cultures were positive in only 13, and one of these was judged to be "insignificant." The high "false-negative" rate observed in this and similar studies makes it difficult to conclude that the failure to isolate bacteria in patients diagnosed with aseptic loosening can be taken as definitive evidence of the absence of infection. The question remains, then, whether aseptic loosening is not aseptic in a significant number of patients. Arthroplasty is a complicated procedure aimed at "fixing" an artificial implant in a structurally stable manner to living tissue, and there is certainly no reason to assume that this process cannot lead to changes at the implant–bone interface that ultimately result in failure in the absence of infection. That is particularly true since most implants are done in older patients. However, there is also no reason to assume that bacteria do not have the capacity to adopt a lifestyle that compromises both the structural stability of the implant and the ability to identify them as the culprit. The implication is that viable bacteria may persist on or around orthopedic implants in a form that precludes their detection. Certainly this would be true of biofilm organisms because they would be attached to the implant, tissue, or both, and would not be easily sampled for positive culture identification. If that is true, then it becomes necessary to understand how that occurs and how we can overcome the diagnostic barriers such bacteria living as biofilms present.

There would seem to be two obvious possibilities that might account for the failure to confirm infection despite its presence at a level that compromises an implanted orthopedic device. Both arise from the straightforward observation that bacteria have the capacity to tightly adhere to artificial surfaces implanted in the human body. The first possibility is that bacteria adhere so tightly that they cannot be removed by conventional microbiological methods, which are most often limited to aspiration of joint fluid or a swab of the implant and adjacent tissue, or both. The second is that bacteria are capable of adopting a lifestyle that precludes their detection by conventional cultural methods. These are not mutually exclusive possibilities, and perhaps the primary example that emphasizes this is the formation of a biofilm. Based on these possibilities, considerable effort has been directed toward developing alternative means of bacterial recovery (e.g., sonication of explanted devices) and alternative means of bacterial detection that are less reliant on the ability to culture viable organisms (e.g., polymerase chain reaction (PCR) and in situ methods of bacterial detection). In the following sections, we summarize these studies in an effort to answer the question of whether a significant number of cases of aseptic loosening may in fact have an infectious etiology. We then turn our attention to the adaptive response required for persistence within a biofilm and how that adaptive response might contribute to the inability to detect viable bacteria. Because the staphylococci are the predominant musculoskeletal pathogens, we focus this discussion of the adaptive response of the staphylococci to the biofilm lifestyle. We then conclude by discussing alternative possibilities that are not necessarily related to biofilm formation but may also affect the ability to recover viable bacteria using conventional microbiological methods. Included among these latter possibilities are formation of small colony variants (SCVs) and the presence of intracellular bacteria.

3 Alternative Means for the Detection of Bacteria on Explanted Devices

As noted earlier, one possible explanation for the failure to detect the presence of bacteria on a failed implant is that such bacteria are tightly adherent and therefore cannot be efficiently removed for cultural analysis. Two options to address this issue are alternative means of removal and direct detection on the surface of the implant without the need for culture. Although the latter does not necessarily demonstrate the presence of viable bacteria, it does address the issue of bacteria having been present on the implant at some point, and as discussed earlier, this may be sufficient to initiate the biological processes leading to implant failure even if the bacteria responsible for the primary event have been eradicated. We discuss these two approaches together because in many cases they have been used concurrently to address not only the possibility that aseptic loosening may not always be aseptic but also why many cases of apparent infection are culture-negative.

One of the earliest studies that attempted to address these issues directly was that of Gristina and Costeron (1985), who examined samples from 25 patients with

biomaterial-related infections. This included 17 patients with orthopedic implants. Samples included the implants themselves and associated tissue samples. In the former case, the explanted devices were placed in phosphate-buffered saline and then sonicated in an attempt to remove adherent bacteria. Tissue samples were first homogenized in a tissue grinder and then sonicated. Samples were then cultured both aerobically and anaerobically. What made this study unique is that duplicate samples were also processed for both scanning and transmission electron microscopy. Of the 17 patients with orthopedic biomaterial-related infection, 10 were found to be positive for glycocalyx-enclosed adherent bacteria by electron microscopy. Of these 10, only 8 were positive by culture. Although this may not seem like a dramatic difference, it does represent a 20% failure rate as defined by standard culture techniques. Moreover, this study was done in the relatively well-defined context of patients with clinical evidence of infection, and it is reasonable to suggest that the failure rate would be even higher in the less definitive clinical context of aseptic loosening. Dobbins et al. (1988) subsequently used a similar approach and found that 20 of 26 metallic scrapings taken from explanted devices were culture-positive after sonication. Seventy-five percent of the isolates were coagulase-negative staphylococci, all of which produced an extracellular glycocalyx. Importantly, only six of 28 swabs obtained from adjacent tissues revealed Gram-positive cocci, and only three yielded staphylococci on culture. It is also important to note that, unlike the study of Gristina and Costeron (1985), the implants included in this study were removed for reasons other than infection. This led the authors to conclude that "staphylococcus may persist in these sites for long periods without producing clinically apparent problems."

In a more recent report, Tunney et al. (1998) evaluated the relative ability to detect infection using the alternative methods of (1) culture of implants after sonication, (2) culture of adjacent tissue samples after homogenization, and (3) detecting the presence of inflammatory cells in the adjacent tissues. Bacteria were detected in 26 of 120 (21.7%) explanted devices after sonication. In contrast, standard culture techniques yielded only five positive cultures. This represents an 80% failure rate (21 of 26) of standard culture. Sufficient tissue was available for histopathologic analysis from 18 of the 26 patients who were culture-positive after sonication, and examination of tissues from these 18 patients confirmed the increased presence of inflammatory cells. Twelve of these 18 specimens were from patients with a preoperative diagnosis of either aseptic loosening or dislocation while the other six had a history of infection. However, four of the six patients with suspected infection were culture-negative from joint fluid aspirated preoperatively and from tissue samples removed during surgery. These results clearly emphasize the inadequacy of standard culture techniques and suggest the possibility that at least some cases of aseptic loosening may be misdiagnosed because of the failure to recover viable bacteria. Additionally, hospital records were available for 50 of 94 patients with culture-negative implants and a preoperative diagnosis of aseptic loosening or dislocation, and 87% of these patients also showed evidence of inflammatory cell infiltration in the adjacent tissues. In a subsequent study, Tunney et al. (1999) used standard culture techniques, immunofluorescence microscopy (IFM), and the

polymerase chain reaction (PCR) to examine an additional 120 samples from patients undergoing total hip arthroplasty. Based on the results of their previous study (Tunney et al. 1998), immunofluorescence was done with a monoclonal antibody specific for *Propionibacterium acnes* and a polyclonal antiserum prepared by immunizing rabbits with whole cells of *Staphylococcus epidermidis*. Importantly, the latter was subsequently shown to be cross-reactive with other staphylococcal species. IFM result was positive in 63% of the samples tested. Similarly, the result of their PCR protocol, which was targeted to a highly conserved region of the bacterial 16S ribosomal RNA (rRNA) gene, was positive in 72%. Inflammatory cell infiltration was observed in 73%. In contrast, culture of tissue samples and samples from implants taken after sonication were positive in only 22%. The authors concluded on the basis of their IFM and PCR results that the bacterial infection rate of hip prostheses is grossly underestimated by standard culture techniques even as applied after sonication.

One potential problem with studies employing highly sensitive techniques such as PCR is that the bacteria that are most often isolated from infected implants are also among the most common commensal species. This means that they may also be detected as contaminants, particularly when extraordinary measures are required to facilitate detection. Indeed, Trampuz et al. (2006) recently concluded that, although sonication can improve recovery, it also carries a risk of contamination particularly when done using bags to contain the explanted device. Additionally, PCR protocols are most often done with broad-range primers that will amplify a gene target (usually the 16S rRNA gene) that is highly conserved in diverse bacterial species. Although this increases sensitivity and coverage in terms of detecting different bacterial pathogens, it also increases the likelihood of false-positive results. Such protocols also fail to provide critical diagnostic information with respect to the identity of the offending pathogen. At the same time, it is possible to take additional steps to characterize the PCR products and thereby obtain additional diagnostic information. For instance, Fenollar et al. (2006) recently described the PCR-based analysis of 525 clinical samples obtained by needle aspiration and/or surgical biopsy from patients with suspected bone and joint infection. This included DNA sequencing of the PCR products, which allowed the investigators to identify the bacterial species present in each sample. Interestingly, Fenollar et al. (2006) detected several bacterial species that are rarely associated with human disease, and four species that had never been reported in association with human infection. This is important because it suggests that at least some culture-negative results may reflect the failure to use culture conditions appropriate for these species. Overall, PCR and standard culture yielded identical results in 475 samples (90.5%). This suggests that culture is relatively reliable even by comparison to a highly sensitive approach such as PCR. At the same time, every type of discrepancy (e.g., false-positives and false-negatives by both PCR and culture) was also observed with roughly equal frequency. Nevertheless, the collective evidence suggests that the sensitivity of PCR-based methods may be useful in establishing an infectious etiology even in culture-negative cases as long as appropriate controls are included (Kabayashi et al. 2006; McDowell and Patrick 2005), and this may offer an important

advantage with respect to determining definitively whether a significant number of patients diagnosed with aseptic loosening may in fact have an underlying infectious etiology.

To date, most studies aimed at examining alternative means of bacterial recovery and/or detection have been done in the clinical context of presumed infection, and relatively few have been carried out in the specific context of aseptic loosening. In an effort to address this issue, we carried out a study in which we examined explanted orthopedic devices from 21 patients, all of whom were carefully selected to exclude those with clinical evidence of infection (Nguyen et al. 2002). Specifically, patients were excluded if they had (1) revision for any reason other than presumed aseptic loosening, (2) prior periprosthetic infection in the index joint, (3) suspected infection at any other site based on clinical and laboratory findings (fever >100.4°F, signs or symptoms of skin or soft tissue infection, urinalysis suggestive of urinary tract infection, leukocytosis (WBC >12,000 mm^{-3}), or abnormal erythrocyte sedimentation rate or C-reactive protein), (4) use of antibiotics in the 2 weeks prior to surgery, (5) operative findings consistent with infection, or (6) revision arthroplasty that did not include removal of the femoral component. We also included a sterile, unused control device that was processed along with the explanted device at the time of removal. Specifically, the control and explanted devices were processed simultaneously by surgeons employing surgical isolator suits in an operating room with laminar airflow. Implants were individually placed into sterile bags containing sterile phosphate-buffered saline and sonicated using relatively mild sonication intensity (60 Hz for 30 min). This intensity was chosen because preliminary experiments confirmed that it was the maximum intensity that did not result in reduced bacterial viability at least as defined for *S. aureus*. Bacteria in the sonicate were then harvested by filtration through sterile, 0.45-μm filters. The filters were then placed "face-down" on sheep blood agar. Sonication, filtration, and plating were all done in a laminar flow hood that was presterilized using UV light. Culture results were recorded after 5 days at 37°C. Control studies using the same volume (250 mL) of phosphate-buffered saline containing known numbers of a *S. aureus* musculoskeletal isolate demonstrated that we could detect as few as 50 colony-forming units (cfu) per implant. Using this protocol, bacteria were detected on 2 of 21 (9.5%) explanted devices. However, we also cultured bacteria from one of the control samples. These results suggest that the incidence of undetected infection is much lower than previously reported, but it must be emphasized that we used very strict exclusion criteria and that our study involved a relatively small number of patients (which was unavoidable given the exclusion criteria). Also, our protocol would not have detected subclinical bacterial infections with fewer than 50 cfu per prosthesis. We also limited our study to aerobic culture, and there are reports suggesting that processing samples anaerobically can increase detection even when the infection is caused by bacterial species that are not strictly anaerobic (Tunney et al. 1999).

More recently, Bori et al. (2006) used intraoperative histology and culture to evaluate periprosthetic tissue samples from 61 revision arthroplasties suspected of aseptic loosening. Cultures were considered positive only when the same bacteria were isolated from at least two samples. These authors identified 12 culture-positive

cases, 11 of which were coagulase-negative staphylococci. Assuming none of these were false-positive results, the likelihood of which is increased by the criteria that the same bacteria be recovered from at least two samples, this would represent an infection rate of 19.6% despite an initial diagnosis of aseptic loosening. These authors also noted that intraoperative histology can be effective in cases in which infection is suspected, while it was less effective in the clinical context of aseptic loosening. Specifically, they found that only 6 of 12 culture-positive cases had clear evidence of inflammatory cell infiltration.

PCR-based studies have also been used to address the specific issue of aseptic loosening. Ince et al. (2004) described a study in which PCR was used to examine samples from 24 patients undergoing revision arthoplasty, none of whom had any clinical evidence of infection, and all of whom had negative culture results from preoperative synovial fluid. Intraoperative biopsy samples were examined by routine culture and by 16S rRNA PCR. Control samples were taken from 9 patients undergoing primary hip arthroplasty. These authors stated that "PCR and routine culture showed no microorganisms in either group, with the exception of 1 patient in the loosening group," and they concluded that PCR offered no diagnostic advantage when compared to routine bacteriologic culture. They also concluded that "loosening of cups ¼ do not usually result form nonculturable periprosthetic infection." However, identifying bacteria in even one of 24 patients represents a 4.2% infection rate, and given the number of patients undergoing revision based on a diagnosis of aseptic loosening, this could ultimately represent a significant number of patients with an underlying infectious etiology. Further evidence for this hypothesis comes from the results of Clarke et al. (2004), who used PCR to compare 113 specimens from 31 total hip revison arthoplasties diagnosed as aseptic loosening with 105 specimens from 28 primary hip arthroplasties. Bacterial DNA was found in 46% of the revision samples; however, it was also found in 21.4% of the primary samples. These experiments included multiple samples from the same patients, and it was ultimately concluded that samples from 52% of patients undergoing revision arthroplasty and 29% of patients undergoing primary arthroplasty contained bacterial DNA. It was assumed that positive samples from primary procedures represented contamination, which highlights the concern that the extreme sensitivity of PCR-based methods can lead to false-positive results (McDowell and Patrick 2005). However, even taking these false-positives into account, there was a significantly higher occurrence of positive samples in patients undergoing revision arthroplasty, and this suggests the presence of infection in a significant proportion of patients diagnosed with aseptic loosening.

As a final comment about PCR in the context of aseptic loosening, it should be emphasized that conventional PCR cannot distinguish between viable and nonviable bacteria. To the extent that "aseptic loosening" may be the ultimate outcome of physiological changes initiated by bacterial products even in the absence of an ongoing infection, this could be considered an advantage. At the same time, the fact that such changes were initiated by nonviable bacteria or their products does not directly address the need for antimicrobial therapy, and in that context it makes sense to develop methods that offer the advantages of PCR in terms of speed and

sensitivity but that can also be used to detect ongoing infection. One approach is the use of reverse-transcription PCR (Stoodley et al. 2005), which is based on detection of mRNA rather than DNA. Because bacterial mRNAs have a short half-life, their detection implies recent gene expression, which in turn implies viability and an active infection. It remains unclear, however, whether this approach offers a significant advantage in the context of aseptic loosening because one explanation for the absence of clinical evidence of infection in such cases is the presence of viable but metabolically inactive bacteria. This emphasizes the need to select the bacterial targets used to define active gene expression carefully in order to increase sensitivity as much as possible. Indeed, this is one advantage of studies aimed at defining patterns of gene expression in the specific context of growth and/or persistence within a biofilm.

A second alternative diagnostic approach that also does not address the issue of viable vs. nonviable bacteria, but does eliminate the need to recover bacteria from implanted devices, is the use of in situ methods of detection. For example, Krimmer et al. (1999) used a fluorescently labeled DNA probe corresponding to the bacterial 16S rRNA gene for in situ hybridization. They demonstrated the utility of this "FISH" (fluorescence in situ hybridization) approach with *S. epidermidis* within a biofilm and with SCVs of *S. aureus*. This is important because both of these are possible explanations as to why bacteria are not recovered from patients diagnosed with aseptic loosening. They also examined tissue taken during surgery from one patient with presumed infection but suspect culture results, and they were able to confirm the presence of *S. epidermidis* in situ. A recent adaptation of this approach is the use of fluorescently labeled peptide nucleic acids (PNA), which are hybrid molecules consisting of a peptide-based backbone linked to nucleic acid bases. This offers the advantage of reducing the charge on the molecule, which promotes entry into cells, without sacrificing the specificity of nucleic-acid hybridization. Gonzalez et al. (2004) showed that "PNA FISH" was both sensitive and accurate in the context of positive blood cultures, but this approach has not yet been evaluated in the context of orthopedic infection in general and aseptic loosening in particular. Although not directly relevant to the issue of in situ detection of bacteria, Nekhotiaeva et al. (2004) recently demonstrated that PNA technology could be used to limit gene expression in *S. aureus* and, by targeting the PNA to an essential gene, to inhibit growth. Assuming the genes most relevant to biofilm formation can be identified, this raises the possibility that PNA technology could be used not only diagnostically but also to limit biofilm formation in vivo. Although not specifically related to implant-associated infections, Sanderson et al. confirmed this FISH approach with tissue samples from patients afflicted with chronic rhinosinusitis (Sanderson et al. 2006). Using this molecular approach, they identified *H. influenzae* biofilms resident on the human nasal sinus tissue in over 80% of the samples tested, while none were culture-positive. As more data are obtained utilizing these novel approaches, it is likely clinicians may rely more heavily on such standards than on misleading, standard culture techniques.

Other possible methods of detecting infection without the need to recover or culture bacteria include direct visualization by immunoflourescence or confocal

microscopy, or both. This can be done using either generalized bacterial stains (Neut et al. 2003) or specific antibodies (McDowell and Patrick 2005; Tunney et al. 1999). McDowell and Patrick (2005) recently described a protocol in which the prosthesis was removed and sonicated to dislodge adherent bacteria. The sonicate was then concentrated, fixed directly to the wells of a glass slide, and examined by IFM using antibodies specific to the pathogens of interest. These authors found that this approach allowed not only for the efficient recovery of implant-derived bacteria but also for the discrimination of these bacteria from contaminants obtained during processing of the explanted device. Specifically, contaminants were consistently observed as single cells or small aggregates while bacteria derived from implant-associated biofilms were present as large, easily distinguished aggregates. The ability to distinguish between contaminants and implant-associated bacteria is a fundamentally important consideration with respect to addressing the specific issue of whether a significant number of cases of aseptic loosening do in fact have an infectious etiology. However, it is also possible that the sonication procedure dislodged some biofilm bacteria from the sample and it was these that were then labeled contaminants. Nonetheless, this was an interesting study that merits further attention from those in the field.

Both nucleic-acid-based and microscopy-based methods have proven useful in addressing the question as to why many cases of presumed infection are culture-negative, and, by careful design of DNA reagents and/or antibody preparations, both can be adapted to introduce enough diagnostic specificity to help define appropriate antimicrobial therapy even in the absence of culturable bacteria. Presumably, either or both could also be used to address the issue of whether a significant proportion of patients diagnosed with aseptic loosening are infected. However, to date, none of these approaches have been adequately evaluated in that specific clinical context. One problem in that regard is that, almost by definition, adequate evaluation will require evaluating a large number of samples, and many of these techniques are not readily amenable to such large-scale studies. Nevertheless, given the potential significance of the problem, it is certainly worth investigating these alternative methods on a larger scale and in the specific context of aseptic loosening. With that said, we now turn our attention to factors that might account for the diagnostic failure of culture-based methods, based on the assumption that understanding these factors might lead to improved bacteriological methods that may also be useful in definitively answering the question of whether a significant proportion of cases diagnosed as aseptic loosening may in fact have an infectious etiology.

4 Does the Biofilm Lifestyle Limit the Ability to Detect Infection in Aseptic Loosening?

The most obvious possibility to explain why aseptic loosening may often be misdiagnosed is the formation of a bacterial biofilm. This is true both because it could limit the ability to recover tightly adherent bacteria and because it could compromise detection by standard culture techniques. By way of explanation, it is generally

accepted that the biofilm lifestyle can be divided into several relatively discrete steps. We believe the following steps warrant consideration. The first is some form of non-specific, easily reversible form of attachment mediated by general surface characteristics of both the bacterium and the substrate. The second is a more specific mechanism of tight attachment. This step is defined by characteristics that allow the bacterium to attach to the substrate itself, and it is distinguished from the third step, which promotes interactions between bacterial cells. It is this third step that results in the accumulation of bacteria in the multilayered organization characteristic of bacterial biofilms. These second and third stages are mediated by specific adhesins produced by the bacteria themselves, and it is presumably at these stages that adherence would progress to the point that recovery would be compromised. The fourth stage is an adaptive response that allows the bacterium to persist within the biofilm. The fifth and final stage is a means of detachment resulting in the seeding of new sights. As with the discussion of aseptic loosening itself, there are several recent reviews that discuss various aspects of these different stages (Fitzpatrick et al. 2005; Mack et al. 2004; Gotz 2002). The specific mechanics of tight adherence are less important to this discussion than whether or not these mechanisms ultimately reduce the ability to remove bacteria for culture, and we have already addressed this to the best of our ability in the preceding discussion. At the same time, it is possible that bacteria dislodged from biofilms have attributes that limit our ability to detect them using standard culture techniques, and in this respect it is the nature of the adaptive response to persistence within a biofilm that is most relevant.

Most reports examining staphylococcal biofilm formation have been aimed at identifying factors that contribute to the attachment and accumulation phases, and these were discussed in the previous chapter. Relatively few studies have attempted to define the nature of the adaptive response to the biofilm lifestyle. One of the first of these was the study by Beenken et al. (2004), which used microarray-based technology to define global changes in gene expression associated with the persistence of *S. aureus* within a biofilm. This study used a comprehensive, genome-scale microarray to compare gene expression patterns in a mature biofilm with those observed in exponential and postexponential phase planktonic culture. The results confirmed that biofilms represent a unique lifestyle by comparison to conventional culture methods. Specifically, this study identified 48 genes in which expression was induced at least twofold in a biofilm by comparison to both exponential and postexponential phase planktonic culture. In contrast, 84 genes were identified that were expressed at reduced levels by comparison to both planktonic conditions. A subsequent study that used the same experimental approach but a different strain of *S. aureus* reported very similar, although not identical findings (Resch et al. 2005). Proteome analysis also found that ~72% of the proteins that were present in altered amounts in lysates prepared from biofilm-harvested cells could be correlated with changes in gene expression observed by transcriptional profiling (Resch et al. 2006). In all cases, differences were observed in genes and proteins that fall into essentially every metabolic category, but overall the largest number were assigned functions in central intermediary metabolism. This is consistent with the hypothesis that persistence within a biofilm requires a dramatic change in bacterial cell physiology and metabolism.

Although many questions remain, one central theme that emerged from the studies of Beenken et al. (2004) was that persistence within a biofilm requires an adaptive response that limits the deleterious effects of the acidic products that result from carbohydrate metabolism in an anaerobic environment. This is consistent with the results of a subsequent study (Weinrick et al. 2004) in which it was demonstrated that growth under mildly acidic conditions resulted in changes in gene expression much like those observed in a biofilm. An example is the dicistronic operon *alsSD*, expression of which was induced both in a biofilm and under mildly acidic growth conditions. The *alsS* and *alsD* genes encode acetolactate decarboxylase and acetolactate synthase respectively. These enzymes function sequentially to convert pyruvate to acetoin and ultimately to 2,3-butanediol. Production of acetoin and 2,3-butanediols rather than the more acidic products of carbohydrate metabolism has been shown to be important for both acid tolerance and biofilm formation in other bacterial species (Kovacikova et al. 2005), and in a subsequent study, Cassat et al. (2006) confirmed that this is also the case in *S. aureus* by demonstrating that an *alsSD* mutant has a reduced capacity to produce acetoin and a reduced capacity to form a biofilm. Interestingly, an *alsSD* mutant has also been shown to have a defect in stationary phase survival at least as defined by planktonic culture (Yang et al. 2006). This defect is most evident in the presence of exogenous glucose, which is consistent with the hypothesis that the inability to adapt to the acid production associated with anaerobic carbohydrate metabolism plays an important role in the adaptive response of *S. aureus* to persistence within a biofilm. However, these studies were done under in vitro conditions, and whether the survival defect is relevant to persistence within a biofilm in vivo or the inability to culture bacteria in cases of aseptic loosening remains to be determined. In preliminary studies, we have demonstrated that an *alsSD* mutant also has a reduced capacity to form a biofilm in vivo, but this did not preclude the isolation of viable bacteria even after 10 days of infection (Cassat et al. 2006). Nevertheless, these results remain important for three reasons. First, they emphasize the importance of the adaptive response of bacteria to the biofilm lifestyle, and it cannot be assumed that this adaptive response does not compromise the ability to culture viable bacteria in the "real world" of aseptic loosening. Second, they introduce the possibility that specific culture techniques could be developed to overcome this compromise. Third, they suggest that genes such as *alsSD* could be targeted for the development of effective therapeutic agents that specifically target the biofilm lifestyle.

The studies discussed above clearly indicate that the persistence of *S. aureus* within a biofilm requires an extensive adaptive response, and we believe that understanding this response is important both in the general context of understanding the pathogenesis of biofilm-associated infection and in the specific context of addressing the possible role of bacterial biofilms in aseptic loosening. With respect to the latter, a primary concern is that the nature of the adaptive response would limit the ability to culture the offending bacteria. This could be the case if such bacteria were viable but metabolically inactive. Indeed, "viable but nonculturable" bacteria have been described in a number of species (Oliver 2005), and it is certainly possible that they are present within biofilms associated with orthopedic implants. However, the

transcriptional profiling and proteomic studies done to date also establish that the vast majority of genes were expressed in biofilms at levels comparable to planktonic culture, and this suggests that *S. aureus* is not metabolically inert within a biofilm. In this respect it is important to recognize that most studies done to date have been limited to some form of in vitro biofilm model. In the studies done by Resch et al. (2005, 2006), bacteria were harvested from dialysis membranes placed on agar plates. Beenken et al. (2004) used flow cells in which bacteria were required to remain adherent even under the shear force of continuous flow. Both of these proved useful as evidenced by the identification of differentially expressed genes. Nevertheless, it remains unclear whether these models fully mimic in vivo conditions. More directly, viable bacteria are easily harvested from such model systems, but that is not necessarily the case with implant-associated biofilms in vivo. One approach to this problem is to carry out similar studies using RNA and/or cell lysates harvested from biofilms grown in vivo. This is technically challenging because it is difficult to isolate enough of the relevant sample (RNA or bacterial proteins) in a sufficiently pure form to carry out these experiments. An alternative approach was described by Brady et al. (2006), who examined the host immune response during biofilm-associated infection as a means of identifying bacterial proteins produced in vivo. Specifically, these authors harvested *S. aureus* from in-vitro-grown biofilms and prepared whole cell lysates. The same *S. aureus* strain was used to initiate osteomyelitis in a rabbit model. Serum was then harvested at various intervals postinfection and used to screen the cell lysates by Western blot. This allowed identification of a number of proteins that elicited a host antibody response. This implies that the relevant genes were expressed at some point in vivo, but it does not necessarily indicate active gene expression or viability in that it is possible that the immune response was directed toward bacterial products released from dead or dying bacteria rather than viable bacteria themselves. However, viable colony-forming units were also recovered during these studies, and so it is more likely that the immune response was generated because of viable bacteria within the animals. The results remain important, however, in that they suggest that the corresponding *S. aureus* proteins may also be viable targets for antibiofilm immunotherapy. The corresponding antibodies may also prove useful with respect to the development of highly specific IFM protocols capable of detecting adherent bacteria in situ.

5 Alternative Explanations for Diagnostic Failure in Aseptic Loosening

Two other possibilities that may also contribute to the failure to recover bacteria using standard culture techniques are formation of SCV and the presence of intracellular bacteria. These are not mutually exclusive alternatives either with respect to each other or with respect to biofilm formation. For instance, SCVs can persist intracellularly (von Eiff et al. 1997a,b) and they can form biofilms (Krimmer et al.

1999; Haussler 2004). Indeed, it may well be a combination of all of these attributes that ultimately results in the failure to confirm infection in a significant proportion of patients diagnosed with aseptic loosening.

Variant subpopulations of bacteria that grow very slowly have been recognized in the staphylococci and other bacterial species for many years. Such variants form colonies on agar medium that are generally about one-tenth the size of normal colonies (Proctor et al. 2006, von Eiff et al. 2006a,b). This means that they can be easily overlooked in the clinical laboratory, and it is in this respect that they may have direct relevance to the failure to detect infection in patients diagnosed with aseptic loosening. Their slow growth also has several relevant consequences, including reduced susceptibility to host defenses and antimicrobial therapy (Baumert et al. 2002). In general, the SCV phenotype is reversible, and until recently this made it difficult to study. Stable variants carrying mutations in specific genes have now been generated, and this has allowed more detailed characterization of the SCV phenotype. For instance, von Eiff et al. (2006) recently demonstrated that *S. aureus menD* and *hemB* mutants exhibited a stable SCV phenotype and were defective in utilizing a variety of carbon sources and Kreb's cycle compounds required to generate ATP via electron transport. Similar studies by Kohler et al. (2003) led to the conclusion that such mutants can only generate ATP from glucose or fructose by substrate level phosphorylation. At the same time, a *hemB* mutant was shown to have an enhanced capacity to bind fibrinogen and fibronectin (Vaudaux et al. 2002), which could be important in terms of promoting the attachment of SCVs to implanted orthopedic devices. Such mutants have also been shown to persist intracellularly (von Eiff et al. 1997) and to be capable of causing various forms of infection, including septic arthritis (Jonsson et al. 2003) and endocarditis (Bates et al. 2003). Perhaps most importantly, there are reports describing the isolation of SCVs from patients with various forms of musculoskeletal infection, including device-related infections, and it has been suggested that the appearance of SCVs is actually a consequence of growth in vivo (Proctor et al. 2006; von Eiff et al. 2006). This suggests that the SCV phenotype reflects the adaptive response of bacteria to in vivo conditions irrespective of whether it is directly correlated to persistence within a biofilm. There are also reports demonstrating that the SCV phenotype is induced in patients undergoing local antibiotic treatment with beads containing gentamicin (von Eiff et al. 1997). This is important in that local antibiotic delivery using gentamicin-impregnated poly(methyl methacrylate) beads is a common approach in the treatment of osteomyelitis and implant-associated infection (Lew and Waldvogel 2004). Taken together, these studies suggest that SCVs can cause recurrent bone and implant infection and that the failure to account for their unique properties, particularly with respect to their reduced growth rate, may yield a "false-negative" culture result that may ultimately lead to a diagnosis of aseptic loosening.

A final possibility that may also contribute to diagnostic failure in presumed cases of aseptic loosening is the presence of intracellular bacteria. Although the staphylococci were long considered to be among the classic extracellular pathogens, recent reports demonstrate that *S. aureus* can be internalized by various host cells, including osteoblasts, and that bacteria can survive within the intracellular

milieu (Bayles et al. 1998; Ellington et al. 2006). Interestingly, Vesga et al. (1996) has demonstrated that internalization of *S. aureus* by cultured endothelial cells induces the small colony phenotype. In the intracellular environment, *S. aureus* would be protected from both host defenses and antimicrobial agents (Ellington et al. 2003). Moreover, using a tissue culture system, Ellington et al. (1999, 2003) also showed that dead or dying osteoblasts release viable bacteria into surrounding tissues. This suggests that *S. aureus* may exist in an intracellular, quiescent state, and this may be a contributing factor in the pathogenesis of recurrent infections. It may also further complicate diagnosis based on standard culture techniques. Internalization of *S. aureus* and other bacterial pathogens has also been shown to have significant consequences with respect to host cell cytokine production (Alexander and Hudson 2001), and this may be important with respect to inducing changes in host physiology that ultimately result in implant failure irrespective of whether viable bacteria can be cultured at the time of revision.

6 Conclusion

It is difficult to definitively answer the question as to whether a significant number of cases diagnosed as aseptic loosening may in fact have an infectious etiology. Some investigators have concluded that the question itself is not important in that, in the absence of clinical evidence of infection, the recovery of bacteria from explanted devices is of little clinical significance (Moussa et al. 1997). We are not convinced that is the case. Indeed, the collective studies done to date support the hypothesis that a significant number of cases diagnosed as aseptic loosening may in fact have an infectious etiology, and we believe that failing to appropriately diagnose and treat these infections does have significant clinical consequences for at least some patients. On the basis of this, we also believe that additional study that takes all of the issues discussed in this chapter into consideration is in the best interest of the growing number of patients that rely on arthroplastic surgery to maintain an independent and productive lifestyle. Further justification for this effort comes from the observation that a significant number of patients who do have clinical evidence of infection are culture-negative, and in such cases the only therapeutic option is empiric rather than determinative antimicrobial therapy. While empiric therapy is often effective, it is clearly not the preferred approach, and this also demands a thorough investigation of alternative methods to both detect and diagnose infection even in its most insidious forms.

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Biofilms and Ventilation

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Abstract The need for mechanical ventilation (MV) has paralleled the growth of the ICU for a ageing population with multiple disease entities, particularly those associated with pulmonary diseases. As the process of ventilation has improved, the etiology of Ventilator-Associated Pneumonia (VAP) has moved from the instrumentation to the colonization of the patient, focusing most recently on the link of normal oral flora as the initial, catalyzing insult in patients at risk for pneumonia through a biofilm in the lumen of the endotracheal tube (ETT).

The ETT, particularly the lumen, is an unrecognised reservoir for biofilms in the evolving discussion of indwelling medical device (IMD)-associated infections; yet it is the perfect environment for such communities to form. The endotrach lumen is protected from systemic therapeutic interventions. The environment is rich with the parameters associated with biofilm development.

Risk factors for ventilator-associated pneumonia and development of ETT luminal biofilms are multi-factorial including: length of hospitalization, surgery, prior antibiotic usage, stress ulcer prophylaxis, host factors such as ARDS, upper aspiratory colonization, and poor oral hygiene. The most significant risk factors, however, appear to be length of mechanical ventilation and prior exposure to antibiotic therapy.

1 Introduction

1.1 Background and Magnitude of the Problem

Ventilator-associated pneumonia (VAP) is now the most costly and important nosocomial infection in hospitalized patients in the USA. Eight to 28% of mechanically ventilated patients will develop VAP. The rate increases with duration

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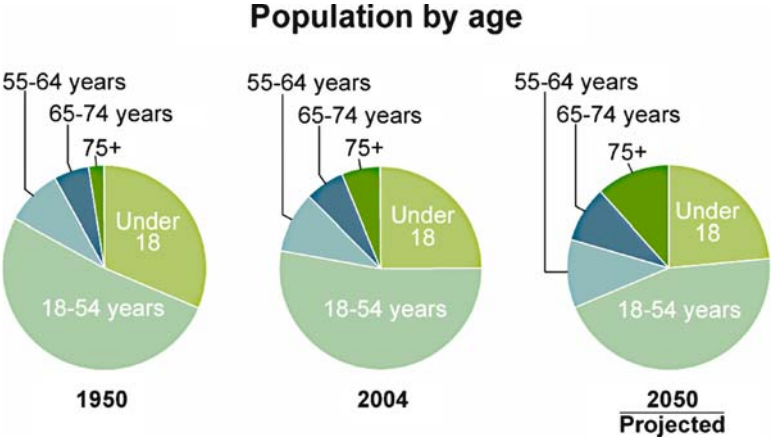

Fig. 1 Target population demographic changes predicted for 2050

of mechanical ventilation (MV; 5% at 5 days, 68% at 30 days, cumulative). This high infection rate is the result of the interface between two events, the increased use of MV and the increase of older patients with comorbidities who need longer support for respiratory function. At the bifurcation of these two events is the biofilm, which acts as the reservoir and key ingredient for the ICU environment and the microbial population.

Figure 1 shows the demographics of the US population with the potential increase in patients at risk for MV.

1.2 The Cost of VAP

Measuring the cost of VAP is multifactorial. It is clearly dependent upon length of stay (LOS) and comorbidity. A literature review suggests an increase in total patient costs of ~$40,000 per case (Rello et al. 2002). On an average, MV increases LOS in the ICU by 100%. Specifically, the average LOS in the ICU is 5.6 days without ventilation, compared to 11.5 days with ventilation. The average LOS in the hospital without ventilation is 14 days. The LOS may increase to 25 days with MV. Rello et al. 2002 stated that the overall incidence is 18–60% with an average of 30%.

The Institute for Healthcare Improvement states that VAP adds an estimated cost of $40,000 to a typical hospital admission.

The cost of MV goes beyond charges and costs. Mechanical ventilation in the ICU is associated with 20–34% of all hospital costs and 7–8% of total health care. Additionally, prolonged MV in the ICU consumes 50% of the ICU resources.

1.2.1 Diagnostic Related Group (DRG)

Another way of addressing cost is through Diagnostic-Related Group (DRG) calculations established by the federal government. Table 1 ranks those systemic diseases linked to oral biofilms, indicating that of the six systemic consequences, VAP costs represent greater than 80% and have the greatest total days, number of discharges, and average total days.

1.2.2 Additional Cost/Antibiotic Resistance/Burden of Disease

The impact of a disease is multifactorial. Table 2 highlights the integration of these factors and links them to total burden of disease: mortality, quality of life, and cost beyond immediate care, i.e., antibiotic resistance. VAP has the highest relative burden of disease, compared with two other costly diseases: sepsis and surgical site infection.

1.2.3 Multi Drug Resistance

Antibiotic resistance and the problems with multidrug-resistant isolates in MV are another cost burden on the health care system. As Neiderman points out, "All studies show that modifying an initial inadequate therapy according to microbiology results in severely ill patients with VAP does not translate into a better outcome. It is imperative to change strategies that impact on the ICU floor to reduce the selective pressures." Niederman et al. VAP and Tracheobronchitis Sponsored Program (chiron).

Tables 1–4 indicate the consequence of the selective pressures created by the current strategies and their effect on ultimate cost. Fifty percent of all antibiotics

Table 1 Burden of oral-biofilm-associated disease with supporting characteristics: Number of discharges and average total days hospitalized

Disease	DRG	Total charges ($)	Medicare reimbursement ($)	Total days	Number of discharges	Average total days
VAP	475	8,006,357,380	2,588,756,835	1,293,553	121,328	10.7
Atherosclerosis	132, 133	1,284,089,604	326,207,998	303,839	108,759	2.50
Otitis media	68,69,70	284,252,979	70,518,948	89,605	24,522	3.07
Infective endocarditis	126	266,061,846	81,152,691	58,940	5,498	10.7
Trush/ Streptococc.	73	131,574,508	36,272,799	36,023	8,391	4.3
Periodontitis	185, 186	111,470,304	30,585,714	28,764	6,381	4.5
Total		10,083,806,621	833,494,985	1,810,724	274,879	5.11

Centers for Medicare and Medicaid Services Inpatient Hospital National Data for Fiscal Year 2005*VAP* ventilator-associated pneumonia, *DRG* Diagnostic-Related Group

Table 2 The three main components of burden of disease incorporating clinical and social outcomes

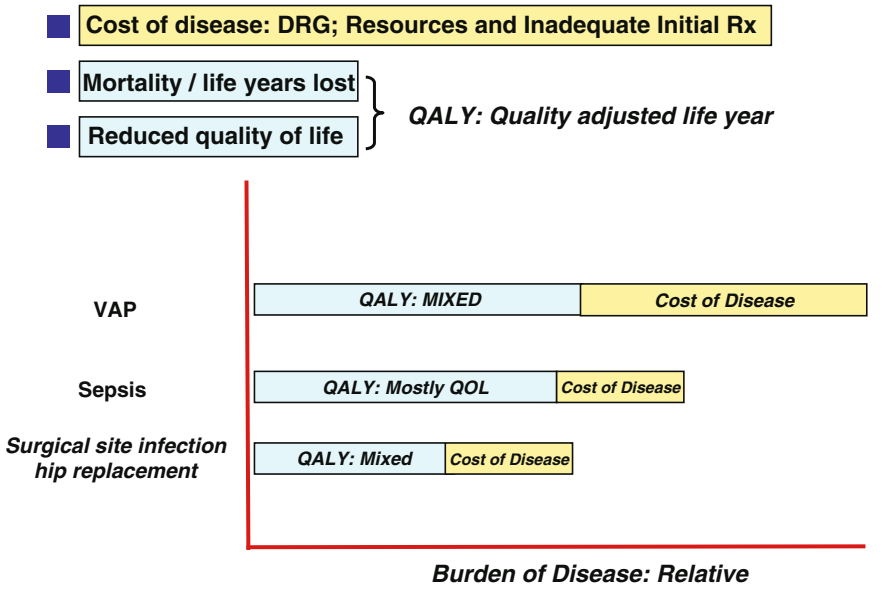

Table 3 Clinical burden of three selected infectious diseases in the USA

	Hospitalizations	Mortality rate (%)	Mortality	Cost per patient ($ n
Severe sepsis	660,000	23	150,000	22,000–70,000
Community acquired	395,000	13	50,000	
Hospital acquired	265,000	38	100,000	
Adequate initial Rx[a] (70%)	185,000	28[a]	51,000	
Inadequate initial Rx[a] (30%)	80,000	62[a]	49,000	
Pneumonia	1,300,000	9	115,000	12,000–22,000
Community acquired	1,000,000	2.4	24,000	
Hospital acquired	300,000	30	90,000	
Ventilator associated pneumonia	135,000	45	61,000	41,000
Adequate initial Rx[b] (56–75%)	75–100,000	10–20[b]		
Inadequate initial Rx[b] (25–44%)	35–60,000	40–60[b]		

In an "average" 500 bed hospital studies indicate that patients with acquired infections will receive inadequate initial antimicrobial therapy per year:

- *About 40 with hospital acquired sepsis*
- *Between 20 and 30 with ventilator associated pneumonia*

[a] Ibrahim et al. (2000). © 2000 American College of Chest Physicians
[b] Chastre and Fagon (2002), Sanchez-Nieto et al. (1998), Ruiz et al. (2000) and Dupont et al. (2001)

prescribed in the ICU are administered for treatment of lower respiratory infection (Kollef 2005). Table 3 compares Burden of Disease and therapeutic consequences per case in the USA, while Table 4 tabulates cost for an average 500-bed hospital.

Table 4 Clinical burden of three selected infectious diseases in an "average" 500-bed hospital, highlighting impact of inadequate initial therapy

	Hospitalizations	Mortality rate (%)	Mortality	Cost per year ($m)
Severe sepsis	350	23	80	7.5–24
Community acquired	200	13	26	
Hospital acquired	140	38	55	3–10
Adequate initial Rx[a] (70%)	100	28[a]	28	
Inadequate initial Rx[a] (30%)	40	62[a]	25	
Pneumonia	670	9	60	8–15
Community acquired	500	2.4	12	
Hospital acquired	170	30	50	7
Ventilator associated pneu.	70	45	30	2.8
Adequate initial Rx[b] (56–75%)	40–50	10–20[b]		
Inadequate initial Rx[b] (25–44%)	20–30	40–60[b]		
Surgical site infections (2.6%)	360	4.3	15	4.3

The annual additional burden of hospital acquired infections from sepsis, pneumonia and surgical site infections in an "average" 500 bed hospital:
Additional cost of $14 mill to $21 mill
About 120 patients will die from these infections

[a] Ibrahim et al. (2000). © 2000 American College of Chest Physicians
[b] Chastre and Fagon (2002), Sanchez-Nieto et al. (1998), Ruiz et al. (2000) and Dupont et al. (2001)

2 Epidemiology/Demographics

2.1 Impact of VAP

VAP is the most common hospital-acquired infection in patients requiring MV (Rello et al. 2002). Approximately 8–28% of patients receiving MV are affected (Chastre and Fagon 2002). The mortality rate due to VAP ranges from 20 to 30%, and can be as high as 50–70%. Mortality rates are higher for older patients, patients with a depressed level of consciousness, and patients who have received prior antibiotic therapy. VAP is associated with an increased LOS in the hospital of 11.5 additional days, 9.6 additional days on MV, and 6.1 additional days in the ICU. Each case of VAP is associated with a direct cost of $40,000 (Rello et al. 2002). One component of this cost is related to antimicrobial therapy. Fifty percent of all antibiotics prescribed in ICUs are administered for the treatment of lower respiratory infection (Kollef et al. 2005).

2.2 Prevalence of VAP

The cumulative risk of developing VAP is concentrated within the first days after intubation: 3% risk per day in the first week of MV, 2% per day in the second week, and 1% per day thereafter (Cook et al. 1998). Since most MV is

short-term, approximately half of all episodes of VAP occur within the first 4 days of MV (Kollef 2005). There is a 68% cumulative rate at 30 days (Hoffken and Niederman 2002).

2.3 Risk Factors

Endotracheal intubation remains the most important risk factor for the development of nosocomial pneumonia (Rello et al. 2002). Other risk factors include chronic obstructive pulmonary disease (odds ratio [OR], 18.3), organ system failure (OR, 10.2), coma (OR, 40.3), acute respiratory distress syndrome (OR, 9.7), and head trauma (OR, 5.2). Patients who are older than 60 years (OR, 5.1) and are of the male gender (OR, 2) are at greater risk for VAP. Other risk factors include neurosurgery (OR, 10) and reintubation (OR, 5.94) (Bonten et al. 2004).

2.4 Link Between VAP and Oral Health

There is growing evidence that the epidemiology of VAP is more intimately linked to the oral health than previously recognized. Recently, greater than 700 species were identified in the oral cavity by using molecular methods, and as hypothesized, a select population of these are the primary colonizers in the lumen of the endotrach and may present a network that enhances attachment of the more traditional pathogens associated with late-onset VAP. This awareness is generally described in the oral/systemic connection and includes a growing list of references. Diseases associated with the oral flora and imbalance of selected pathogens include caries (65.6%), periodontal disease (5.6 billion per year), arterial sclerosis (5 billion per year), low birth weight babies (2 billion per year), otitis media (5 billion per year), infective endocarditis (50,000 per infected patient), and VAP (26 billion per year). Figure 2 illustrates the relationship between oral flora and systemic disease. The impact of floral colonization and potential pathogenecity is highlighted by the difficult challenges presented to nursing in maintaining a reduced bioburden during MV. There are limited protocols and although these have been standardized, routine use is difficult.

3 Clinical Infection

3.1 Definition of VAP

There is a general consensus that VAP is a clinical diagnosis and therapeutic options are directed by clinical presentations. For patients at risk, these include (1) newer, persistent pulmonary infiltrates on X-ray and (2) at least two of the following:

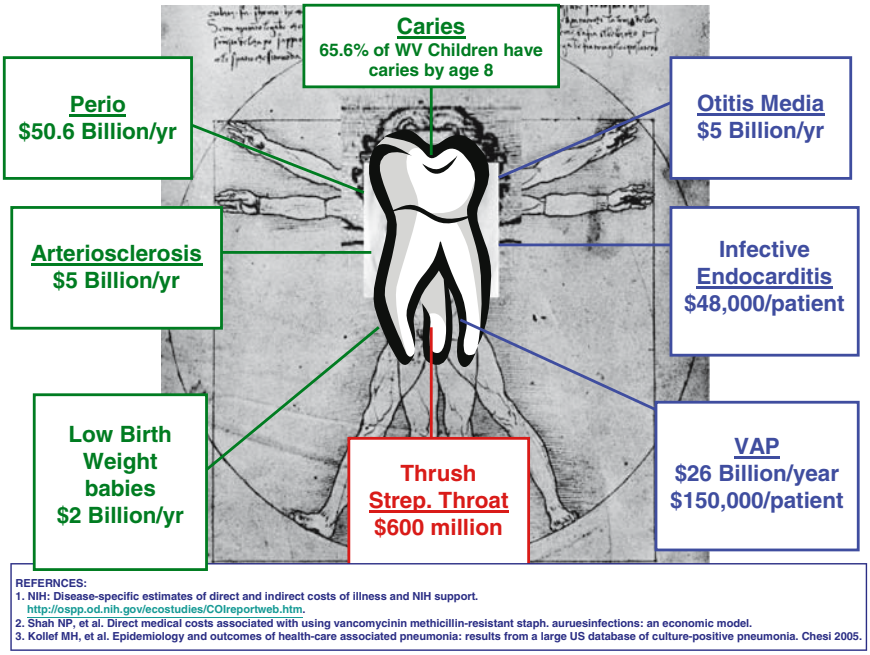

Fig. 2 Total cost burden to health care with six orally linked diseases

temperature below 36 or above 38° C, WBC count less than 5,000 or greater than 10,000, or purulent endotracheal aspirate (Citation).

3.2 VAP vs. HAP

VAP is one of several classifications of pneumonia. The variations in classification show the expansion of disease to the lung, the involvement in supportive care, plus the place of acquisition or exposure. Hospital-associated pneumonia (HAP) may address community-acquired pneumonia and have significant etiologic variation due to MV.

3.3 Early-Onset vs. Late-Onset VAP

For management and ease in classification, VAP is divided into early and late phases, and in comparison to wound infections, it is a biphasic disease. "Early disease" is generally classified as pneumonia of less than 5 days' duration, whereas late disease is associated with 7 days or more. During the transition from early to late, the microbial flora undergo a significant alteration from Gram-positive to Gram-negative. Clinically, it is imperative to extubate the patient before 5 days to reduce

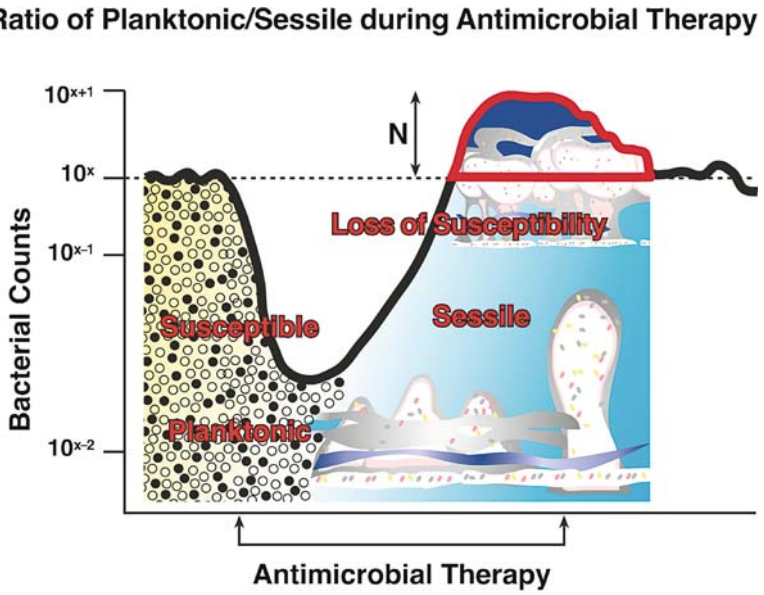

Fig. 3 Relative time graph highlighting biphasic shift from planktonic to biofilm bacterial burden as a result of antibiotic selective pressures

the risk factors in significant long-term disease. Figure 3 shows a depiction involving the ratio of planktonic isolates to biofilm phenotype that can also be characterized as early and late. A significant consequence of the biofilm in late disease is the increased bioburden that the biofilm phenotype enhances.

4 Microbiology

4.1 Organisms Involved in Early- vs. Late-Onset VAP

The microbiology of VAP is influenced by a number of risk factors, including age, geographic location, and comorbidity. On the other hand, there is a cadre of organisms that remain universally the same throughout the disease process. They can be divided into microorganisms associated with early-onset (less than 5 days) VAP and those associated with late-onset (more than 7 days) VAP.

4.2 Importance of Synergy in Pathophysiology of VAP

These organisms are listed in Table 5. Generally, early-onset organisms are Gram-positive core ones and are often associated with oral flora while late-stage VAP pathogens are core organisms plus gastrointestinally associated, Gram-negative rods.

Table 5 Organisms involved in early- vs. late-onset ventilator-associated pneumonia (VAP)

Early-onset VAP: core organisms	Late-onset VAP: core organisms *plus*
Haemophilus influenza	*Pseudomonas aeruginosa*
Methicillin-susceptible *Staph. aureus*	Methicillin-resistant *Staph. aureus*
Streptococcus pneumoniae	*Actinobacter* species
Enteric Gram-negative bacilli (nonpseudomonal)	*Candida albicans*
	Stenotrophomonas multiphilia

The proportion of organisms varies and it is important to recognize that the biphasic nature is not limited to monospecies but also occurs among mixed flora. There is growing evidence that synergy plays an important role among selective organisms either early or late in the biphasic disease. Most recently a number of ancillary organisms have begun to play significant roles in preliminary or empiric antibiotic therapy. These include community-acquired, methicillin-resistant *S. aureus* with the PVL locus. *Klebsiella pneumoniae* may be of growing significance, as is multidrug-resistant *P. aeruginosa* in late disease. *Actinobacter* seems to be incredibly geographic. Some reports suggest that it is 100% associated with late-stage disease, others find it very infrequently.

4.3 Synergistic Relationships Exist Between Pathogens and Oral Flora

There is also growing awareness of the importance of mutualism or synergy among significant oral colonizers. This highlights the role of the plaque hypothesis or ecologic hypothesis emphasizing significant pathogens that coadhere or cross-communicate. Significant evidence has emerged for several organisms that coadhere. These organisms include *P. aeruginosa* and *C. albicans*, *Streptococcus mutans* and *C. albicans*, and *P. gingivalis* and *St. gordonii*. These symbiotic relationships emphasize the potential importance of the oral flora.

4.4 Quorum Sensing is a Key Factor for Survival in Biofilms

Synergism is most beneficial for the existence of organisms in a biofilm community. Biofilms can be thought of as a survival mode for bacteria. They particularly address the features of selected organisms to adhere preferentially to abiotic or biotic surfaces. Often early colonizers in the biofilm are of a Gram-positive nature, emphasizing their historic perspective of inanimate attachment. This will recruit secondary colonizers, which may start the cascade and move the community towards complex biofilms with late-stage colonizers being of a Gram-negative origin. These latter organisms are particularly associated with late-stage VAP. Organism survival in the biofilm community is dependent upon a number of

factors. Perhaps the most important factor is quorum sensing, although inherent antibiotic resistance and host resistance are key factors to long-term survival. The molecules elaborated by organisms allows for intra- and interspecies communication. Their primary purpose is to respond to stress in the environment. It is now apparent that these molecules share many antibiotic characteristics. In fact, their primary activity may have been antibacterial in nature to serve as a survival scheme. The fact is that quorum-sensing molecules have a much larger job description than being antiinfectives or antibiotics.

5 Role of the Endotracheal Tube

5.1 Association of VAP with MV

The history of VAP is directly linked to MV and its evolution. Figure 4 correlates this evolution with the change from negative pressure to positive pressure with a highlight of the endotracheal tube (ETT) bypassing the epiglottis. With the advent of positive pressure in the 1970s, considerable investigation linked VAP to

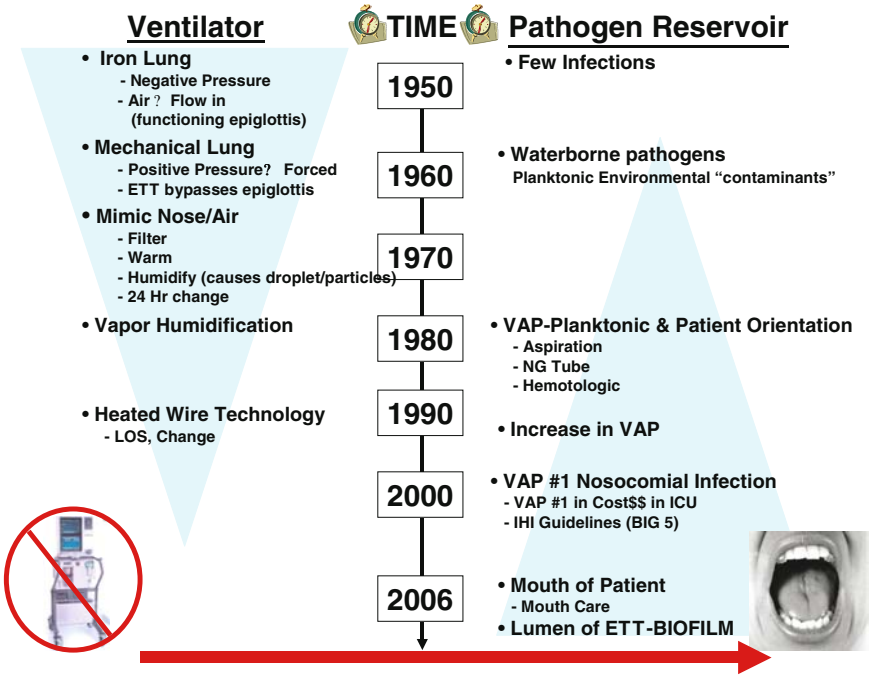

Fig. 4 Changing paradigms: pathophysiology of ventilator-associated pneumonia and shift from ventilator to oral colonization

contamination of the water reservoir and circuits, and to the nonsterile conditions over time. Engineering skills addressed each of these through the 1980s and 90s until it was quite apparent by 1990 that VAP was a misnomer and that MV was not the cause of "ventilator"-associated pneumonia.

5.2 *Characteristics of the Lumenal Biofilm*

The unmasking of biofilms and the importance of the abiotic surface to a biofilm phenotype helped close the circuit on the importance of endotrachs, particularly the lumen of the implanted device. Most early information concerning the endotrachs focused on pooling, and it is still one of the major routes of potential contamination. However, more recent literature has been emphasizing the importance of the lumenal biofilm and the movement of social communities with multispecies by rolling or rippling activity. Biofilms are characterized by a number of features that include three-dimensional structure and the ability to form communities under static and shear forces. The bidirectional shear associated with ventilation, ranging from 12–18,000 Reynolds units, is the single reason why luminal biofilm growth is perhaps the greatest contributor to movement of organisms into the respiratory field of the intubated patient. Figure 5

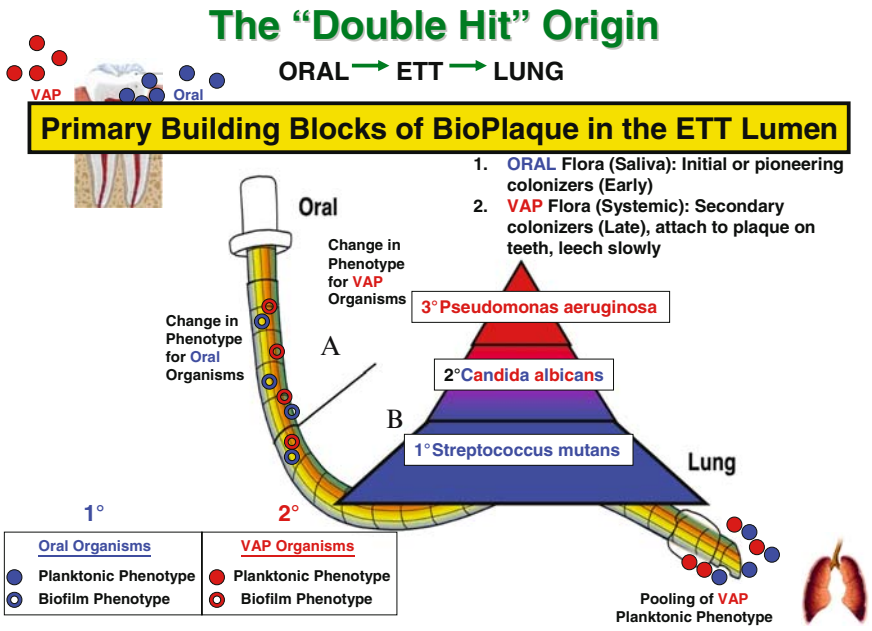

Fig. 5 A "working hypothesis" integrating published data and ventilator-endotrach-lung (VEL) results, emphasizing three synergistic organisms as key partners in oral origin

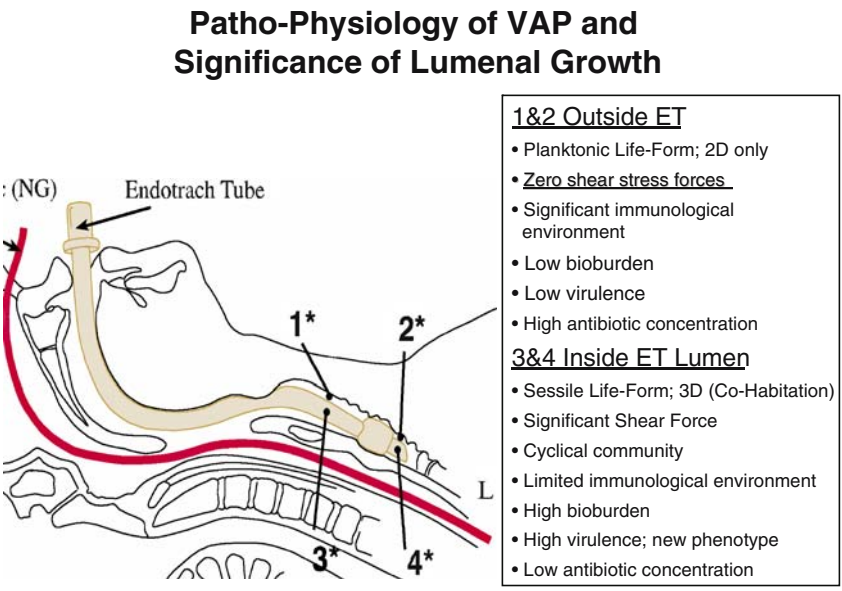

Fig. 6 Significance of lumenal biofilms and protection from humoral factors important in host defenses

integrates the most recent information and presents a hypothesis for organism movement and inoculation into the lung field. It is called the Double Hit Hypothesis, where two pools of organisms, an oral flora and a more traditional VAP flora, define early- and late-stage VAP. Dental plaque serves as a major reservoir of early disease and of colonizers of the endotrach. This provides a significant reservoir for *S. mutans*, which may have the most important role in initial colonization by creating a dental plaque environment on the lumen of the endotrach. Secondary colonizers including *C. albicans* and subsequent *P. aeruginosa* enhance the complexity and three-dimensional architecture of the plaquelike material on the endotrach lumen.

The importance of the endotrach in the neutralization of planktonic material, but not biofilm bacteria, is seen in Fig. 6. It should be noted that lumenal biofilm has the significant advantage of reduced immunologic stress. The pressures of white cells, antimicrobials, and other antiinfectives are markedly reduced.

A number of methods have been utilized to control the biofilm both on the outside and on the inside of the device, including the cuff. Silver has been found to be the most universally applicable agent, and a number of schemes to attach silver as an ion or an element have been addressed. (Please see Sect. 10.)

5.3 The Ventilator-Endotrach-Lung Model

We have evaluated the impact of ventilation on the growth of lumenal biofilm by using a five-construct, engineered Ventilator Endotrach Lung model. This uses existing material from the ICU and is built around the parameters that define the biofilm structure. These parameters are outlined in Fig. 7 and were incorporated into the Model, which is described in Fig. 8. Following a 50-patient analysis, ventilation parameters were selected to continually evaluate the growth of the biofilm over 14 days utilizing a continuous flow nebulization of five selected organisms. These organisms were added in selected order given the history of VAP at West Virginia University Hospital (Table 6).

Of the many parameters defining a biofilm in the lumen, three were found to be most critical: (1) the organism population, (2) the nutritional component, and (3) the stress. We used chronic obstructive pulmonary disease as our primary investigational environment recognizing the percent oxygen at 60% with Reynolds forces equaling 12,000. Analysis was based on bidirectional forces with imaging of the undisturbed lumenal biofilm enhanced by confocal laser scanning microscopy coupled with COMSTAT, a metric analysis of exact proportions (http://www.im.dtw.dk/COMSTAT).

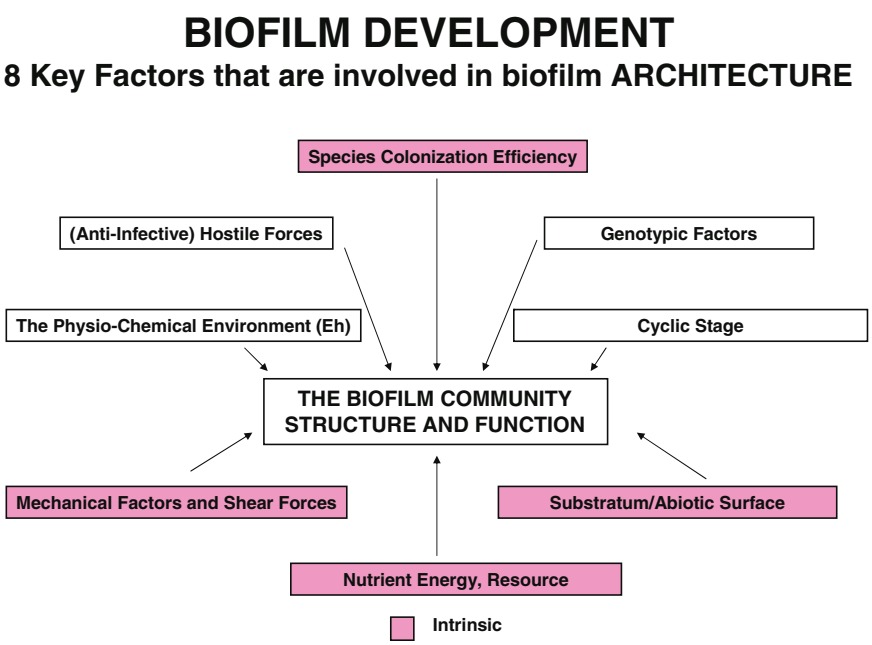

Fig. 7 Eight key factors that are involved in biofilm architecture

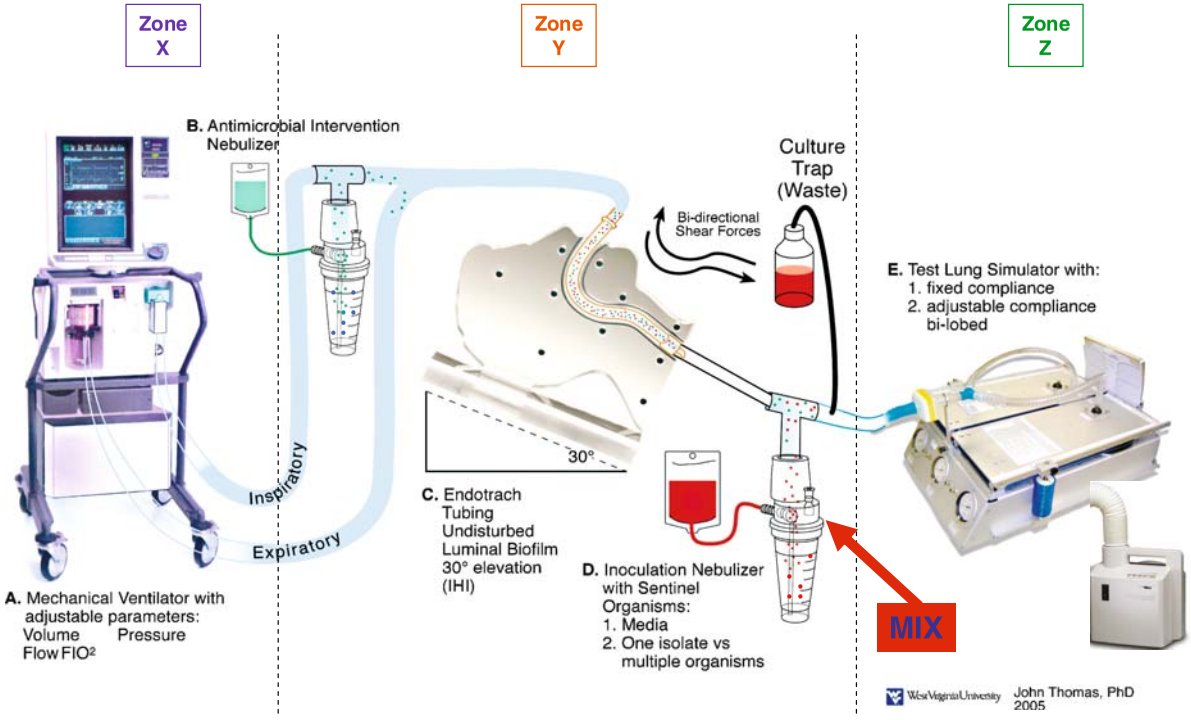

Fig. 8 Complex biofilm development in an engineered, closed ventilator-endotrach-lung (VEL) model describing the three main components and instrumentation: zone X, zone Y, and zone Z

Table 6 Five selected organisms monitored in the ventilator-endotrach-lung (VEL) model

Methicillin-susceptible *Staph. aureus*
Candida albicans
Methicillin-resistant *Staph. aureus*
Pseudomonas aeruginosa
Stenotrophomonas multiphilia

6 Pathophysiology

6.1 Immune Mechanisms of the Host

In a normal adult, the upper airways are usually heavily colonized while the lower respiratory tract is historically thought to be sterile. Multiple host mechanisms exist to prevent infection, including anatomic airway barriers, antimicrobial agents in saliva, an intact cough reflex, mucus production, and mucociliary clearance (Davis 2006). The humoral and cellular immune systems are also essential components of host defense.

6.2 Effect of MV on the Immune System

In mechanically ventilated patients, many of the normal host defense mechanisms are compromised. Intubation breaks the normal protective barrier, disrupts the cough reflex, compromises mucociliary clearance, and injures the tracheal epithelium. This combination of events introduces a direct conduit for bacteria from the oropharynx into the lower respiratory tract (Hoffken and Niederman 2002).

6.3 Pooling of Secretions Around Endotracheal Cuff

Critically ill patients often have a depressed level of consciousness and an impaired gag reflex, leading to pooling of contaminated secretions in the posterior part of the oropharynx. Between 100 and 150 mL of secretions can accumulate within a 24-h period. Microaspiration of these oropharyngeal secretions is a major risk factor for nosocomial pneumonia. The cuff on the ETT does not prevent the passage of infected material into the airways. The contaminated secretions pool above the cuff and gain access to the trachea along the folds in the cuff. In 85% of nosocomial pneumonia infections, the causative organism was detected in the subglottic secretions. This infected material is then propelled into the distal airways by inspiratory flow.

6.4 Chronic Illness Affects Host Immune Response

Critical illness, comorbidities, and malnutrition cause further immune dysfunction, compromising the ability of the immune system to effectively respond. Illness also causes changes in normal flora, with an increase in Gram-negative organisms and *S. aureus*, along with an increase in their ability to adhere due to alterations in the oropharynx (Bauer et al. 2002). The structure and function of the alveoli change during acute lung injury and acute respiratory distress syndrome, which are common complications in acute illnesses. Neutrophil function is impaired. Pulmonary edema and alveolar hemorrhage provide a favorable environment for bacteria (Dhanireddy et al. 2006).

6.5 MV Produces an Inflammatory Response

In addition to contributing to the aspiration of pooled secretions, MV produces an inflammatory response in the host. It causes a local and systemic increase in inflammatory cytokines associated with hepatic and renal dysfunction. MV exacerbates both pulmonary and systemic inflammation in response to bacteria, increases alveolar capillary permeability to proteins, and contributes to the pathogenesis of acute lung injury and multiple organ dysfunction syndrome (IMAI et al. 2003). Approximately 25–40% of VAP cases are due to the aspiration of gastric materials (Chastre and Fagon 2002).

6.6 Importance of Oropharyngeal Organisms

The oropharynx appears to be the most important source of microorganisms (Bonten et al. 2004). The 2003 Guidelines from the Centers for Disease Control and Prevention (CDC) reported that 63% of patients admitted to the ICU have oral colonization with a pathogen associated with VAP. Additionally, 63% of the patients admitted with an oral pathogen associated with VAP acquire a second bacterial pathogen in the oral cavity. In 76% of VAP cases, the organisms colonizing the mouth and the lung were the same. *P. aeruginosa* was the most prevalent species, followed by enterobacteria and *Staphylococcus aureus.*

6.7 Adherence to ETT, Followed by Biofilm Formation

The 2003 CDC Guidelines implemented a comprehensive oral hygiene program to prevent the oropharyngeal colonization. The organisms responsible for VAP in orally intubated patients reside in dental plaque and the oral mucosa (Tablan et al.

2004). These oropharyngeal organisms gain access to and adhere to the conditioning film made up of saliva, mucus, proteins, etc., that coats the inner surface of the ETT. Adherence is the first step in the formation of a biofilm, followed by cell growth, proliferation, and production of a glycocalyx. Cells are able to migrate and spread to form microcolonies. They can also move by rolling and saltatory movement along a surface (Cason et al. 2007). As the biofilm layer on the inner surface of the ETT increases, the lumenal diameter decreases. This results in increased airway resistance and produces more embolization of material into the lower respiratory tract (Inglis et al. 1995).

6.8 Biofilm Development Controlled by Quorum Sensing

This series of events is controlled by quorum sensing, an intrabacterial communication mechanism composed of signaling molecules such as the acyl-homoserine lactones found in Gram-negative organisms. These molecules are secreted in response to cell density, osmolarity, and nutritional supplies, resulting in radical changes in gene and protein expression and result in a variety of biofilm phenotypes. It is clear from many studies that a single biofilm phenotype does not exist.

6.9 Characteristics of a Biofilm

Bioflms are three-dimensional, heterogeneous structures consisting of numerous microenvironments and niches that promote synergistic cooperation between bacterial phenotype. The organisms produce a protective extracellular matrix polymer whose major component in staphylococcal biofilms is polysaccharide intercellular adhesion. The polymer matrix provides resistance to antimicrobial agents as well as resistance to clearance by the immune system (Donlan and Costerton 2002). The biofilm represents a top-to-bottom gradient of decreasing oxygen, nutrient, cell density, temperature, pH, and osmolarity. In the interior of the biofilm, bacterial metabolic activity decreases and the microenvironment becomes anaerobic. The bacteria are still viable, metabolically active, structurally intact, and capable of reproduction, but they have lost their cultability. This is a reversible change to a sessile phenotype produced by the activation of stress-response genes and sigma factors in response to environmental changes. The phenotypic change of *P. aeruginosa* to small colony variants increases their ability to form biofilms and effects antimicrobial tolerance. Exposure to subinhibitory antibiotic concentrations induces mucoid phenotypes that generate thicker biofilms with additional matrix components such as colanic acid in *E. coli*, which supports biofilm maturation, and alginate in *P. aeruginosa*, which protects the bacteria from leukocyte phagocytosis (Leid et al. 2005).

7 Risk Factors/Predisposing Factors

7.1 Introduction

The most important steps in preventing VAP in a hospital setting are to adhere to infection control measures and to identify hospital-specific pathogens through routine surveillance (Fagon and Rello 2006; Kollef 2007; Leone et al. 2007).

7.2 Endotracheal Intubation is a Risk Factor for VAP

Endoctracheal intubation is an important risk factor for the development of nosocomial pneumonia (Davis 2006). The ETT blocks the body's natural defense against inhaled agents and blocks the cough reflex, making patients more susceptible to infections. The ETT is also an important area for biofilm development.

7.3 Early-Onset vs. Late-Onset VAP

Stratifying patients by risk factors is important when choosing initial medication regimens for the treatment of VAP. Consideration of the time of onset of pneumonia is an important component to ensure that appropriate pathogens are covered. The risk of VAP is highest early in the course of hospital stay. Early-onset VAP carries a better prognosis and is more likely to be caused by antibiotic-sensitive bacteria. Late-onset VAP is more likely to be caused by multidrug-resistant pathogens and is often hard to treat.

7.4 Risk Factors for VAP

Patients with burns, trauma, central nervous system disease, witnessed aspiration, underlying respiratory disease, and underlying cardiac disease are at increased risk for nosocomial pneumonia. Male gender has been identified as an independent risk factor for the development of VAP. Patients who suffer trauma and/or undergo surgery of the head, neck, thorax, or abdomen have been shown to be at an especially increased risk (Davis 2006). These risk factors are listed in Table 7.

7.4.1 Prior Antibiotic Exposure

The most significant risk factors appear to be the length of MV and prior exposure to antibiotic therapy. Specific pathogens and risk factors associated with these can be found in Table 8. Identifying patients who are at a high risk for multidrug-

Table 7 Risk factors for ventilator-associated pneumonia

Prolonged hospitalization
Mechanical ventilation
Endotracheal tube
Reintubation
Tracheostomy
Nasogastric tube
Surgery
Prior antibiotic therapy
Stress ulcer prophylaxis with H2 blockers
Sinusitis
Supine position
Host factors
Acute respiratory distress syndrome
Upper respiratory tract colonization
Oral hygiene

Table 8 Risk factors linked to VAP-associated organisms

Organism	Risk factors
Streptococcus pneumoniae	COPD, smoking, absence of prior antibiotic therapy
Methicillin-sensitive *Staphylcoccus aureus*	Young age, traumatic coma, neurosurgical problems
Haemoophilus influenzae	COPD, absence of prior antibiotic exposure
Methicillin-resistant *Stapylococcus aureus*	COPD, prolonged MV, history of steroid use, prior antibiotic therapy
Pseudomonas aeruginosa	COPD, prolonged MV, prior antibiotic therapy
Acinetobacter	Neurosurgery, ARD, head trauma, prior ceftazidime therapy, poor hand hygiene
Anaerobic bacteria	Patients with altered levels of consciousness, and patients with greater severity of illness
Legionella	Immunosuppression, smoking, alcoholism, COPD, chronic renal failure

VAP ventilator-associated pneumonia, *COPD* chronic obstructive pulmonary disease, *MV* mechanical ventilation, *ARD* acute respiratory distress

resistant infections is key to appropriate treatment. The following group of patients are at high risk for MDR and microbial nosocomial hospitalization.

1. Patients who have received an antibiotic within 90 days.
2. Hospitals or communities with high rates of antibiotic resistance.
3. Nursing home residents.
4. Patients receiving home infusions.
5. Patients receiving dialysis.
6. Patients with a family history of multidrug-resistant pathogens.
7. Patients receiving wound care.
8. Patients with immunosuppressive diseases (Davis 2006; Fagon and Rello 2006; Kollef 2007; Leone et al. 2007).

8 Host Response

8.1 Introduction

The inflammatory response to pneumonia, VAP, and lung injury involves three separate, but interrelated mechanisms: (1) the immune response to bacteria and endotoxins, (2) the inflammatory response to hypoxia, and (3) the inflammatory response to MV itself.

8.2 Inflammatory Response to Bacteria and Endotoxins

The lung is composed of the vascular compartment and the airway compartment. Endothelial cells in the arteries, veins, and capillaries line the vascular system and are the cells most actively involved in an inflammatory response. These cells may be regarded as the corresponding cells in the respiratory compartment. Airway epithelial cells also function as immune effector cells and secrete adhesion molecules, cytokines, and chemokines. The presence of lipopolysaccharide induces acute pulmonary edema as well as neutrophil recruitment by binding to CD14 and Toll-like receptors, resulting in the transcription of inflammatory mediators such as tumor necrosis factor α. Adhesion molecules play a major part in the inflammatory process by mediating adherence of leukocytes to the endothelium and initiating extravasation. Intercellular adhesion molecule 1 is a ligand for the β-2 integrins CD11a/CD18 and CD11b/CD18 on leukocytes. Endotoxin-induced lung injury occurs via both endothelial and epithelial intercellular adhesion molecule 1 (Madjdpour et al. 2000; Beck-Schimmer et al. 2002, 2004).

8.3 Inflammatory Response to Hypoxia

In addition to the inflammatory response to lipopolysaccharide and bacterial products, decreased alveolar oxygen levels (hypoxia) also induce lung inflammation. Hypoxia has been shown to alter the tachykinin system. Tachykinins are a family of peptides expressed in the lung and around the pulmonary vasculature, as well as in sensory nerves, located near the airways. Substance P is the tachykinin peptide expressed at the highest levels in the lung and it acts primarily through the NK-1 receptor. This ligand–receptor pair mediates neurogenic inflammation in the airways via cytokines, including IL-1, IL-6, and tumor necrosis factor α. Hypoxia increased the expression of NK-1 in the pulmonary microvasculature and in alveolar macrophages. It also increased Substance P levels in the lung by 48 and 72 h. These conditions could contribute to pulmonary edema (Zee et al. 2006).

Alveolar hypoxia was shown to induce macrophage recruitment, to increase albumin leakage, and to enhance the expression of macrophage-dependent inflammatory mediators. Alveolar macrophages appear to have a prominent role in the inflammatory response in hypoxia-induced lung injury and the related recruitment of inflammatory mediators (Madjdpour et al. 2003).

8.4 Inflammatory Response to MV

The administration of MV results in lung damage termed ventilator-induced lung injury (Charles et al. 2004). MV is an important cofactor in the development of acute lung injury. In the setting of prior injury or infection, MV can lead to inflammation, causing injury to the alveolar epithelial membrane and contribute to the development of acute lung injury (Dhanireddy et al. 2006).

In a murine model, MV enhanced lung inflammation when the animals were primed with systemic or intrathecal endotoxin. Neutrophil counts, MPO activity, and proinflammatory cytokine levels were increased by the combination of MV and bacterial products. MV was shown to be a contributing factor in the progression from pulmonary bacterial infection to acute lung infection/acute respiratory distress syndrome and multiple organ dysfunction syndrome (Dhanireddy et al. 2006).

The lung bacterial burden and the histological evidence of pneumonia were shown to be increased in rabbits receiving MV than in the spontaneously breathing animals (Charles et al. 2004). Bacterial translocation and systemic lung infection occurred more frequently during MV administration (Kollef 2002).

MV was also shown to contribute to epithelial cell apoptosis in distal organs such as the kidneys and small intestine. This apoptotic activity could contribute to the development of multiple organ dysfunction syndrome (Dhanireddy et al. 2006).

9 Diagnosis

9.1 Obtaining and Preparing Microbiological Specimens for Culture

9.1.1 Planktonic vs. Sessile Phenotype

In culturing and recovering microorganisms that are in the biofilm phenotype, dispersal and disruption have proven beneficial for recovery of such organisms. These are not the best, but at the moment the options are limited. There are several common, simple laboratory procedures that have been effective for a variety of biofilm phenotypes, as well as biofilms composed of monospecies and multispecies organisms and organisms associated with normal flora. However, the fact that these communities

are, by definition, attached to a surface, makes them recalcitrant to standard clinical microbiological culture techniques.

9.1.2 Disruption/Dispersal by Sonication

Biofilm configurations and 3-D architecture do not withstand the energy deposition associated with sonication. Hence, sonication has been a useful tool to disperse/disrupt/deaggregate the biofilm structure from explanted tissues or devices. Several observations need to be kept in mind. Probe sonicators are not as effective as water bath sonicators. Sonication should be done at three fourths of the instrument's total power and for a minimum of 3 min; 5 is optimum. It is important to utilize the energy measurements of the instrument in relation to ergs. In evaluating the usefulness of either the probe sonicator or the water bath sonicator, it is critical to assess, using dark-field microscopy, that the aggregates of the biofilm structure have been broken up into single morphotypes. Vortexing is a necessary complement to sonication. Two minutes is not uncommon.

9.1.3 Disruption by Scraping

Removal or loosening of the biofilm from the substratum is maximized by scraping. A variety of devices such as rubber "policemen" from tissue culture, blades, and other metallic devices may be useful in removing the biofilm before sonication and vortexing. Another unique adaptation of physical force to remove the biofilm is the lumenal brush. Although controversial, it has received considerable attention in Europe as the primary mode to ascertain the presence of biofilm in the lumen of PICC catheters. Data suggest that it has a place, if not in patient management, then clearly in laboratory for recovery of organisms bound securely to the lumen of the catheter. Given that it was meant to be used in a sterile patient environment, it is quite expensive for this task.

9.2 Antibiofilm ("Loss of Susceptibility")

One of the key functions of a diagnostic microbiology laboratory is its ability to measure, track and report changes in antibiotic profiles of planktonic isolates. Over the years, it has probably become the number one feature for clinicians, administrators, and pharmacists, recognizing the associated costs in treating resistant pathogens. These patterns are all on single, pure culture isolates grown in a luxurious, nonstressful environment, representing a planktonic phenotype only. Since it was established earlier that 65–80% infections in the ICU are associated with biofilms, the organisms that need evaluation are those in the biofilm phenotype.

In contrast, it is not the individual organism's resistance that is being evaluated, but rather the overall community's loss of susceptibility that is the phenotypic marker of the biofilm community. Thus, it is a significant challenge particularly for those biofilm communities that are multispecies and that reflect a phenotypic marker of the heterologous community.

9.3 Methodology for Differentiation between Planktonic and Biofilm Phenotypes

A number of unique and adventurous methodologies have been used, none of them standardized nor recognized by NCCLS. However, they have attempted to show the difference between the planktonic and the biofilm phenotypes, and at least recognize that more often than not, biofilms are composed of multispecies representing the normal flora of the colonized site and have significant loss of susceptibility.

9.3.1 Calgary Biofilm Device (MBEC)

Calgary biofilm device is the most recognized and well-established method for defining the biofilms' ability to resist anti-infectives. This device was developed to simulate requirements necessary for the NCCLS, which has standardized inoculum concentrations and utilizes a recognized platform on a 96-well plate. The MBEC (minimal biofilm elimination concentration) assay is illustrated in Fig. 9. It is commercially available, adapting a standard MIC methodology, emphasizing biofilm formation in sheer and in nutrient depravation, if necessary. The unique feature of this device is its corresponding MBEC. It is laborious and time-consuming and takes essentially 5 days; its value cannot be underestimated in patients who fail therapy and in whom biofilm elaboration is the major feature of chronic disease. It is important to remember that biofilms are often associated with chronic diseases and that in contrast to the planktonic phenotype, an immediate definitive MIC analysis by the laboratory is not necessarily a critical feature of long-term management. However, the ratio of MBEC to MIC may have clinical significance. The Calgary biofilm device has several modifications which allow one to use it either as a clinical tool or for evaluating anti-infectives in a more research setting. Results emphasize the difference between MIC and MBEC, often presented as a ratio (see Fig. 10).

9.3.2 Three-Profile Method for Antibiofilm Evaluation

In our laboratory we have established three profiles for antibiofilm evaluations, each of which has a subset. This was done in concert with clinical pharmacy, selecting the anti-infectives that might be most efficacious for the biofilm in the disease associated with the profile. It is important that selected non-anti-infectives may

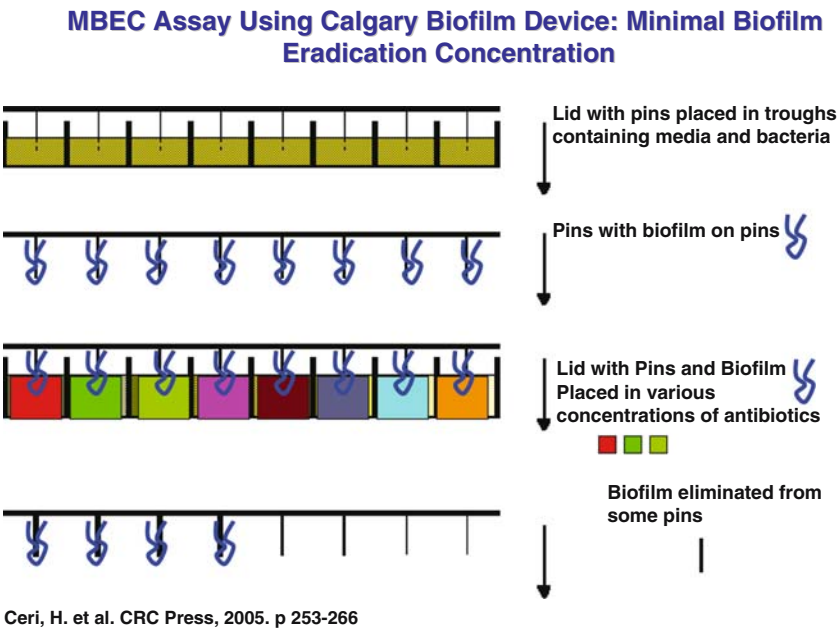

Fig. 9 Minimal biofilm eradication concentration (MBEC) assay using Calgary biofilm device

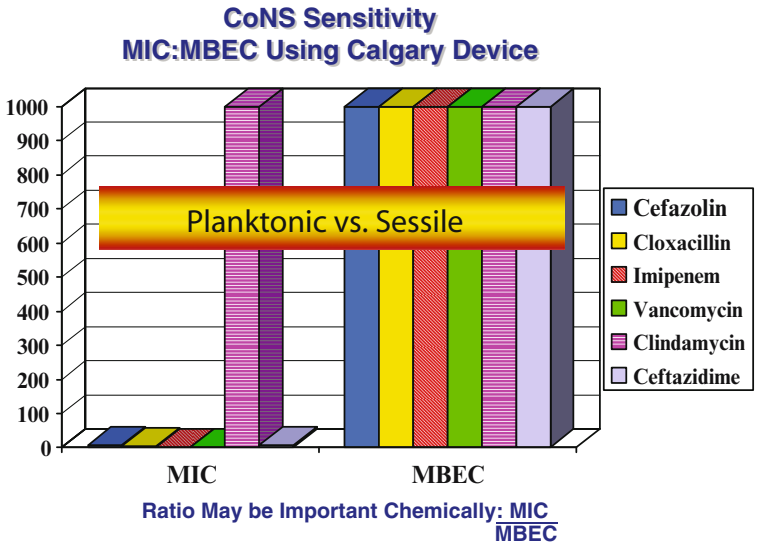

Fig. 10 The ratio of two population susceptibility endpoints may have clinical significance and predict poor outcomes with selected antibiotic interventions

have antibiofilm properties, and hence, the importance of azithromycin, a Gram-positive antimicrobial, used for patients with cystic fibrosis, or those primarily colonized with Gram-negative isolates. The three profiles are Bacteriology, Fungus, and Dental with the antibiotics in associated ranges. The four catagories utilized for Bacteriology include respiratory, blood, chronic wound, and urinary isolates. The Dental recognizes the Calgary biofilm device covered with calcium hydroxyl appetite, which replicates the tooth surface and allows for adherence of periodontal and cariologic microorganisms from the oral environment.

9.3.3 Poloxamer Technique

Recently the reverse gel or poloxamer has found unique applications in clinical microbiology. Reverse gels are liquids at temperatures below 15 C and solids at higher temperatures, i.e., 37 C. Planktonic isolates are upregulated in poloxamers (Gilbert et al. 1998), which allows for antibiotic disc or elution methods to measure biofilm loss of susceptibility when incubated at elevated temperatures. The method holds significant promise, as evidenced by a number of publications addressing its clinical value (Clutterbrick et al. 2007).

9.4 Overview of the Evolution of Diagnostic Microbiology

Table 9 highlights the evolution of diagnostic microbiology in the biofilm era. The newer methods for diagnosis, shown in the table, indicate that the majority of prokaryotes are viable but noncultable using standard methods and may be detected only by molecular-based systems or direct imaging, emphasizing fluorescence in situ hybridization.

Table 9 Evolution of laboratory and clinical tools for detection of biofilm phenotypes and associated diseases

Traditional diagnostic methods
Bronchial alveolar lavage
Tracheal aspirate
Protected specimen brush
• Quantitative vs. qualitative culture
Newer methods for diagnosis
Biofilm-directed diagnosis
• Cultable vs. viable but non-cultable organisms
Direct E-test
Molecular-based detection
Chemiluminescence-based detection
Fluorescence in situ hybridization
Poloxamer
New diagnostic/prognostic markers
Procalcitonin
C-reactive protein
S-TREM-1 (soluble triggering receptor expressed on myeloid cells)-1

10 Therapeutics

10.1 Introduction

Our current guidelines for the treatment of VAP focus on early detection and identification of the organism (American Thoracic Society; Infectious Diseases Society of America 2005). Although these guidelines are in place to help reduce treatment resistance and improve morbidity and mortality rates, they have not been completely successful. There is still a high rate of morbidity and mortality associated with VAP, and new therapeutic modalities are targeting prevention of biofilm formation. Current guidelines and therapeutic strategies as well as new research for the prevention of biofilm formation will be discussed in this section.

10.2 Antibiotic Strategies for the Treatment of VAP

After the diagnosis of VAP has been made, it is important to initiate antibiotics immediately. Appropriately choosing antibiotic regimens based on risk factors, using broad-spectrum agents, and adequately dosing antibiotics will help improve overall outcomes. If a patient has recently received an antibiotic the empiric regimen should include antibiotics from a different class. Once confirmation of the organism is received, a de-escalation of antibiotics should be considered. Patients who receive the appropriate antibiotic regimen and have a good clinical response may require a shorter duration of therapy (7–8 days) (American Thoracic Society; Infectious Diseases Society of America 2005).

10.2.1 Organisms Associated with Early-Onset VAP/Recommended Antibiotic Treatment

When choosing empiric antibiotic treatments, the first step is to evaluate time of onset and risk factors for drug resistant pathogens. In patients with early onset and no known risk factors for drug-resistant pathogens, the suspected organisms include *Streptococcus pneumoniae*, *Haemophilus influenzae*, methicillin-sensitive *Staphylococcus aureus*, and antibiotic-sensitive enteric Gram-negative bacilli. The recommended antibiotics are ceftriaxone, fluroquinolone, ampicillin/sulbacatm, or ertapenem (American Thoracic Society; Infectious Diseases Society of America 2005).

10.2.2 Organisms Associated with Late-Onset VAP/Recommended Combination Treatment

Patients with late onset or risk factors for resistant pathogens should be treated with broad-spectrum antibiotics. Suspected organisms include *P. aeruginosa*,

Table 10 Management options for ventilator-associated pneumonia pathogens associated with multidrug resistance (MDR)

MDR pathogen	Medication of choice
Pseudomonas aeruginosa	Combination therapy with β-lactam and a fluroquinolone or aminoglycoside
Acinetobacter	Carbapenems, sulbactam, colistin, polymixin, extended spectrum β-lactamase, carbapenems
Methicillin-resistant *Staph. aureus*	Linezolid, vancomycin

K. pneumoniae, *Acinetobacter* species, *Legionella pneumophila*, and methicillin-resistant S*taphylococcus aureus*. Antipseudomonal cephalosporin (cefepime, ceftiazidime), antipseudomonal carbepenem (imipenem or meropenem) or β-Lactam/β-lactamase inhibitors (piperacillin-tazobactam) plus antipseudomonal fluoroquinolone (ciprofloxacin or levofloxacin) or aminoglycoside (amikacin, gentamicin, or tobramycin) plus linezolid or vancomycin are the recommended antibiotic combinations. Decisions regarding antibiotic choice should be based on hospital surveillance data. Table 10 lists the pathogens that are often associated with multidrug resistance and the recommended therapeutic options for these patients (American Thoracic Society; Infectious Diseases Society of America 2005).

10.3 Aerosolized Antibiotics

Aerosolized antibiotics may be an effective adjunct to systemic antibiotic therapy. Small studies evaluating the use of aerosolized aminoglycosides have found them to be beneficial, but overall improvement in long-term morbidity and mortality is lacking (Mandell 2007). There has been concern that aerosolized antibiotics may increase the risk for resistance and have been used cautiously.

10.4 Alternative Therapeutic Options

10.4.1 Colistin

Multidrug-resistant bacteria have led to a need for more therapeutic options. Colistin is a polymyxin antibiotic that is effective against most Gram-negative bacilli. It has been used both intravenously and aerosolized for the treatment of VAP. Intravenous colistin has been shown to be effective in 25–62% of patients with Gram-negative infections (Linden and Paterson 2006). Less is known about the use of aerosolized colistin. Nephrotoxicity is common with colistin and patients should be monitored appropriately.

Reducing bacterial colonization in the airways has been a treatment consideration for many years. New modalities are now being studied to determine their usefulness in prevention of biofilm formation and treatment of bacterial colonization.

10.4.2 Chlorhexidine Gluconate

Chlorhexidine gluconate is a broad-spectrum antiseptic that is both bacteriostatic and baceteriocidal. It is thought to work by disrupting the membrane of the bacteria. Chlorhexidine is an active ingredient in mouthwash designed to kill dental plaque and other oral bacteria. Studies have used chlorhexidine to reduce the number of microorganisms in the mouth and reduce the risk of developing VAP. Small studies have demonstrated that chlorhexidine may be an effective agent in reducing the concentration of oral bacteria, thus reducing the incidence of VPA. Larger controlled studies need to be conducted to confirm these findings (DeRiso et al. 1996; Fourrier et al. 2000; Grap et al. 2004; Houston et al. 2002).

10.4.3 Protegrins

The use of topically applied antimicrobials has been studied for both the treatment and prophylaxis of VAP. Protegrins are a family of naturally occurring, small, broad-spectrum antimicrobial peptides. Iseganan is an antimicrobial peptide under development in multiple areas, including oral mucositis and VAP. In vitro data suggest that iseganan acts against aerobic and anaerobic Gram-positive and Gram-negative bacteria and yeasts. In 2003, the Food and Drug Administration granted fast track designation for iseganan for the prevention of VAP. A multicenter, randomized, double-blind trial was conducted to determine the occurrence of microbiologically confirmed VAP in patients receiving iseganan vs. placebo. Patients were randomized within 24 h of MV to receive 3 mL of iseganan oral solution or placebo six times per day for up to 14 days. Although not statistically significant, the study results showed 4% fewer VAP cases and 4% more deaths in the iseganan arm. The study did not support the hypothesis that topical administration of iseganan would reduce the incidence of VAP (Kollef 2006).

10.4.4 Silver-Coated Endotracheal Tubes

A phase III trial is currently enrolling patients to determine whether silver-coated ETTs are effective in decreasing the incidence of VAP (Rello et al. 2006). In vitro studies have supported the broad-spectrum activity of silver. Silver has been shown to block biofilm formation in animal studies. A recent study examined the feasibility and safety of using silver-coated ETTs in patients who required MV. The silver-

coated ETT was well tolerated and delayed colonization on the inner tube surface and prevented lung inflammation at a higher rate when compared with the control group (Pacheco-Fowler et al. 2004).

10.4.5 Quorum-Sensing Inhibitor

Biofilms are often antibiotic resistant and capable of producing toxins. Bacteria produce these toxins through a cell-to-cell communication known as quorum sensing. By interfering with the quorum sensing it may be possible to prevent certain types of infections. A number of researchers and companies have embarked on the development of such compounds, but to date, none have shown broad promise as therapeutic agents. Nonetheless, there is hope that such a therapeutic approach, which is primarily targeted to the biofilm lifestyle, will produce compounds that can be translated quickly into the clinical setting.

Treatment of biofilms with standard antibiotic therapy is difficult. Usually long courses of antibiotics are required and are often unsuccessful. The use of long courses of antibiotics increases the risk for drug resistance. New classes of medications are needed to help improve the morbidity and mortality associated with VAP. New drugs in the pipeline may offer a unique mechanism of action for treating infections that cannot be treated with conventional therapies.

11 Path Forward

VAP is now the most costly, mortality-associated disease for hospitalized patients. It has become a hallmark of disease progression in spite of changed antimicrobials, changed strategies and changed schemes. Recently, the focus has shifted away from the traditional use of antimicrobials and antiinfectives to the primary problem, which is the patient and the microbial population. The Institute for Healthcare Improvement initiative started in 2004 has focused on patient care and developed a scheme of four measured interventions that are outlined in Table 11. The initiative has had significant intervention potential to date.

Table 11 The emergence of nontherapeutic interventions to stabilize microbial burden historically associated with ventilator-associated pneumonia pathogenesis

Institute for healthcare improvement (IHI) initiatives: The ventilator bundle
Elevation of the head of the bed
Daily “sedation vacations” and assessment of readiness to extubate
Peptic ulcer disease prophylaxis
Deep venous thrombosis prophylaxis

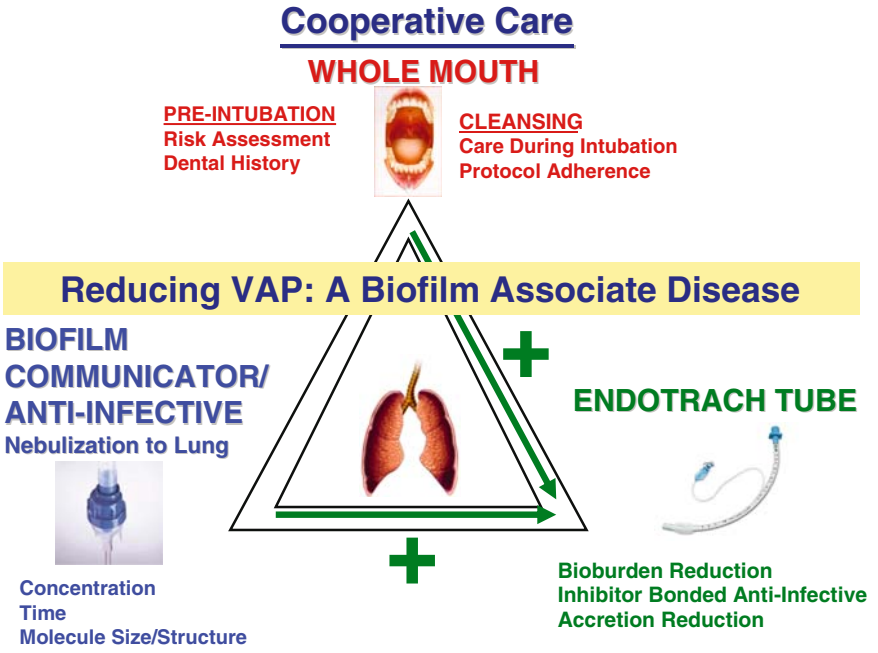

Fig. 11 Interrelating the need for partnering or linking therapies and interventions to reduce ventilator-associated pneumoniaVAP

Key, however, is the emerging theme that no single therapy or intervention will be completely successful. It must involve multiple schemes. Figure 11 addresses cooperative care and interventions including (1) whole mouth care; (2) biofilm reduction and bioburden reduction (utilization of the ecologic hypothesis) using forces that favor nonpathogenic organisms in the oral flora which will reduce the potential for lumenal inclusion of selected pathogens; and (3) the utilization of endotrach materials that will alter the surface tension, and reduce hydrophobicity and the ultimate coadhesion of selected markers. Intervention should also focus on the work of breathing and reduction of accretion buildup, which is the macroscopic portion of a microscopic process.

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Biofilm Formation on Natural Teeth and Dental Implants: What is the Difference?

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Abstract Dental plaque is a milieu of microbes embedded within a polymeric layer of glycoproteins precipitated from the saliva onto tooth enamel, dental implants, cementum, and epithelium. This dental biofilm can extend below the gingival margin into the socket where the microorganisms are concealed from the bacteriostatic components in the saliva and the shearing forces exerted by chewing and normal brushing. Gingivitis and periodontal disease occurs when more virulent microbes colonize the subgingival pocket and evade eradication by the host defenses. A distinctive shift in microbial colonization is observed with the progression of disease highlighted by the expansion of gram negative and strictly anaerobic bacteria. Host response and inflammation meant to eliminate the microbial insult are counterproductive and generate a deeper pocket within the dental socket that permits the microbial access to the alveolar bone. Dental implants form another nidus for bacterial infection following surgery when the surface and socket are exposed to the oral environment. Dental implantation may be a direct consequence of periodontal disease; therefore the underlying microbial infection could threaten successful osseointegration of the implant if a microbial biofilm develops. Periodontal pockets and other oral niches, such as the mucosa and tonsils, are reservoirs for the microbial pathogens that initiate the inflammation of the marginal soft tissue around the dental implants that may lead to implant failure. Reduction in peri-implantitis rates are shown when patients delay implant surgery after tooth extraction and rigorous preoperative and postoperative antibiotic regiments as well as improved dental hygiene are incorporated into the post-operative treatment.

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1 Introduction: General Principles of Biofilm Formation on a Natural Dentition

Dental plaque is a diverse microbial community, embedded in a matrix of host and bacterial polymers, growing on teeth as a biofilm. Dental plaque develops naturally and contributes to the host defenses by preventing colonization by exogenous species. The composition of dental plaque varies at distinct surfaces as a result of the inherent biological and physical properties at these sites; the balance of the predominant bacterial populations shifts in disease. Plaque has an open architecture, with channels traversing from the biofilm surface through to the enamel. This structure affects the movement of molecules within plaque, and gradients in key determinants develop. Bacteria growing on a surface display a novel phenotype; one consequence of which is an increased resistance to antimicrobial agents (Marsh 2003).

In the history of oral microbiology, Antonius Van Leeuwenhoek set the stage in the seventeenth century while describing several bacteria present in dental plaque. He detected mobile and immobile bacteria and the influence of oral hygiene on the bacterial composition of the plaque present. The mouth is an open growth reservoir for bacteria because of access to nutrients through the presence of saliva and the crevicular fluid. This is why over 700 different species of microorganisms have been identified through molecular approaches as inhabitants of the oral cavity.

All surfaces in the oral cavity are continuously covered with a pellicle. Pellicle is a selective precipitation of glycoproteins from the saliva onto the hard surfaces like tooth structures and other surfaces (e.g., implant surfaces) and epithelium. These negatively loaded globular structures are attracted to the positively loaded structures such as calcium ions present in the hydroxyapatite of enamel. In as little as 2 h, the pellicle on tooth surface is 100 nm thick. Bacterial attachment to this pellicle takes place in two phases. First an initial reversible attachment occurs due to equilibrium between the van der Waals attraction and the electrostatic rejection. A second irreversible attachment then occurs when bacteria bridge with their pseudopods to the surface when located in the primary minimum, the closest location to the surface they can get. Motile bacteria can also use their own energy to bridge the distance and adhere to the surface. Higher surface-free energy and surface roughness are important factors to facilitate primary adhesion. Most likely the first bacteria that will adhere are the facultative anaerobic bacteria, for example, streptococci. As colonization progresses, more Gram negative species will colonize, consisting primarily of anaerobic rods and cocci together with fusiform bacteria. After 6–9 days of undisturbed plaque formation, motile bacteria, for example, spirochetes often join the biofilm. One gram of supragingival plaque contains 10^{11} bacteria.

1.1 Oral Microbial Successions

The histology of a tooth structure dictates that not only will supragingival plaque be formed but that the biofilm will also extend below the gingival margin. A healthy

pocket, the socket that creates a perfect niche for biofilm formation, normally contains 10^3–10^5 bacteria. There, the bacteria adhere not only to the enamel but also to the pocket epithelium and the exposed cementum. Subgingival flora differs from the supragingival flora not only because the nutrients are different (crevicular fluid) but also because these microorganisms are better protected from the bacteriostatic components in saliva and from external removal forces of the tongue and cheeks, chewing forces, and normal brushing techniques. A pocket can remain healthy as long as the local immune defense of the host cope with the situation presented. Whether a pocket is healthy or diseased is relative. Crevicular fluid is blood serum with a high concentration of plasma proteins. This fluid, which includes neutrophils, lymphocytes, and macrophages, originates from the crevicular plexus and leeks through the connective tissue and the pocket epithelium into the pocket. The trilogy of periodontitis, host defense, virulence of the bacteria present, and oral hygiene regimen, dictate whether infection will eventually lead to disease.

1.2 Pathogenesis

1.2.1 Gingivitis

The experimental gingivitis model of Loe and Theilade explains that excluding any oral hygiene regimen will allow for plaque to grow. From the third day on, gingival inflammation could be diagnosed through the Silness and Loe index (Silness and Loe 1966) and detection of Gram negative species, filaments and fusiform bacteria was observed. When optimal oral hygiene was reinstated after 15 days, gingival inflammation disappeared and the colonization of the plaque present had been changed to aerobic cocci and rods. This has been explained through the experimental gingivitis model (Theilade et al. 1966).

1.2.2 Marginal Gingivitis

Because of supragingival plaque growth, the subgingival area will be better protected and a lower concentration of oxygen will be present. A shift in bacteria will take place (Table 1).

Table 1 Shift in bacteria when a pocket turns from a healthy pocket into disease

Healthy pocket	To	Diseased pocket
Gram +ve		Gram −ve
Cocci		Rods
Immobile		Motile
Facultative anaerobe		Strict anaerobe
Fermentative		Proteolytic

An augmentation in the number of *Actinomyces israelii* and *A. naeslundi* and *Capnocytophaga* species is observed. With the induction of gingivitis, the necessary nutrients (haemine) for *Prevotella intermedia* and *P. nigrescens* become present and result in colonization by these organisms.

1.2.3 Necrotizing and Ulcerative Gingivitis

Most probably, a weakening of the immune system in the patient due to stress, smoking, or other underlying disease could be the predisposing factor resulting in necrotizing or ulcerative gingivitis. This allows for rapid growth and invasion of a high number of *Treponema* species and *Prevotella intermedia* in the pocket epithelium, which coincides with foetor ex ore, bleeding, and high fever.

1.2.4 Acute Streptococcus Gingivitis

Overgrowth of *Streptococcus pyogenes* in the pharynx or on the tonsils can cause pharyngitis or tonsillitis, which can spread to the gingival tissues and cause acute streptococcus gingivitis. In contrast to other gingival infections, this is a very specific disease that can be treated with a small spectrum penicilline.

1.2.5 Periodontitis

The role of bacteria in the process of periodontitis has been proven in animal studies in gnotobiotic species. Inducing species of the red complex led to periodontal breakdown that could be stopped through specific antibiotic treatment (Haffajee and Socransky 2000). The genetic proneness and the environmental factors together with the bacteria present determine whether the immune system of the patient can withstand infection or collapse and eventually lead to periodontitis.

The role of bacteria in acute infections could even be more important. The impact of antibiotic treatment in juvenile, rapid progressive, refractory, and acute necrotizing periodontitis can therefore be huge. Periodontitis is a nonspecific disease meaning different bacteria can cause a destruction of the periodontium. Within the microbial species responsible for periodontitius, different subspecies can often be more virulent than others. The lack of effective antibody production against *A. actinomycetemcomitans* could be related to periodontitis at a very early age and has previously been called *Juvenile periodontitis*. This disease clinically presents as a very aggressive alveolar bone loss, where the bone and soft tissues around first molars and incisors are involved first. This localized character can change into a generalized pattern over time. Not only is there a prominent presence of *A. actinomycetemcomitan* but also *Porphyromonas gingivalis* and *Bacteroides forsythus*. Adult periodontitis, however, is the most common form of periodontitis.

The disease is known under different clinical manifestations, all of which lead to the loss of "teeth supporting tissues" when left untreated. The subgingival species reflect a motile character, are mainly anaerobic, and show no proteolitic activity. Spirochetes and rods are most common in such a gingival pocket. The Forsyth institute described several bacteria that increased in number in active periodontal pockets, including, *A. actinomycetemcomitans, P. gingivalis, P. intermedia, B. forsythus, P. micros*, and *S. intermedius*.

1.3 Summary

The concentration of bacteria in the pocket does not differ between a socket with gingivitis or one with periodontitis. Only the depth of the pocket changes, thus the total number of bacteria and the virulence of the bacteria present will be the indicating factor. The type of bacteria present and the virulence factors of those bacteria, in the different forms of disease, can differ. However, in these types of infections in the open environment of the oral cavity, the virulent bacteria will almost always exist as a complex community. Therefore, therapeutics mostly enquires inclusion of broad spectrum antibiotics. The main focus of oral disease care should still be the prevention of community formation.

2 Biofilm Formation on Dental Implants in the (Partially) Edentulous Patient

Implants can be placed using two-stage surgery. The pristine implant surface is torqued in the jawbone and after inserting a cover-screw, the periosteum covers the implant allowing for osseointegration of the implant. Osteoblasts grow towards the titanium surface and because of the osseoconduction capacities of titanium oxide, the pseudopods of the osteoblasts allow complete osseointegration of the dental implant. A residual infection site could cause biofilm formation on the titanium surface at the apex of the dental implant, or the presence of fibroblasts in close contact with the dental implant could allow for fibrous encapsulation of the titanium surface under sterile circumstances. This will prevent the implant from osseointegration. If osseointegration takes place, the implant will be uncovered after 3–6 months according to this protocol. Different implant surfaces might promote the osseointegration and this might lead to faster exposure of the implants. A transmucosal part allows fixation of the superstructure to the dental implant. This transmucosal part is again a pristine surface that will be fixed in the oral cavity. Biofilm formation will take place on this surface exposed to the oral environment, according to the postulate of Koch, immediately after surgery.

Implants can also be placed immediately with the transmucosal part called "single stage surgery." Biofilm formation on the transmucosal parts will thus occur

simultaneously with the osseointegration process of the dental implant. Under these circumstances, the adherence of the biofilm microorganisms and osseointegration process will be two competing processes. Therefore, bacterial load must be low enough such that healing time is sufficient for the osteoblasts to adhere "first" to the implant surface. Enhancing the implant surface characteristics speeds up the process of osseointegration and positively alters the balance in profit of integration (Le et al. 2007).

2.1 Apical Biofilm Formation

Primary endodontic infections are mixed infections dominated by anaerobic bacteria, often characterized by species belonging to genera *Fusobacterium*, *Prevotella*, *Porphyromonas*, and *Actinomyces* (Sundqvist 1992a, b). Many of these microorganisms are recognized or implicated pathogens in periodontal and peri-implant disease. Multiple case reports have raised concerns that implant sites with a history of endodontic infection or proximal to teeth with endodontic infection may increase the risk of implant failure (Shaffer et al. 1998).

Novaes and Novaes (1995) reviewed the success of dental implants in patients, where the implants had been placed immediately following tooth extraction. The authors concluded that successful implant integration is highly predictable, based on their clinical experience, at tooth extraction sites with prior periapical lesions, given appropriate preoperative and postoperative management, including meticulous alveolar debridement. Lindeboom et al. (2006) evaluated the clinical success of implants placed in alveolar sites with chronic periapical infection. Fifty patients were randomized to immediate implant placement after extraction, and 25 to placement after a 3-month healing period. Two implant failures occurred in the immediate implant placement group, whereas none failed in the delayed placement group. Although the most prevalent cultivable bacteria were *F. nucleatum* (70%) and *P. micros* (42%), both commonly found in root canal infections and associated with periodontal disease, over 50% of the cases were characterized by a polymicrobial flora. Collectively, these studies provide only limited evidence on the relationship of endodontic infection to implant failure and suggest, with appropriate surgical and antimicrobial management, that history of endodontic infection does not pose a significant clinical risk. The evidence, however, is limited; several recent studies provide insight into the potential risk of implant failure at alveolar sites with prior endodontic infection. Most probably, the risk of implant failure is associated with the amount of endodontic infection and the manageability of the infected site before implant placement.

2.2 Coronal Biofilm Formation

The formation of a soft-tissue sulcus following surgical placement and wound healing always takes place when transmucosal parts are attached. The depth of the sulcus is

variable and determined primarily by the relationship of the implant platform to the crestal height of bone and the thickness of the overlying soft tissue. As a consequence of the sulcus, oral microbial colonization and biofilm development on dental implants and teeth exhibit shared characteristics, both in health and disease.

The awareness of one of the classic postulates of Koch, the transfer of abscesses, is very important in the oral field of implant dentistry. This means that transfer of bacteria from one locus to another can cause the same disease in the other locus, whether this is between or within subjects. Transfer of bacteria from one surface to another can take place through bacterial crawling or distribution in the saliva.

Bacterial plaque accumulation exhibits an association with inflammation of the marginal soft tissue around dental implants, as with natural teeth (Berglundh et al. 1992). Moreover, healthy gingiva and peri-implant keratinized mucosa exhibit marked similarities in the distribution and phenotypes of inflammatory cells (Tonetti et al. 1995). Although surface properties of implants may affect early bacterial adherence, these features do not appear to be significant in influencing long-term oral microbial successions (Nakazato et al. 1989). The microbial composition of the biofilm and chronicity of exposure represent risk factors for alveolar bone loss, as in periodontal disease.

Studies on bacterial translocation provide strong evidence that teeth with periodontal pockets can serve as reservoirs for microbial pathogens that can colonize implant sites (Ellen 1998; Papaioannou et al. 1996; Quirynen and Listgarten 1990). Similarities in pattern of bacterial colonization between implants and teeth exhibiting inflammatory breakdown support this model of microbial transmission (Cooper and Moriarty 1997). Moreover, given that periodontal pockets are at higher risk of colonization by pathogenic bacteria, such as Gram-negative rods and spirochetes, many clinicians consider partially dentate patients, particularly those with a history of periodontitis, to be at a greater risk for the development of peri-implantitis (Meffert 1996). Recent studies now provide insight into the development and maturation of biofilms associated with dental implants in the edentulous patient. Less information, however, is available on the etiologic role of biofilms in inflammatory peri-implant disease and implant loss in the edentulous patient.

Multiple observational studies support a differential risk between partially dentate and edentulous patients for the development of peri-implant biofilms harboring pathogenic microorganisms and inflammatory soft tissue conditions. Kalykakis et al. (1998), for instance, found that partially dentate subjects accumulated more plaque, exhibited higher crevicular fluid flow rates, and harbored more frequently *P. gingivalis* and *P. intermedia* than edentulous subjects. Apse et al. (1989) found a higher proportion of black pigmenting anaerobes on implants in partially dentate than edentulous patients. George et al. (1994) compared the clinical and microbiological parameters in partially dentate and edentulous patients with a history of periodontal disease. Recovery of *A. actinomycetemcomitans* from peri-implant sites was found to be similar in partially dentate and edentulous patients; however, *P. gingivalis* and *P. intermedia* were identified more frequently from implants in partially dentate than edentulous patients. Consistent with the pathogenicity of these selected bacteria, implant sites harboring *A. actinomycetemcomitans*,

P. gingivalis, or *P. intermedia* were found to exhibit greater marginal soft tissue inflammation. Moreover, a comparison of the frequency of microbial recovery at 1–2 years with 3–4 years revealed that the probability of identifying these bacteria increased significantly the longer an implant was present in the oral cavity (George et al. 1994). Peri-implant probing depths were also shown to increase over this time period, suggesting that clinical pocket formation parallels oral microbial successions.

Further evidence of differential biofilm colonization between partially dentate and edentulous patients comes from studies exploring the predominate cultivable microorganisms associated with peri-implant disease. Augthun and Conrads (1997) examined anaerobic bacteria colonization of inflammatory tissue associated with deep (>5 mm) peri-implant pockets in edentulous patients with failed implants. As often documented in patients with periodontitis, *F. nucleatum*, *Capnocytophaga* spp., *Eikenella corrodens*, *A. actinomycetemcomitans*, and *Bacteroidaceae* spp. were recovered from the deep pockets. *A. actinomycetemcomitans* and *Bacteroidaceae* spp. were commonly cultivated from the peri-implant pockets. Deep peri-implant pockets, therefore, appear to be a greater risk for a disproportionate colonization with selected bacteria, as in the advanced periodontal lesion (Augthun and Conrads 1997). The identification of these bacteria in deep peri-implant pockets is also consistent with their recovery and role in the pathogenesis of peri-implantitis (Hultin et al. 2002).

Within the context of periodontal disease risk assessment and treatment, there is growing recognition that the oral cavity and pharynx possess multiple ecological niches, such as mucosa and lymphoid tissue, which may harbor potentially periodontopathic microorganisms. The presence of such bacterial niches would suggest that dental implants, similar to teeth, are at risk of colonization and biofilm formation – a risk that should increase in susceptible hosts as a function of exposure. Danser et al. (1997) compared the recovery of microorganisms from clinical healthy oral mucosa and peri-implant tissues in edentulous patients with a history of periodontitis. Mucosal and peri-implant tissues were found to harbor *Peptostreptococcus* spp., *Fusobacterium* spp., and other *Prevotella* species. Although low cultivable levels of the putative periodontal pathogens *Bacteroides forsythus* and *Campylobacter rectus* were identified from these sites, *A. actinomycetemcomitans* and *P. gingivalis* were not detected in this patient population. Interestingly, *P. intermedia* was associated at implant sites with peri-implant probing depths of 5 mm or greater, as frequently found in periodontal pockets, as well as an association between *Peptostreptococcus* spp. and marginal soft tissue inflammation. These findings suggest that the oral mucosa can serve as a source for bacterial colonization of dental implants in edentulous patients.

Longitudinal studies of bioflim colonization on implants in edentulous patients provide additional evidence consistent with the notion that other oral niches, such as the mucosa and tonsils, can act as a clinically important repository for periodontal pathogens. Devides and Franco (2006), for example, evaluated the presence of the periodontal pathogens *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* in the mandibular arch of edentulous patients prior to implant placement and 4 and 6 months after the placement of mandibular implant-supported fixed-prostheses. Prior to implant placement, recoverable levels of *P. gingivalis* were not found.

A. actinomycetemcomitans and *P. intermedia* were isolated in a small number of patients (13.3% and 46.7%, respectively). Noteworthy, however, was that the proportion of patients harboring these periodontal pathogens was higher at 4 and 6 months following implant placement. At 6 months, the proportion of patients with cultivable evidence of *P. gingivalis*, *A actinomycetemcomitans*, and *P. intermedia* ranged from about 50–75% for each microorganism. Devides et al. concluded that oral microbial colonization and succession of these microorganisms in the peri-implant sulci occurs as a function of time in the oral environment, even in the absence of clinical or radiographic evidence of peri-implant disease (Devides and Franco 2006). There is emerging evidence to suggest that full-mouth disinfection supports more successful therapeutic management of inflammatory periodontal disease, which may prove the case in treatment of peri-implant disease.

The submarginal microbial composition of plaque associated with clinically stable and functional implants in edentulous patients has been characterized by the presence of Gram-positive or facultative anaerobic cocci (Apse et al. 1989; Bower et al. 1989; Hultin et al. 1998; Mombelli et al. 1988; Pontoriero et al. 1994). Mombelli et al. (1988), for instance, identified *Fusobacterium* spp. and *P. intermedia* in less than 10% of samples from implants in edentulous patients. A recent review and meta-analysis of studies on experimentally induced peri-implantitis in animal models revealed that the period of ligature application (i.e., the duration of plaque accumulation) generally had no influence on the resultant depth of the bone defect (Baron et al. 2000). These findings suggest that other factors, such as microbial composition, may influence more directly the severity of peri-implant bone loss (Baron et al. 2000). Microbial composition, in turn, may also be influenced by implant composition, as suggested by in vitro studies (Berry et al. 1992; Gao et al. 2002).

There is limited evidence to suggest that the host–parasite interaction at the implant site may be different in partially dentate and edentulous persons. Hultin et al. (1998) reported that inflammation around implants induced a stronger neutrophil response (i.e., functional and antigenic elastase) in partially dentate than edentulous patients, despite the absence of significant differences in the microbiota and clinical parameters. Other studies, however, suggest that individual differences in wound healing capacity and inflammatory response, which are influenced by modifiable factors, such as cigarette smoking, contribute to risk for peri-implant disease, as found in periodontal disease (Esposito et al. 1998; Newman and Flemmig 1988; Paquette et al. 2006). Impaired healing ability is considered a common cause of early implant failure, whereas chronic marginal inflammation in conjunction with host characteristics is a major etiological factor in late failure (Esposito et al. 1998).

2.3 Summary

Although the success of implants in partially dentate and edentulous individuals appears comparable (Klinge 1991), the risk of developing peri-implantitis seems to be more pronounced in patients with a history of periodontitis (Klinge et al. 2005;

Roos-Jansaker et al. 2006). The results of multiple studies suggest that the early implant-associated microflora in edentulous patients often lacks *A. actinomycetemcomitans*, *P. gingivalis*, and other periodontopathogens (Augthun and Conrads 1997; Mombelli et al. 1995; Mombelli and Mericske-Stern 1990; Papaioannou et al. 1995; Quirynen et al. 1994). Nevertheless, colonization of implant sites by these pathogens and others, including *F. nucleatum*, *P. micros*, and *E. corrodens*, has been well documented in edentulous patients, particularly at implant sites with pocket formation (Danser et al. 1997; Nakou et al. 1987). Collectively, these observational findings support the concept that microflora colonizing mucosal niches, such as the tonsils, can contribute to microbiota formation associated with the implant and marginal soft tissue (Lee et al. 1999; Quirynen et al. 2002).

3 Biofilm Formation as a Risk Factor for Peri-Implant Disease and Implant Failure

Excessive surgical trauma together with an impaired wound healing, premature occlusal loading, and infection are frequently implicated in early implant failures (an implant that fails before 6 months after transmucosal exposure), whereas marginal infection and excessive occlusal force are factors commonly implicated in the etiology of late failures (Esposito et al. 1998). Late implant failures also cluster in a small subset of individuals (Esposito et al. 1998; Tonetti 1998). In a study by Lee et al. (1999), microbial changes were observed the longer the implants had been in function and in those patients with a history of periodontal or peri-implant infections. A history of periodontitis had a greater impact on the peri-implant microbiota than implant loading time. The major influence on the peri-implant microbiota was, however, the microbiota on remaining teeth. Laine et al. (2005) evaluated the bacterial cultures from implant sites in 17 patients who lost 30 implants. The most common cultivable aerobic and anaerobic bacteria were *Streptococcus milleri* and *Fusobacterium nucleatum*, respectively.

A growing body of evidence indicates that failing implants can be associated with a complex microbiota. Alcoforado et al., for example, found that failing implants were often associated with a complex microflora, which included bacteria such as *Peptostreptococcus micros*, *Wolinella recta*, and *Fusobacterium* species (Alcoforado et al. 1991). Importantly, primarily nonoral microorganisms, including *Candida albicans*, were also associated with failing implants. The investigators conclude that, given the diversity in microbial composition and antimicrobial susceptibility among "peri-implantitis" isolates, antimicrobial therapy should not be implemented early in patient management, even without a prior comprehensive microbiological analysis (Alcoforado et al. 1991).

Leonhardt et al. (1999) examined stable and failing implants in 88 patients. Stable implants with no marginal inflammation were shown to harbor a microbiota similar to that found in periodontal health. In contrast, over 50% of the implants with peri-implantitis were colonized by multiple periodontal pathogens, including *P. gingivalis*,

P. intermedia, and *A. actinomycetemcomitans*. Moreover, the sites often harbored nonindigenous and opportunistic microorganisms less commonly associated with periodontitis, such as *Staphylococcus* spp., enterics and *Candida* spp., suggesting possible differences between natural teeth and implants in risk for colonization and disease progression. Listgarten and Lai (1999) similarly concluded that the detection frequency and levels of recovery of some periodontal pathogens in failing implants are significantly different from that of teeth with periodontitis.

The proportion of spirochetes and motile organisms has been found to be higher in association with implant with deeper probing depths (Papaioannou et al. 1995). Rosenberg (Rosenberg et al. 1991) examined the microflora associated with implants failing from infection or trauma in partially dentate and edentulous patients. Failure was attributed to infection when clinical signs and symptoms, such as bleeding and suppuration, were present. Spirochetes and motile rods comprised about 40% of the total morphotypes associated with implants failing secondary to infection. A high proportion of the cultivable flora was comprised of *P. micros*, *Fusobacterium* spp., enteric gram-negative rods, and yeasts. In contrast, implants failing secondary to traumatic etiology demonstrated a microbiota predominated by streptococci and similar to that found in periodontal health. The latter findings suggest that traumatic occlusion may predispose implants to failure, independent of biofilm formation (Saadoun et al. 1993).

3.1 Genetic and Environmental Risk Factors

Some studies show that a small number of patients lose the majority of their implants (Fardal et al. 1999), consistent with the prevalence of moderate to severe periodontitis. There is growing interest in the role of inflammatory cytokines, such as interleukine 1, in modulating bone resorption in peri-implantitis (Salcetti et al. 1997). Recent genetic studies reveal polymorphisms associated with multiple proinflammatory cytokines that may predispose the host to differential susceptibility to bone loss secondary to biofilm formation; however, the predictive value of these genetic markers for identifying patients at risk for implant failure remain unknown. Currently, systemic and environmental factors that compromise wound healing and immunologic competence, such as smoking and poorly controlled diabetes mellitus, remain the important risk factors for implant failure (Esposito et al. 1998; Paquette et al. 2006).

4 Future

There is a prevailing belief that the most judicious way to prevent peri-implantitis and implant failure is through sound surgical technique, ensuring a zone of keratinized tissue, biomechanical over-engineering, and rigorous follow-up and hygiene instruction (Clarizio 2000). As with periodontal disease, there is a clear recognition

that overall levels of biofilm burden result in an inflammatory lesion that predisposes individuals to periodontal breakdown. Biofims also remain a significant risk factor for peri-implantitis and implant failure in certain individuals. The factors that modulate risk, however, remain incompletely understood. Future studies are necessary to elaborate on that aspect.

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Complicated Urinary Tract Infections due to Catheters

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Abstract Urinary catheters are indwelling medical devices used in both the nosocomial and nursing home settings for relief of urinary retention and incontinence. Due to the frequent- and sometimes unnecessary-use of indwelling catheters in these settings (circa 21–50% of patients), many individuals are placed at risk from sequelae associated with catheter placement. The most notable complication associated with indwelling urinary catheters is the development of nosocomial urinary tract infections (UTIs) known as catheter-associated UTIs (CAUTIs). Over one million CAUTI cases per annum are recorded in the United States; this represents greater than 40% of all nosocomial infections. Both the presence of the catheter itself and the nosocomial setting predispose individuals to CAUTIs. CAUTIS may be caused by a number of etiological agents; the most common of these being *Escherichia coli* and *Proteus mirabilis*. Microorganisms isolated less commonly from CAUTIs include *Staphylococcus aureus*, *Klebsiella* spp., *Pseudomonas* spp. and *Enterobacter* spp. Regardless of the genus, pathogens wishing to survive within the environment of the catheterized urinary tract must possess essential virulence factors including, most importantly, those related to adherence to the catheter itself. This chapter seeks to set out in detail the nature of these virulence factors.

1 Introduction

Indwelling urinary catheters are standard medical devices utilized in both hospital and nursing home settings to relieve urinary retention and urinary incontinence. The urinary catheter used most commonly is the Foley indwelling urethral catheter, comprising a tube inserted through the urethra and held in place by an inflatable balloon to allow drainage of the bladder. Long-term indwelling catheter use is becoming more commonplace.

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Owing to frequent and sometimes unnecessary use of indwelling catheters during hospitalization (21–50% of patients (Jain et al. 1995)), many individuals are placed at risk from associated sequelae. A study of 1,540 nursing home residents determined that the risk of hospitalization, length of hospitalization, and length of antibiotic therapy were three times higher in catheterized residents than in noncatheterized residents (Kunin et al. 1992). The most notable complication associated with indwelling urinary catheters is the development of nosocomial urinary tract infections (UTIs) known as catheter-associated UTIs (CAUTIs).

1.1 Epidemiology of the Infection

CAUTIs, the most common type of nosocomial infection, account for more than 1 million cases annually (Tambyah and Maki 2000) or more than 40% of all nosocomial infections in hospitals and nursing homes (Stamm 1991; Stamm and Hooton 1993; Warren 1997), and constitute 80% of all nosocomial UTIs (Hartstein et al. 1981). Furthermore, catheter-associated bacteremia is estimated to cost ~$2,900 per episode (Saint 2000).

Individuals requiring an indwelling catheter are predisposed to the development of CAUTIs because of the presence of both the catheter itself and potentially pathogenic multidrug-resistant organisms in the hospital setting. This fact is underscored by the fact that the majority of these patients, by the nature of their visit, are also immunocompromised. Most cases of catheter-associated bacteriuria or the presence of bacteria in the urine are asymptomatic. However, when an episode of CAUTI becomes symptomatic, the resulting sequelae can range from mild (fever, urethritis, cystitis) to severe (acute pyelonephritis, renal scarring, calculi formation, bacteremia).

1.2 Pathogenesis of CAUTIs

Similar to other mucosal pathogens, uropathogens use specific strategies to infect the urinary tract, including colonization of a urinary catheter and/or mucosal site (uroepithelial cells), evasion of host defenses, replication, and damage to host cells (Fig. 2). The insertion of a foreign body such as an indwelling catheter into the bladder increases the susceptibility of a patient to UTIs. The majority of uropathogens are fecal contaminants or skin residents from the patient's own native or transitory microflora that colonize the periurethral area (Clegg and Gerlach 1987; Daifuku and Stamm 1986; Leranoz et al. 1997; Old et al. 1983; Yamamoto et al. 1985).

Once firmly attached on the catheter surface or the uroepithelium, bacteria begin to change phenotypically, moving into the sessile, biofilm mode of growth (Fig. 1). Indwelling urinary catheters favor colonization of uropathogens by providing a surface for the attachment of host cell binding receptors that are recognized by bacterial adhesins, thus enhancing microbial adhesion. Upon insertion, urinary catheters may damage the protective uroepithelial mucosa, leading to exposure of

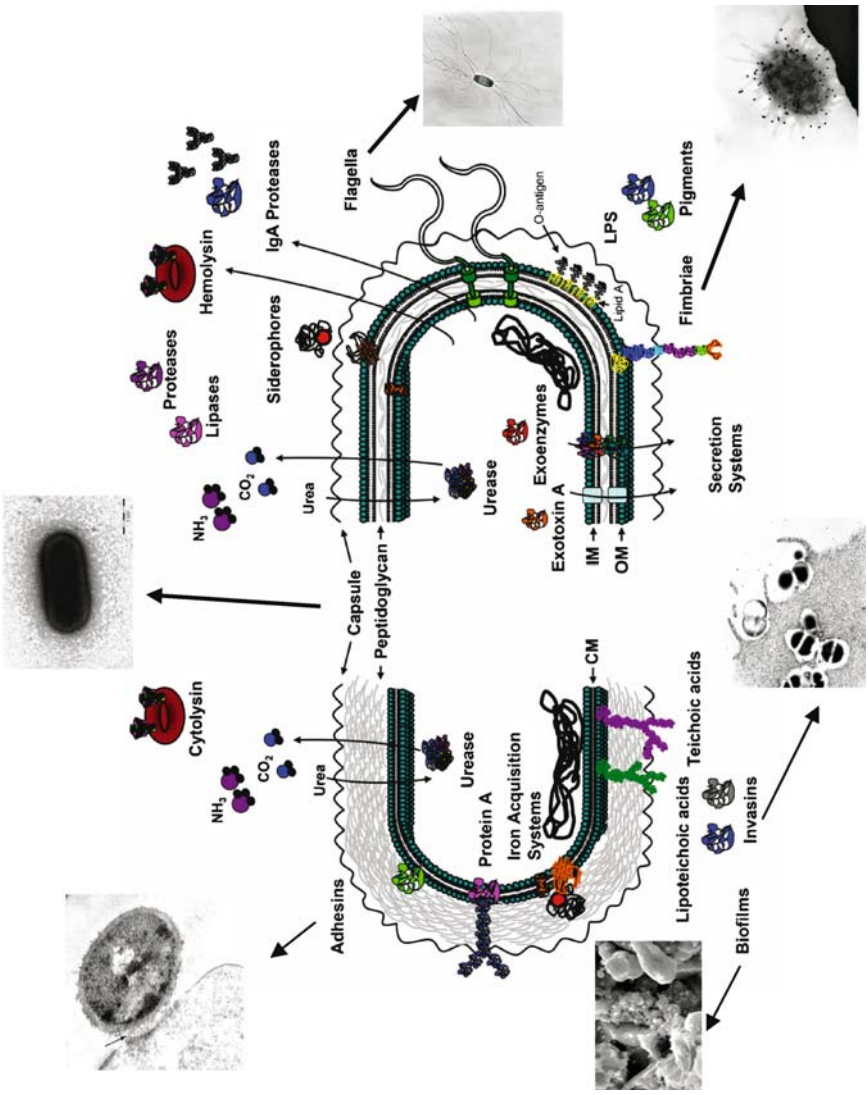

Fig. 1 Virulence factors of uropathogens (Schembri et al. 2005; Jones et al. 2004; Li et al. 1999; Bayles et al. 1998)

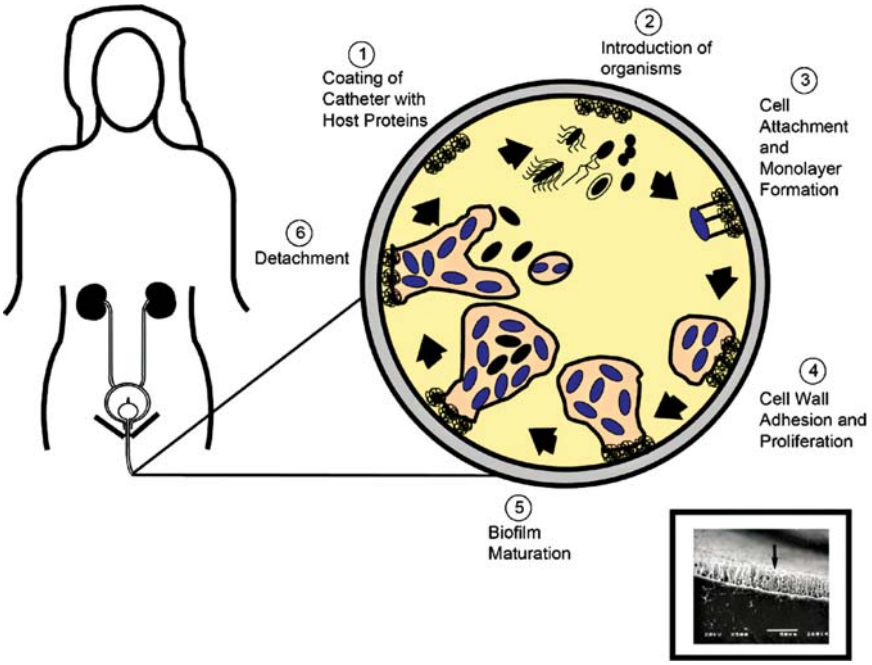

Fig. 2 Pathogenesis of biofilm formation on urinary catheters during CAUTIs. *Insert:* a scanning electron micrograph of a urinary catheter encrusted with *Pseudomonas aeruginosa* (Stickler et al. 1998)

new binding sites for bacterial adhesins (Garibaldi et al. 1980). Finally, the presence of the indwelling catheter in the urinary tract disrupts normal host mechanical defenses, resulting in an overdistension of the bladder and incomplete voiding, which leaves residual urine for microbial growth (Hashmi et al. 2003).

It was surprising indeed to discover during the composition of this chapter how little effort has been directed toward understanding the role of biofilm in the initiation and persistence of CAUTIs. This is particularly so when one considers the large – and growing – body of evidence implicating biofilm growth in the pathogenesis of a wide variety of infections in multiple anatomical sites. It is our view that research involving CAUTIs should be redirected and focused on the biofilm growth of organisms if there is ever to be hope for improved patient health.

2 CAUTIs Caused by *Escherichia coli*

Escherichia coli is a facultative anaerobe and member of the family *Enterobacteriaceae*. These organisms are serotypically diverse, spanning over 250 serotypes based on the O, H, and K antigens (Orskov et al. 1977). UPEC are the most commonly isolated organisms in community-acquired UTIs (70–90%) and

among the most commonly isolated in nosocomial-acquired UTIs (50%), including CAUTIs (Kucheria et al. 2005). *E. coli* has been identified as the causative agent in 90% of all cases of UTI in ambulatory patients (Johnson and Stamm 1989).

2.1 E. coli Virulence Factors Implicated in CAUTIs

2.1.1 Adhesion

E. coli (and, indeed, any other potential uropathogens) must attach to uroepithelial cells and the catheter surface to colonize and initiate CAUTI and express a variety of adhesins to assist in this initial attachment. Adhesins also contribute to the direct triggering of host and bacterial signaling pathways, assisting in the delivery of bacterial products to host tissues, and promoting bacterial invasion into host cells (Mulvey 2002). A study by Reid et al. (1996) suggested that nonspecific adhesins, not specific fimbriae, expressed by UPEC, are responsible for attachment to urinary catheter material, emphasising the central role of biofilm in pathogenesis. Currently, it is unknown which specific adhesin molecules are involved with the colonization of UPEC to catheter surfaces. However, potential adhesins associated with UTIs, including type 1, P, S, FC1 and F9 fimbriae and Iha and Dr adhesins, could possibly play a role during CAUTIs. The most extensively studied adherence factors of UPEC are type 1 and P fimbriae (Mulvey 2002); an in-depth description of these structures has been reviewed elsewhere (Fernandez and Berenguer 2000; Mulvey 2002).

Since UPEC are more commonly associated with infections of the intact urinary tract, it is thought that less virulent organisms are capable of causing CAUTIs. These bacteria may express fewer and perhaps different virulence factors during this process, compared with organisms that are able to infect structurally and functionally normal urinary tracts (Mobley et al. 1987). It has been implied that *E. coli* strains that infect the catheterized urinary tract have a reduction in the expression of P fimbriae and possibly other factors such as hemolysin, serum resistance, colicin production, and certain H, O, and K serotypes (Mobley et al. 1987). This suggests that the presence of a urinary catheter and a neuropathic bladder increases susceptibility to colonization of the urinary tract (Benton et al. 1992). These data also strongly suggest that the ability to colonize and exist as a biofilm community on the urinary catheter surface is central to the pathogenesis of CAUTIs.

Type 1 fimbriae are critical for the interaction of UPEC with uroepithelial cells during colonization of the bladder (Connell et al. 1996; Langermann et al. 1997; Mulvey et al. 1998; Thankavel et al. 1997). FimH of type 1 pili are involved in the adherence of these organisms to the bladder epithelium through the recognition and binding of mannosylated integral membrane glycoproteins uroplakin Ia (Zhou et al. 2001) and Ib located on superficial epithelial cells. These pili have been suggested to be expressed for the initial interactions between *E. coli* and various surfaces; thus it is speculated that type 1 pili could be involved in the initial interactions with the catheter surface or in interactions with uroepithelial cells during CAUTIs caused by this microorganism.

P fimbriae or pyelonephritis-associated pili (*pap*) are the second most common virulence factor associated with *E. coli* uropathogenesis. *E. coli* expressing P fimbriae attach to globoside residues present on human kidney epithelial cells and play a role in the pathogenesis of pyelonephritis as well as ascending UTI (Dodson et al. 2001; Plos et al. 1995). However, experimental evidence suggests that these adhesins have a less important role in colonizing abnormal or obstructed urinary tracts (Jantunen et al. 2000; Tseng et al. 2001). Based on these findings, it is thought that P pili may have either no role or a limited role during CAUTIs caused by *E. coli*.

Recently, Ulett et al. (2007) described a novel fimbria for UPEC strain CFT073 known as the F9 fimbria. These fimbriae were suggested to play a role during biofilm formation and are found in other UPEC and pathogenic *E. coli* strains. UPEC is capable of expressing other surface adhesins, including S pili (Mulvey 2002), F1C pili, IrgA adhesin, and Dr adhesins (Mulvey 2002). The precise role of these surface structures during CAUTI is yet to be elucidated.

E. coli expresses a number of virulence factors that assist in its ability to persist in the urinary tract. However, there is limited research on how this organism adheres to catheter surfaces. It can be speculated that some of the known adhesins UPEC uses during UTIs may be expressed during CAUTIs caused by this organism as host cell components attach to the catheter surface to provide binding sites. However, more extensive research on adherence of *E. coli* during CAUTIs is both warranted and necessary to better understand the pathogenesis of this infection.

2.1.2 Motility

Once *E. coli* is established on the catheter surface, flagellar-mediated motility is important for the ascent of this uropathogen from the catheter to the bladder and subsequently to the upper urinary tract (ureter, kidney). Two recent mutagenesis studies by Lane et al. (2005) and Wright et al. (2005) demonstrated that flagella, although not absolutely required for virulence during UTIs, greatly enhanced persistence and fitness of *E. coli* during this type of infection. Therefore, flagellar-mediated motility should likely be considered as important for movement of *E. coli* on the catheter surface and from the catheter surface to the uroepithelium. This, however, has not yet been demonstrated directly.

2.1.3 Invasion and Formation of Biofilm

Invading bacteria must possess strategies for avoiding the host immune response in order to persist in the urinary tract; these include fimbriae that are subject to phase variation, production of capsular exopolysaccharide, and formation of biofilm. Type 1 fimbriae are subject to phase variation to evade the host immune response and have been implicated in the initial stages of biofilm formation (see earlier text). *E. coli*, as with other uropathogens, has been shown to produce an exopolysaccharide capsule as a means of avoiding the immune response and thereby contributing

to serum resistance. There are over 80 types of these capsular polysaccharides (K antigens), with K1 capsules most frequently observed among urinary and clinical isolates (Johnson 1991; Orskov 1978). These capsules are thin, highly anionic structures that tend to aggregate spontaneously, aiding in biofilm formation (Jorgensen et al. 1976). These acidic polysaccharide capsules assist in avoiding phagocytosis and complement activation (Johnson 2003). Currently, there are no studies that have investigated the role of these structures during CAUTIs caused by *E. coli*.

The ability of *E. coli* strains to persist in the urinary tract has been demonstrated to be related to the formation of intracellular biofilm structures known as intracellular bacterial communities (IBCs); (Justice et al. 2004). These IBCs are formed in a sequential manner. First, during murine cystitis, the invading bacteria are attached to the cell surface by type 1 fimbriae and then invade the uroepithelium (Martinez and Hultgren 2002; Martinez et al. 2000) 1–3 h after the initial inoculation. Localized actin rearrangements occur and engulf the bound organism via a zipper-like phagocytosis (Martinez and Hultgren 2002). After being internalized in the murine superficial bladder cell, UPEC replicates rapidly forming clusters known as early IBCs (Justice et al. 2004). Recently, type 1 fimbriae have been shown to have an additional intracellular role during this stage of IBC formation (Wright et al. 2007). As IBCs mature around 6–8 h postinoculation of the murine bladder (Justice et al. 2004), they more closely resemble classical biofilm structures (Dunne 2002) where the bacterial doubling time is increased (from ~30 to 60 min) and the bacterial cell length is shortened (0.7 μm vs. 3 μm). At this stage, podlike protrusions are observed on the surface of murine bladder epithelial cells (Anderson et al. 2003). Around 12 h postinoculation, bacterial detachment is observed (Justice et al. 2004) as either a whole community or individual highly motile cells that burst out of the murine bladder lumen in a process referred to as fluxing, comparable to classical shedding of biofilm flocs (Anderson et al. 2004). IBCs and biofilms likely contribute to the persistence of these organisms due to the increase in resistance to antibiotics and the host immune response. IBC formation has not yet been demonstrated in humans.

Several factors contribute to the formation of biofilms by *E. coli*. These include fimbriae, curli, flagella, antigen 43 (Ag43), and extracellular matrix molecules, including cellulose, colanic acid, and poly-β-1,6-*N*-acetyl-D-glucosamine (Danese et al. 2000, 2001; Davey and O'Toole 2000; Donlan and Costerton 2002; Wang et al. 2004; Zogaj et al. 2001). Specifically, biofilm formation mediated by type 1 fimbriae may assist in colonization of urinary catheter surfaces (Mulvey 2002).

There have been recent studies examining biofilm formation on catheters by UPEC. Ferrières et al. (2007) demonstrated that certain catheter materials such as silicone and silicone–latex actually select for and promote biofilm formation for the most virulent UPEC strains whereas asymptomatic bacteriuria strains form better biofilms on polystyrene and glass. Koseoglu et al. (2006) revealed that UPEC type O4 had formed mature biofilms after 12–24 h and developed biofilms completely in almost all latex/silicone balloon catheters samples after 4–7 days, as examined by scanning electron microscopy.

Thus biofilm formation is critical for initiating and maintaining CAUTIs. Any factors involved in this process are likely important virulence factors during CAUTIs caused by UPEC. However, there are currently very few studies that have examined biofilm formation, structure, and importance in these infections. Our understanding of the pathogenesis of – and ability to treat – CAUTIs would most likely be enhanced greatly by further research in this area.

3 CAUTIs Caused by *Proteus mirabilis*

Proteus species, members of the family *Enterobacteriaceae* (Penner 1984), are distinguishable from most other genera by their ability to swarm across an agar surface. These organisms are widely distributed in the environment and in the intestinal tract of humans and other mammals. *Proteus* bacilli are more commonly associated with UTIs in those individuals with structural or functional abnormalities, especially ascending infections in patients undergoing urinary catheterization (Warren et al. 1982, 1987). Colonization of the intestinal tract allows *Proteus* to establish reservoirs for transmission into the urinary tract by intermittent colonization of the periurethral region. This intermittent colonization can lead to subsequent contamination of the catheter, thus allowing nosocomial infections to develop (Chow et al. 1979).

Proteus-associated UTIs are difficult to treat and the bacterium persists because of complications associated with this type of infection, including bladder and kidney stone formation (urolithasis), that can lead to obstruction of catheters and the urinary tract (Krajden et al. 1987; Scott 1960; Warren et al. 1982). The three species of *Proteus* associated with UTIs are *Proteus mirabilis*, *Proteus vulgaris*, and *Proteus penneri*. While UTIs caused by *P. vulgaris* (Senior and Leslie 1986) and *P. penneri* (Krajden et al. 1984, 1987) have been identified, *P. mirabilis* is the third most common cause of complicated UTI (12%) and second most common cause of catheter-associated bacteriuria in long-term catheterized patients (15%) (Warren 1996). To establish and maintain infections of the urinary tract and colonization of catheters, *Proteus* species must adapt to the catheterized urinary tract and produce an arsenal of strictly regulated virulence factors.

3.1 P. mirabilis Virulence Factors Implicated in CAUTIs

3.1.1 Adhesins

Indwelling urinary catheters serve as the initiation site of CAUTIs by introducing uropathogens such as *Proteus* spp. into the urinary tract and providing a surface for coating by host cell debris and protein that may be recognized by bacterial adhesins. *P. mirabilis* strains tend to attach to catheters with a greater propensity, compared

with other Gram-negative bacteria (Roberts et al. 1990). Studies have demonstrated that *P. mirabilis* attaches to a number of catheter polymers, including ethylene, propylene, polystyrene, sulfonated polystyrene, silicone, and red rubber (Hawthorn and Reid 1990; Roberts et al. 1990).

To facilitate binding to these different surfaces, *P. mirabilis* must be capable of producing a variety of adherence factors, such as fimbriae and hemagglutinins, that play an important role in the establishment of CAUTIs. *Proteus* species have been shown to produce various fimbriae and hemagglutinins involved with colonization of the urinary tract and possibly catheter surfaces, including mannose-resistant/Proteus-like (MR/P) fimbriae (Old and Adegbola 1982), mannose-resistant/Klebsiella-like (MR/K) hemagglutinin (Old and Adegbola 1982), uroepithelial cell adhesin/nonagglutinating fimbriae (UCA/NAF) (Wray et al. 1986), *P. mirabilis* fimbriae (PMF) (Bahrani et al. 1993), and ambient temperature fimbriae (ATF) (Massad et al. 1996).

MR/P fimbriae are perhaps the best-understood fimbriae expressed by *P. mirabilis* strains during UTIs. These fimbriae are thick channelled (7–8 nm) and are classified as mannose-resistant fimbriae (Brinton 1965; Silverblatt and Olek 1978). Expression of MR/P fimbria is subject to phase variation (Bahrani and Mobley 1993, 1994). Many studies have suggested that MR/P fimbriae play a role in the virulence observed during UTIs caused by uropathogenic *P. mirabilis*. In the CBA model of ascending UTI, infection with *P. mirabilis* elicited a strong immune response to MrpA, the major structural subunit of MR/P fimbria, indicating that MR/P fimbriae were expressed *in vivo* (Bahrani et al. 1991). Isogenic mutants incapable of expressing MR/P fimbriae were attenuated when examined in this mouse model (Bahrani et al. 1994; Li et al. 1997, 1999, 2002). A mutant constitutively expressing MR/P fimbria outcompeted the wild-type strain in the murine bladder but not the kidneys in a cochallenge experiment, thereby establishing MR/P fimbria as an important colonization factor of the bladder for *P. mirabilis* (Li et al. 2002). Tissue-binding studies by Sareneva et al. (1990) revealed the propensity of this fimbrial type to adhere specifically to the human renal tubular epithelial cells and to the exfoliated uroepithelial cells of urinary sediment.

Experiments conducted by Jansen et al. (2004) suggest that MR/P fimbriae dictate the localization of bacteria in the bladder and contribute to biofilm formation, a process essential for the establishment of CAUTIs. A *P. mirabilis* HI4320 construct engineered to constitutively express MR/P fimbriae colonized the luminal surfaces of murine bladder umbrella cells and formed significantly more biofilms after 2 days of growth in urine ($P = 0.05$), compared with a deletion mutant. Although studies have associated the expression of MR/P fimbriae with virulence during UTIs caused by *P. mirabilis*, there is no direct evidence substantiating a role of these fimbriae during CAUTIs.

On the other hand, MR/K fimbriae have been linked with the attachment of organisms to catheter surfaces and with persistence of catheter-associated bacteriuria (Mobley et al. 1988; Rozalski et al. 1997; Yakubu et al. 1989). Expression of these thin (4–5 nm) nonchanneled type 1 mannose-resistant fimbriae (Brinton 1965; Silverblatt and Olek 1978) enables *P. mirabilis* to attach tightly to the Bowman's

capsule of the host kidney glomeruli and to the tubular basement membranes (Sareneva et al. 1990). Although more associated with *P. penneri* strains (Yakubu et al. 1989), it is speculated that MR/K fimbriae play a possible role in the initial adherence to catheter biomaterials during *P. mirabilis* CAUTIs.

Besides MR/P and MR/K fimbriae, the other fimbriae produced by *P. mirabilis* during UTIs may contribute to attachment to the catheter surface. Surface adhesins determined not to be involved in the hemagglutination caused by MR/P and MR/K fimbriae have been identified in *P. mirabilis*, including UCA/NAF (Bahrani et al. 1993; Bijlsma et al. 1995; Cook et al. 1995; Tolson et al. 1995; Wray et al. 1986), PMF (Massad et al. 1994a,b), and ATF (Massad et al. 1994a,b). Wray et al. (1986) characterized UCA, a NAF from *P. mirabilis* HU1069 that was demonstrated to weakly attach to exfoliated human desquamated uroepithelial cells. The 540-bp *ucaA* gene that encodes the major fimbrial subunit of UCA has nucleic acid homology to the *f17a* gene of *E. coli* F17 pilin (58%) (Cook et al. 1995) and was identified in all 26 *P. mirabilis* strains tested (Bijlsma et al. 1995). Owing to the homology of UCA to the F17 pilin of *E. coli* (Cook et al. 1995), these fimbriae might be involved in the colonization of the intestines (Coker et al. 2000). Based on studies conducted by Bahrani and colleagues (Bahrani et al. 1991, 1993; Bahrani and Mobley 1993), there was some ambiguity as to which fimbrial types were identified as UCA (Wray et al. 1986) since thin (4 nm) and thick (6 nm) fimbrial filaments were observed by electron microscopy and multiple bands were isolated on sodium dodecyl sulfate polyacrylamide gel (Tolson et al. 1995). These fimbrial subunits were isolated and characterized from a *P. mirabilis* strain 7,570 isolated from a patient with struvite urolithiasis and renamed nonagglutinating fimbriae (NAF) by Tolson et al. (1995). The N-terminal sequence of this fimbrial subunit was confirmed to be identical to the N-terminal sequence from *P. mirabilis* HU1069 strain of the Wray study and not homologous to the N-termini of MR/P, ATF, or PMF fimbrial subunits (Tolson et al. 1995). Bacteria expressing NAF adhered strongly to a number of cell lines *in vitro*, including uroepithelial cell (Tolson et al. 1997), MDCK (Madin-Darby canine kidney) (Altman et al. 2001; Lee et al. 2000), and EJ/28 urinary tract tumor cell lines (Latta et al. 1998). Purified NAF from *P. mirabilis* binds to a number of glycolipids such as asialo-GM1, asialo-GM2, and lactosyl ceramide (LacCer), as demonstrated by thin layer chromatography overlay assays and solid-phase binding assays (Lee et al. 2000). Because of its homology to fimbriae that assist in intestinal tract colonization, it is possible that these fimbriae play a role in the initiation of CAUTIs by allowing *P. mirabilis* to attach and establish in the intestines and thus form a reservoir of organisms that can potentially cause CAUTIs. However, there have been no definitive studies examining this possibility.

P. mirabilis fimbriae are encoded by the genes located in the *pmf* gene cluster. There are conflicting results as to the function these fimbriae during UTI. In a study by Massad et al. (1994a,b), PMF were demonstrated to play a role during colonization of the bladder since an isogenic mutant in the *pmfA* gene of *P. mirabilis* HI4320 was 83-fold more attenuated when compared with the wild-type strain during independent challenge in the CBA mouse model of ascending UTI (Massad et al.

1994 a,b). However, in this same study, PMF could not be shown to be involved with attachment to human uroepithelial cells since attachment to this cell type is similar in both the wild type and the *pmfA* mutant. These conflicting results require resolution.

Ambient-temperature fimbriae were classified as a new fimbrial type as examined by electron microscopy and immunogold labeling (Massad et al. 1996) and were identified in all eight *P. mirabilis* strains analyzed. An allelic replacement *atf* mutant colonized the murine urinary tract at a comparable gene to the wild type in independent challenge and outcompeted the wild type in cochallenge experiments in the murine model of ascending UTI (Massad et al. 1996). As nonclinical strains of *Proteus* express AtfA as observed by Western blot analysis (Zunino et al. 2000), it was suggested that these fimbriae are involved in colonization of *P. mirabilis* in the environment and are most likely not involved in CAUTIs.

Currently, only MR/K fimbriae are known to be associated with the process of attachment during CAUTIs. Clearly, additional studies must ascertain whether known factors or currently uncharacterized factors are involved in adherence as this process is essential for these types of infections. The identification of novel adherence factors as well as other virulence factors will be facilitated by the recent annotation of the *P. mirabilis* HI4320 genome by the Sanger Centre in conjunction with the Mobley Laboratory.

3.1.2 Motility

In general, flagella on the surface of bacterial pathogens assist in host colonization and dissemination, initial attachment, and sensing the extracellular environment (Belas and Suvanasuthi 2005). For *Proteus* species, these surface structures are important in the process known as swarming, a distinct characteristic of these organisms. Therefore, flagellar motility and potentially swarming are important during CAUTIs as the ability of *P. mirabilis* to disseminate from the initial site of colonization on the catheter surface to the uroepithelial cells of the urinary tract is critical for establishment of infection.

Swarming is a surface-induced, multicellular differentiation process that allows organisms to move in a coordinated manner and expand the population to new locations over solid surfaces (Mobley and Belas 1995; Rather 2005; Williams and Schwarzhoff 1978). During growth in liquid medium, *Proteus* species assume the form of an infectious single-cell, motile, 1.0–2.0 μm-long that displays a distinct phenotype including the presence of peritrichous flagella on its cell surface and swimming behavior. However, when transferred onto solid medium, these swimmer cells differentiate into hyperflagellated, multinucleated, nonseptated, elongated swarmer forms, measuring 20–80 μm in length. These differentiated swarmer cells migrate out from the original inoculation site in a rapid and highly coordinated manner that is dependent upon multicellular interactions and cell-to-cell signaling (Belas, 1992).

Swarmer cells align themselves in multicellular rafts and are enveloped in the colony migration factor Cmf extracellular slime material that is required for and

facilitates translocation through a reduction in surface friction (Gygi et al. 1995; Jones et al. 2004; Stahl et al. 1983). The swarming process continues until cell numbers are reduced by cell loss or when the bacterial mass changes direction of motion (Belas, 1992). The cessation of movement, known as consolidation, is accompanied by the dedifferentiation and replication of swarmer cells into vegetative swimmer cells. This periodicity distinguishes *P. mirabilis* swarming from other swarming processes. For a more in-depth description on the process of *Proteus* swarming, refer to the reviews by Rather (2005) and Rozalski et al. (1997).

Since swarming is such a dominant characteristic of this genus, any factors that affect or regulate this phenomenon would likely affect the fitness of the organism. The swarming phenomenon is a metabolically complicated and demanding process that must genetically coordinate the expression of more than 50 genes (Belas et al. 1991), including those involved in the production, assembly, and operation of flagella and virulence factors such as flagellin, urease, hemolysin, and the ZapA metalloprotease (Allison et al. 1992; Belas et al. 2004).

The definitive roles of flagella and swarmer cell differentiation in the virulence of *P. mirabilis* during UTIs remain controversial but include dissemination of *P. mirabilis* from the initial site of infection to other sections of the catheter or to the urinary tract and avoidance of the host immune response. Flagella are believed to contribute to the virulence of swimmer cells by allowing motility from the catheter to the bladder epithelium and onward, ascending into the ureters and kidneys. An isogenic nonpolar, nonmotile, flagella-negative mutant in *flaD* of *P. mirabilis* WPM111 was attenuated 100-fold when compared with the wild type in the CBA model of ascending UTIs, indicating the importance of flagella in murine UTIs (Mobley et al. 1996).

The biosynthesis of flagella is a key process in both motility and swarming and involves numerous genes on the *Proteus* chromosome (Belas and Flaherty 1994). Flagellin is encoded by *flaC,* and the *flaD* gene encodes for the flagellar filament capping protein (Belas 1994). Studies suggest that the major flagellin protein for *P. mirabilis* is subject to antigenic variation through homologous recombination as three copies of flagellin-determinant gene (*flaA*, *flaB*, *flaC*) that reside on the *P. mirabilis* genome with only one copy that is actively expressed (Belas 1994; Murphy and Belas 1999). It was proposed that flagellin gene rearrangement is a mechanism for host immune system evasion by *P. mirabilis* and is extremely relevant for *Proteus* infections since flagella are highly immunogenic. As a result, any antigenic change could increase the survival of *Proteus* species in the urinary tract through the evasion of sIgA directed toward flagella during colonization in the bladder (Belas 1994). Owing to its relevance during UTIs, it is probable that antigenic variation via flagellin gene rearrangement is a method of host immune response evasion by *P. mirabilis* during CAUTIs.

Swarming cell differentiation is important for the virulence of *P. mirabilis* during UTIs since several virulence factors, including flagellin, urease, hemolysin HmpA, and IgA metalloprotease ZapA, are upregulated in the differentiated swarmer vs. swimmer cells (Allison et al. 1992; Fraser et al. 2002). Mutants in FlhA synthesis, proteins required for flagellar synthesis, are nonmotile because of the loss of *fliC*

transcription, but also have a reduced transcription of *hpmA* hemolysin (Gygi et al. 1995). Therefore, on the basis, in part, of the evidence of coordinate expression of virulence factors during swarming cell differentiation, factors involved in the swarming process are critical in pathogenesis and a similar signal must be regulating both swarming and virulence (Allison et al. 1992, 1993; Chippendale et al. 1994; Mobley and Belas 1995). Interestingly, however, swarmer cells are rarely observed in the murine model of ascending UTI, bringing into question the relevance of this morphotype in the absence of a catheter (Jansen et al. 2003).

Swarming may play a role in the migration of *Proteus* strains on catheter materials. Swarmer cells of *Proteus* species are capable of migrating across 1 cm-long sections of Foley catheters made of hydrogel-coated latex, hydrogel/silver-coated latex, silicone-coated latex, or all-silicone *in vitro* (Sabbuba et al. 2002). Swarmer cells of *P. mirabilis* have been observed to migrate through populations of *E. coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis*, and then continue to migrate with little or no reduction over hydrogel-coated latex catheter sections (Sabbuba et al. 2002). Nonswarming mutants lost the ability to migrate over these catheter sections (Jones et al. 2004). However, upon their introduction into models of the catheterized bladder, these mutants were just as capable as the wild type of encrusting and blocking catheters (Jones et al. 2005a,b). It seems that although swarming might have a role in the initiation of infection, facilitating the passage of the cells from the urethral meatus to the bladder, it is not necessary for the rapid formation of crystalline biofilm, after bacteria have colonized the residual urine in the bladder.

3.1.3 The Role of Biofilm in *P. mirabilis* CAUTIs

During CAUTIs, after initial colonization of the catheter surface, *Proteus* species, as with other uropathogens, form distinctive crystalline biofilm structures (Fig. 3). Biofilm assists in persistence of *P. mirabilis* in the urinary tract by protecting these organisms from antibiotics (Stewart & Costerton 2001) and the host immune response (Leid et al. 2005) and obviously contributes to adhesion to surfaces. Urinary stone formation during *Proteus*-mediated UTI is characteristic of this type of infection and is critical for the development of crystalline biofilms. Bacterial-derived stones account for up to 30% of all urinary tract stones worldwide and account for ~75% of those urinary stones classified as staghorn calculi (Hochreiter et al. 2003). Crystalline biofilms are especially problematic during CAUTIs since catheters become blocked because of encrustation caused by the formation of these structures.

It should be recognized that physical and chemical factors are involved in the initiation and development of the crystalline biofilms that block catheters. Experiments in parallel-plate flow cells showed that when urine cultures flow over polymer surfaces, the pH of the urine can be a major factor in determining bacterial adhesion. For example, some polymers with strongly electron-donating surfaces will resist colonization by cells until the pH of the urine rises above the pH at which

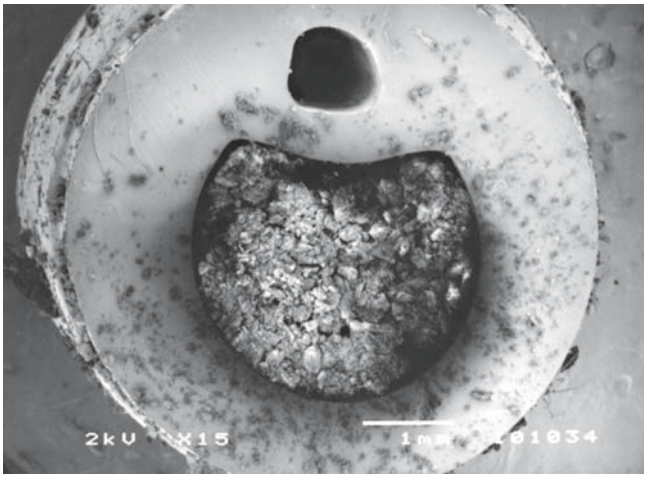

Fig. 3 A cross-section of a silicone catheter removed from a patient after blockage. Crystalline material can be seen completely occluding the catheter lumen

calcium and magnesium phosphates precipitate out of solution. In the alkaline urine, macroscopic aggregates of cells and crystals form in the urine, settle on the polymer surface, and initiate crystalline biofilm formation (Stickler et al. 2006). These observations indicate that to stop biofilm formation on devices in the urinary tracts of patients infected with *P. mirabilis*, it is essential to prevent the rise in urinary pH and the crystallization of apatite and struvite.

Bacterial urease and capsule polysaccharides are known to be involved with urinary crystal formation and, hence, crystalline biofilm formation in *P. mirabilis* (Musher et al. 1975). Since urea is present in concentrations of up 500 mM in human urine (Breitenbach and Hausinger 1988; Jones and Mobley 1987), it is not surprising that bacterial ureases play a pivotal role in the *Proteus*-associated UTI. Urease contributes to the development of urinary stones due to urease-mediated hydrolysis of urea to ammonia and carbon dioxide, which alkalinizes the local environment. This increase in urinary pH causes the local supersaturation and precipitation of calcium phosphate and magnesium ammonium phosphate from urine to form crystals of carbonate apatite [$Ca_{10}(PO_4)_6CO_3$] and struvite ($MgNH_4PO_4.6H_2O$), respectively (Griffith et al. 1976). These crystals accumulate in the biofilms of catheters and urinary epithelial surfaces and eventually obstruct the flow of urine through the catheter and from the bladder or kidney.

The importance of urease as a virulence factor of *Proteus*-associated UTIs as well as CAUTIs has been demonstrated in the CBA mouse model. An insertion mutation in the *ureC* gene abolished urease activity and was attenuated in the murine model (Jones et al. 1990). This urease-negative construct colonized the bladder and kidneys in 100-fold fewer bacteria than did the urease-positive strain 2 days postinoculation and caused no urolithiasis during infection (Johnson et al.

1993). A study by Li et al. (2002) revealed that catheterized CBA mice were more susceptible to infection by the wild-type *P. mirabilis* strain after 7 days, compared with uncatheterized CBA mice. However, although catheterized CBA mice were more susceptible to bladder colonization by the *ureC* insertion mutant than were uncatheterized mice, the mutant was unable to colonize the kidneys under any circumstances (Li et al. 2002). These results suggest that even though urease is important during colonization of the urinary tract during uncatheterized infection, it is not necessary for the initial colonization of the bladder during CAUTIs in the mouse model.

Besides bacterial urease, capsular structures assist in crystalline stone formation observed during UTIs and CAUTIs associated with *P. mirabilis*. These structures are believed to accelerate struvite crystal growth (Clapham et al. 1990; McLean et al. 1991) by aggregating precipitated components of urine into stones (McLean et al. 1988). *Proteus* capsular polysaccharides (CPS) tend to be acidic because of the presence of uronic acid, pyruvate, or phosphate groups, thus, enabling this structure to bind to metal cations such as Ca^{2+} and Mg^{2+} (Rozalski et al. 1997). Purified partially anionic CPS of *P. mirabilis* ATCC 49565 added to artificial urine at a pH of 7.5–8.0 induced more struvite formation than did other CPS types, as examined by particle counting (Coulter counter) and by phase-contrast microscopy (Dumanski et al. 1994). With the exception of one polymer (curdlan) that did not bind Mg^{2+} ions, enhancement of struvite crystallization by CPS polymers was inversely proportional to their Mg^{2+} binding ability. Therefore, it is suggested that weak binding of Mg^{2+} ion by the partial anionic structure of *P. mirabilis* CPS enhances struvite crystallization by enabling the weakly concentrated Mg^{2+} ions to be readily released from lipopolysaccharide (LPS) for crystal formation (Dumanski et al. 1994; Rozalski et al. 1997).

Once a mature biofilm develops on the surface of uroepithelial cells or catheters, organisms within the mushroom-shaped structure communicate with each other utilizing diffusible chemical signals that regulate a variety of cellular functions, including glutamine (Allison et al. 1993), autoinducer-2 (Schneider et al. 2002), cyclic dipeptides, and putrescine (Sturgill and Rather 2004). However, the role of these signaling molecules in the process of biofilm formation or swarming during *P. mirabilis* UTIs or CAUTIs is unclear.

In short, crystalline biofilms are known to form during CAUTIs associated with *P. mirabilis* and are responsible for some of the more severe sequelae experienced. Only a few of the proteins involved with this process in *P. mirabilis*, including bacterial urease, have been identified and studied. Therefore, more extensive studies need to be completed, including the identification of these potential proteins.

3.1.4 Avoidance of Host Immunity

Besides the formation of crystalline biofilms, *P. mirabilis* uses several immunoavoidance factors to persist in the urinary tract, including antigenic variation, capsule production, IgA proteases, and LPS. The flagellin protein of *P. mirabilis* may

undergo antigenic variation as a means of avoiding an antibody response. Capsular polysaccharides, also referred to as slime material or glycocalyx, are a highly hydrated polymer present on the bacterial cell surface. These structures have several known functions during UTIs and CAUTIs caused by *P. mirabilis*, such as a role in crystalline stone formation (McLean et al. 1988) which was discussed in the previous section. Other known functions of CPS include protection against the host immune response and antibiotics, attachment to surfaces (Hoyle et al. 1992), and potentially for swarming. Little is known about these structures in *Proteus* species. Studies of certain O antigens of *P. mirabilis* (O6, O57) and *P. vulgaris* (O19) demonstrated that capsular antigen structures are produced that are identical to the O-specific chains of their LPS (Beynon et al. 1992; Perry and MacLean 1994; Uhrin et al. 1994). One capsular structure of *P. mirabilis* (ATCC 49565) has been identified as an acidic CPS consisting of a high molecular weight polymer of branched trisaccharide units composed of 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine), 2-acetamido-2,6-dideoxy-L-galactose (*N*-acetyl-L-fucosamine), and d-glucuronic acid (Beynon et al. 1992).

The colony migration factor Cmf, an extracellular slime material, from wild-type *P. mirabilis* WT19 is an acidic CPS composed of a tetrasaccharide repeating unit. A mutant in *cmfA* was attenuated in a model of experimental uropathogenicity as compared to the wild type, thereby demonstrating a reduced ability to colonize the urinary tract (Allison et al. 1994). Therefore, it is proposed that this factor contributes to the uropathogenicity of *P. mirabilis* during CAUTIs by facilitating translocation of differentiated cell populations on catheter surfaces.

To combat invading microbes, during the course of UTIs, secretory IgA, the dominant immunoglobulin form in mucus secretion, is produced by the host immune system against antigenic components of uropathogens. Proteases targeting immunoglobulins and other host defense components such as complement (C1q and C3) and antimicrobial peptides (human beta-defensin 1, human cathelicidin LL-37) protect uropathogens from the host response (Belas et al. 2004).

All the clinical strains of *P. mirabilis* and *P. penneri* and many of the strains of *P. vulgaris* examined produce an EDTA-sensitive protease (Loomes et al. 1992; Senior et al. 1987, 1988). This protease cleaves the heavy chain of serum immunoglobulin IgA into two fragments (Senior et al. 1987). The substrate specificity of the protease isolated from a chronic UTI isolate *P. mirabilis* 64676 was expanded to include the cleavage of subclasses of IgA (secretory IgA1, IgA2) and IgG, both present in urine (Rozalski et al. 1997), and the nonimmunoglobulin substrates such as gelatin, secretory component, and casein (Loomes et al. 1990). Cleavage of IgG occurs at the hinge region as a two-stage process that involves a pepsin-like activity and then a papain-like one to yield Fab and Fc fragments (Loomes et al. 1990). It is produced during swarmer cell differentiation and is stimulated by divalent cations ($Ca^{2+} > Mg^{2+}$) (Walker et al. 1999).

To summarize, *P. mirabilis* can persist in the urinary tract during UTIs or CAUTIs by evading the host immune response and are known to express several immunoavoidance factors. Currently, there is no direct evidence confirming that many of these factors are produced during CAUTIs and are important during these infections. Biofilms

have been shown to protect organisms against the host immune response (Leid et al. 2005) and are assumed to protect *P. mirabilis* against this response during CAUTIs. As *P. mirabilis* is known to produce a capsule and capsules are one mechanism of evading the host immune response, it has been suggested that these structures protect these uropathogens against the immune system during UTIs and CAUTIs. The colony migration factor Cmf, an acidic CPS, is also proposed to assist in swarming across catheter surfaces. As the IgA protease ZapA has been demonstrated to be expressed during swarming and swarming has been shown to occur during the migration of *P. mirabilis* across catheter sections, it can be suggested that this protease is expressed during CAUTIs caused by these organisms.

4 CAUTIs due to Other Uropathogens

The organisms most commonly associated with the development of CAUTIs in patients are *E. coli* and *Proteus* species, in particular *P. mirabilis*. However, a number of other bacterial species, although less commonly associated with CAUTIs, also cause a significant proportion of these infections and include *Enterococcus* species (*E. faecalis*), *Klebsiella* species (*K. pneumoniae*), *Enterobacter* species (*E. cloacae*), *Citrobacter* species (*C. freundii*), *Morganella morganii*, *Providencia* species (*P. stuartii*), *Serratia* species (*S. marcescens*), *Pseudomonas* species (*P. aeruginosa*), and *Staphylococcus* species (*S. aureus*, *S. epidermidis*). These bacterial species will be discussed in terms of monomicrobial infections, even though *E. coli* and *P. mirabilis* usually cause polymicrobic biofilm infections. The understanding of these complex bacterial populations in CAUTIs remains rudimentary.

4.1 Enterococcus spp.

Enterococcus species are the third leading cause of bacteremia in the USA (Emori and Gaynes 1993). These pathogens are commonly isolated from chronic or recurrent UTIs, particularly those infections associated with catheterization (Guzman et al. 1989; Lewis and Zervos 1990). The *Enterococcus* species that cause the majority of human infections (80–90%) are *E. faecalis*, followed by *E. faecium* (Moellering 1992; Murray 1990; Ruoff et al. 1990). Adherence factors have been shown to be important in the pathogenesis of entercoccus CAUTIs; an analysis of 30 enterococcal urinary isolates revealed that *E. faecalis* and *E. faecium* strains displayed a higher capacity to adhere to urinary catheters *in vitro* than did other enterococcal species, as examined by sonication quantitative culture technique and scanning electron microscopy (Dworniczek et al. 2003). There are conflicting views on the relevance of certain enterococcal virulence factors (Esp, AS, cytolysin, GelE) and their role in adherence to epithelial cells or catheters (Archimbaud et al. 2002; Joyanes et al. 2000).

Clinical isolates of enterococci have been shown to form biofilms in vitro (Baldassarri et al. 2001; Sandoe et al. 2003; Toledo-Arana et al. 2001) and in vivo on indwelling medical devices such as urinary catheters (Donlan 2001a,b; Donlan and Costerton 2002; Tunney and Gorman 2002). Urinary isolates of enterococci have been observed to produce the cell surface protein Esp. This protein is homologous to biofilm-associated protein (Bap) of *S. aureus* (Cucarella et al. 2001); however, there are conflicting reports on the relevance of this protein during biofilm formation by enterococci (Di Rosa et al. 2006; Shankar et al. 1999, 2001; Toledo-Arana et al. 2001). Some studies suggest that Esp assists in biofilm formation and colonization of the urinary tract of CBA mice (Shankar et al. 1999, 2001) and abiotic surfaces (Toledo-Arana et al. 2001). Analysis of 152 clinical isolates of enterococci correlated biofilm formation to the presence of Esp, as examined by microtiter plate biofilm assays; however, upon further examination, mutants in *esp* were still capable of biofilm formation (Toledo-Arana et al. 2001).

Besides Esp, other enterococcal components suggested to be involved in biofilm formation are SalB, autolysin, the *fsr* quorum-sensing (QS) system, gelatinase, serine protease SprE, cytolysin, enterococcal polysaccharide antigen Epa, and LTA. In general, the role of these factors in CAUTI pathogenesis remains unclear.

4.2 Klebsiella spp.

Klebsiella are Gram-negative nonmotile bacilli found ubiquitously in the environment and the mucosal surfaces of mammals. The most common site of *Klebsiella*-associated nosocomial infection is the urinary tract, causing 6–17% of all nosocomial UTIs, and ranking it as one of the top five causative agents for this type of infection (Bergogne-Berezin 1995; Ullmann 1986). The species most commonly associated with *Klebsiella*-associated nosocomial UTIs are *K. pneumoniae* and *K. oxytoca* (Tarkkanen et al. 1992). *Klebsiella* produce a variety of virulence factors, including adhesins, capsule, LPS, siderophores, serum resistance, and enzymes (Podschun and Ullmann 1998).

Adherence in the urinary tract by *Klebsiella* species is mediated by the production of a number of adhesins classified as either fimbrial or nonfimbrial. Despite the presence of type 1 fimbriae in urinary isolates of *K. pneumoniae*, it is unknown what role, if any, these play during CAUTIs. A major factor in adherence to abiotic surfaces is thought to be the type 3 pilus; transformation of an afimbriated *K. pneumoniae* strain with the gene cluster for type 3 pili increased the strain's ability to adhere to abiotic surfaces and increased biofilm formation (Di Martino et al. 2003). Currently, it is not known what role the third *Klebsiella* fimbria – KPF-28 – plays in *Klebsiella*-associated CAUTIs. Nonfimbrial adhesins of *Klebsiella* spp. include the CF29K protein (Darfeuille-Michaud et al. 1992) and the capsule-like extracellular material (Favre-Bonte et al. 1995).

The capsular material contributes to *Klebsiella* spp. virulence during UTIs in other ways. It likely protects the organism from the bactericidal components of

serum (Williams et al. 1983) and from phagocytosis by polymorphonuclear granulocytes (Podschun and Ullmann 1992; Simoons-Smit et al. 1986); it also plays a role in late biofilm development (Schembri et al. 2005).

4.3 *Enterobacter spp.*

Enterobacter, Gram-negative motile coccobacilli (Acolet et al. 1994; Gallagher 1990; Gladstone et al. 1990; Karpuch et al. 1983) commonly isolated from water, plants, and animals (Falkiner 1992; Gaston 1988; John et al. 1982; Lindh et al. 1991), reside in 40–80% of human intestinal tracts (Gaston 1988). It is the fifth most isolated pathogen from the urinary tracts of ICU patients (6.1%) (Jarvis and Martone 1992) and has been identified as the causative agent of 6–7% of the total cases of nosocomial UTIs in the USA (Jarvis and Martone 1992; Schaberg et al. 1991). The species most commonly isolated from patients with UTIs are *Enterobacter cloacae*, *Enterobacter aerogenes*, and *Enterobacter agglomerans* (Acolet et al. 1994; Gallagher 1990; Gladstone et al. 1990; Karpuch et al. 1983).

A general screen of virulence factors associated with *E. cloacae* strains revealed that these organisms were resistant to the bactericidal effects of serum and were capable of producing the siderophore aerobactin and a mannose-sensitive hemagglutinin (Keller et al. 1998). Furthermore, all of the strains examined could adhere to and invade human laryngeal epithelial cells (HEp-2 cells) (Keller et al. 1998). In a study by Podschun et al. (2001) of 72 clinical *E. cloacae* strains, 64 isolates (89%) expressed type I fimbriae; furthermore, 36 isolates (50%) produced type III fimbriae. *Enterobacter* species have been shown to form biofilms on abiotic surfaces (glass, poly(vinyl chloride)), and studies indicate the presence of two different types of acylated homoserine lactones (3-oxo-C6-HSL, 3-oxo-C8-HSL) produced by these organisms, which may play a role in cell-to-cell communication (Lehner et al. 2005). As with other Gram-negative organisms and similar to that observed with *Klebsiella* species, *E. aerogenes* produces CPS, structures that are known to assist in the avoidance of phagocytosis.

4.4 *Morganella and Providencia spp.*

Formerly classified as members of the *Proteus* group, these organisms are distinguished as separate genera (Buttiaux et al. 1954; Rauss 1936) and are commonly found in the human intestinal tract as part of the normal microflora (Ewing 1986; O'Hara et al. 2000). The infrequent identification of these organisms as the causative agents in UTI despite their abundance in the urine of long-term-catheterized patients may be due to differences in commercial laboratory procedures (Damron et al. 1986).

Morganella and *Providencia* species produce a number of adhesion molecules that allow potential binding to uroepithelial cells (Mobley et al. 1986) and catheters including type 3 or MR/K (mannose-resistant/Klebsiella-like) hemagglutination (Gerlach et al. 1989; Mobley et al. 1988; Old and Adegbola 1982). Old and Scott (1981) demonstrated that *Providencia* strains produce at least five different patterns of hemagglutination (MR/K, mannose-sensitive hemagglutinin, mannose-resistant/Proteus-like (MR/P) hemagglutinin) and at least six distinct types of fimbriae, as examined by electron microscopy. Expression of MR/K hemagglutination by the *P. stuartii* strains isolated from patients experiencing bacteriuria was observed more frequently during long-term catheterization (74%) than during short-term catheterization (26%, $P = 0.004$) (Mobley et al. 1988).

Once attached, these organisms can migrate on catheters and subsequently ascend into the urinary tract through the utilization of flagella and subsequently produce urinary crystals (Keefe 1976; Oka et al. 1985; Silverman and Stamey 1983). Their ability to form crystals can be potentially linked to their capacity to hydrolyze urea through the production of urease (Farmer 1999; Jones and Mobley 1987; Lim et al. 1998; Mobley et al. 1986; Mulrooney et al. 1988); however, their role in catheter encrustation is still in question (Kunin 1989; Stamm 1991; Stickler et al. 1998a,b).

4.5 Serratia *spp.*

Serratia species are commonly found inhabiting the environment (soil, water). These opportunist pathogens cause complicated UTIs associated with or without indwelling catheters (Maki et al. 1973). *S. marcescens* is the main species of *Serratia* isolated from UTIs; however, *S. liquefaciens* has also been identified as a causative agent for this type of infection (Serruys-Schoutens et al. 1984). Little is known about the pathogenesis of *Serratia* species within the host (Hejazi and Falkiner 1997).

Several potential virulence factors have been associated with infections caused by these organisms, including the production of fimbriae, proteins associated with biofilms, O-antigens, LPS, digestive enzymes, hemolysin, siderophores, and bacteriocins, and the ability to swarm.

An analysis of 469 *S. marcescens* strains identified the presence of hemagglutination (63%) and production of type 1 fimbriae (53%) in the serotype O14:K14 strains, commonly associated with clinical infections, compared with 7 and 12%, respectively, of three environmentally associated serotypes (Aucken and Pitt 1998). To initiate infections associated with indwelling urinary catheters, uropathogens such as *Serratia* must establish close contact with various surfaces (catheters, uroepithelia). A number of fimbrial types, including type 1 fimbria, type 3 fimbria, MR/P thick fimbria, MR/P thin fimbria, and FGH MR/P fimbria, have been shown to be expressed by *Serratia* species (Clegg and Gerlach 1987; Grimont and Grimont 1992; Old et al. 1983). Thus far, urinary isolates of *S. marcescens* produce type 1,

type 3, MR/P, and MR/T fimbria, and presumably these fimbria play a role in the adherence of *Serratia* species to catheters and human uroepithelial cells (Clegg and Gerlach 1987; Daifuku and Stamm 1986; Grimont and Grimont 1992; Leranoz et al. 1997; Old et al. 1983; Yamamoto et al. 1985).

The development of *S. marcescens* biofilms is dependent upon QS systems and environmental cues (Rice et al. 2005) and involves a series of defined stages that culminate in a highly porous, filamentous biofilm consisting of cell chains, filaments, and cell clusters (Labbate et al. 2004). This filamentous phenotype is predominant during nutrient-excess conditions, while typical microcolony biofilm phenotype is observed under nutrient-limiting conditions (Labbate et al. 2004). Sloughing of the filamentous biofilm is regulated via QS systems implicating the involvement of these systems in multiple stages of the biofilm life cycle (Labbate et al. 2004). Some *Serratia* strains possess QS systems that are dependent upon *N*-acylhomoserine lactones (AHLs), which subsequently regulate population surface migration, biofilm formation, and carbapenem and prodigiosin production (Wei and Lai 2006). These organisms possess LuxR and LuxI protein homologues, and evidence suggests that the genetic determinants that encode for these systems are transferred laterally, including one example of transfer via transposons (Wei and Lai 2006). *Serratia* species also produce LuxS and autoinducer-2, which apparently function as a second QS system controlling many of the same phenotypes as the LuxR/AHL systems (Wei and Lai 2006). Labbate et al. (2004) described mutants in a signal synthase that developed flat biofilms with the absence of cell chains and clusters and that attach weakly to abiotic surfaces (Labbate, unpublished data). Owing to the presence of these genetic determinants, it seems likely that biofilms are formed during CAUTIs caused by *Serratia* species.

Serratia spp. have the ability to swarm in a way similar to that observed by *Proteus* species (Givskov et al. 1995). *S. marcescens* strains display a characteristic swarming phenotype in which motile short swimmer cells at the colony margin differentiate into elongated, aseptate, and hyperflagellate swarmer cells that coordinately migrate away from the initial site of inoculation upon inoculated onto Luria broth agar plates at 30°C (Eberl et al. 1999; Harshey 2003). QS systems of *Serratia* species regulate swarming motility through the expression of the surfactant serrawettin (Eberl et al. 1999; Lindum et al. 1998). Since this swarming ability has been linked to the upregulation of virulence factors during UTIs caused by *Proteus* species (Allison et al. 1992; Belas et al. 2004), perhaps this same phenotype is observed during some part of the disease process of UTIs associated with *Serratia* species.

4.6 Citrobacter spp.

Citrobacter species are Gram-negative motile enterobacteria typically isolated from a variety of environments including soil and the intestines of fish and cattle (Acres et al. 1975; Depaola et al. 1995). Because untreated infections of the urinary tract

can lead to episodes of bacteremia, CAUTIs associated with *Citrobacter* species are a major health risk because of the significant mortality rate (33–48%) (Drelichman and Band 1985) associated with bacteremia caused by these organisms. The most common species of *Citrobacter* isolated from clinical samples are *Citrobacter freundii* and *Citrobacter koseri*.

Research into the pathogenesis of these organisms during infections such as CAUTIs is minimal. However, these organisms produce virulence factors known to be associated with CAUTIs, including fimbria, flagella, capsules, biofilms, siderophores, bacteriocins, and LPS. Strains of *Citrobacter* produce various adhesins that could potentially assist in the initial colonization of catheter and uroepithelial surfaces as well as in the invasion of epithelial cells. A urinary isolate, *C. freundii* 3009, (Oelschlaeger et al. 1993) expresses a homolog of the type 1 pilus Fim from *Salmonella enterica* sv. *typhimurium*. Besides its potential role in attachment, this Fim homolog is believed to play a role in the invasion of epithelial cells (Hess et al. 2004). Additionally, *Citrobacter* spp. are strongly attracted to fresh human urine, as examined by capillary chemotaxis assay (Herrmann and Burman 1985).

Citrobacter species produce a fibrillar microcapsule that is closely related to the Vi capsule of *S. enterica* sv. *typhi* (Bondarenko et al. 1981; Hess et al. 2004). Since strains of *S. typhi* that produce the Vi capsule resist phagocytosis and the bactericidal action of serum complement (Robbins and Robbins 1984), it is possible that it serves a similar role for *Citrobacter* species that express this capsule during UTIs. Previous work by Bondarenko et al. (1981) has demonstrated that *C. freundii* strains that possess capsules were capable of resisting lysosomal enzymes of guinea-pig macrophages.

The formation of biofilms, critical for the establishment of CAUTIs, has been shown for *Citrobacter* species. These organisms are capable of forming biofilms under lactose limitation with reduced formation exhibited under limiting phosphorus, nitrogen, or glucose conditions, as examined by microscopy (TEM, SEM, confocal laser scanning microscopy) (Allan et al. 2002). The differences in biofilm formation in these conditions are thought to be related to differential expression of fimbriae on the cell surface (Allan et al. 2002). Biofilm formation in *Citrobacter* species has also been associated with the rdar morphotype or the expression of an extracellular matrix (ECM) composed of curli fimbriae and cellulose (Zogaj et al. 2003). This matrix mediates interactions between organisms, adherence of organisms to hydrophilic and hydrophobic abiotic surfaces, and multicellular behavior such as biofilm formation at liquid–solid interfaces (Romling et al. 1998; Zogaj et al. 2003). Expression of curli fimbriae and cellulose has been linked to virulence and transmission such as adherence and invasion of intestinal epithelial cells and chlorine resistance, respectively (Bian et al. 2001; Dibb-Fuller et al. 1999; Gophna et al. 2001; Solano et al. 2002; Sukupolvi et al. 1997). Since *Citrobacter* species are frequently found to cause biofilm-related infections such as CAUTIs, the human gut could serve as a reservoir for dissemination of biofilm-forming isolates (Zogaj et al. 2003).

4.7 Staphylococcus spp.

Staphylococci are nonmotile Gram-positive cocci. These organisms often reside on the mucosal membranes and the epithelial surfaces of mammals as part of the normal human microflora (skin, nose, vagina, anterior urethra) with a carriage rate between 11 and 32% in healthy adults (Millian et al. 1960; Tuazon and Sheagren 1974). Staphylococci, in particular *S. aureus*, have a special predilection to cause infections involving the presence of foreign bodies, including indwelling urinary catheters, perhaps related to their affinity for ECM proteins that can mediate attachment to foreign material (Musher et al. 1994). A study by Uesugi et al. (1996) of staphylococci urinary isolates revealed that the frequency of staphylococci species encountered was about 10% of the total cases from 1984 to 1994.

Most *S. aureus* strains isolated from urinary samples are methicillin-resistant (MRSA) and since ~40–60% of all nosocomially acquired *S. aureus* are resistant to methicillin, these strains are now considered endemic in hospitals (Novick 2003).

Despite its ability to cause infections of the urinary tract and subsequent potentially severe complications, such as cervical spine abscess (Masood et al. 2003), there is limited research examining the mechanisms by which staphylococci cause infections associated with urinary catheterization. Among MRSA strains with enhanced biofilm formation, the presence of three virulence determinants hemolysins *hla* and *hlb*, and *fnbA* predominated, suggesting that colonization and infection of the urinary tract by MRSA strains may be promoted by these gene products. Staphylococci are known to produce a variety of virulence factors all of which will not be discussed here. Instead, those factors that could potentially assist this organism in colonization and biofilm formation will be examined.

Staphylococci, as with other uropathogens, must express a number of factors coordinately to first adhere to the catheter surface and subsequently replicate, accumulate, and develop into a complex biofilm structure. These biofilm communities confer protection to organisms against antibiotics and the host immune response (Leid et al. 2002) due to metabolic changes in the biofilm (Eng et al. 1991). Initial interactions during CAUTIs by staphylococci are accomplished through direct attachment to the catheter surface, through attachment of host ECM proteins bound to the catheter surface, or through cell-to-cell interactions, all dependent upon the utilization of cell-associated and secreted adhesin molecules.

Staphylococci attach to the host ECM proteins that coat the surface of the urinary catheter and colonize the catheter surface through the production of adhesin factors that recognize these proteins as potential receptors (Liang et al. 1995; Park et al. 1991; Schwarz-Linek et al. 2004; Signas et al. 1989; Switalski et al. 1989). These staphylococcal surface adhesins have been collectively designated MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (Foster and Hook 1998; Patti et al. 1994) and some of these molecules recognize and bind to multiple ECM proteins. Many of these proteins are covalently

bound to the staphylococcal cell wall by a C-terminal LPXTG sorting signal (Foster and Hook 1998).

After initial attachment, staphylococci must be capable of permanent attachment by intracellular adhesion since most of the organisms in the biofilm are not in contact with the catheter surface or the uroepithelia. One particular protein involved with this process of cell clustering and permanent attachment in *S. epidermidis* strains has been identified, a β-1,6-linked glucosaminylglycan (Mack et al. 1996a,b) known as the polysaccharide intracellular adhesin (PIA). The genes encoding for the proteins involved in PIA synthesis are located on the intracellular adhesion or *ica* gene locus (*icaADBC*). A homologous *ica* locus has been described in *S. aureus* that is 59–78% amino acid identity to the *S. epidermidis* locus (Cramton et al. 1999). Approximately half (50.8%) of 179 *S. epidermidis* isolates examined by Mack et al. (1996a,b) were capable of forming biofilms, and of these biofilm-producing strains, 86.8% produced PIA, as determined by a specific coagglutination assay. In contrast, 88.6% of the biofilm-negative isolates did not express PIA ($P < 0.001$), suggesting that PIA production is significant for biofilm accumulation in the majority of clinical *S. epidermidis* isolates. PIA also protects the organisms against major components of human innate host defense, including killing by human polymorphonuclear leucocytes and antimicrobial peptides (Vuong et al. 2004).

The biofilm-associated protein (Bap), a high molecular weight bacterial surface protein initially isolated from strains of *S. aureus*, that caused chronic mastitis infections, has several homologs among staphylococcal species. Evidence indicates that this protein has a role in biofilm formation in primary and intracellular attachment independent of the PIA/PNAG exopolysaccharide, as a mutant in the *bap* gene in *S. epidermidis* abolished its capacity to form a biofilm and that the CoNS strains possessing *bap* and not the *icaADBC* operon were capable of biofilm formation (Tormo et al. 2005). Antibodies specific for Bap are produced in animals with confirmed *S. aureus* infections (Cucarella et al. 2004). However, analysis of clinical strains demonstrated that many lacked the *bap* gene, yet were still quite capable of forming biofilms (REF). Therefore, the role of Bap in staphylococcal biofilm formation is still in question.

Staphylococcal CPS have been implicated in attachment to catheter surfaces. One such factor known produced by *S. epidermidis* strains is the capsular polysaccharide/adhesin PS/A that mediates bacterial adherence to polymer surfaces *in vitro* (Tojo et al. 1988). Muller et al. (1993) compared 151 clinical isolates of CoNS for the expression of PS/A and extracellular biofilm or slime and revealed that of the 151 strains analyzed, 103 (68%) produced PS/A and 69 (46%) were found to express extracellular slime, and 87% of the slime-producing isolates were PS/A-producers.

4.8 Pseudomonas spp.

Pseudomonads are nonfermenting aerobic motile Gram-negative bacilli that are ubiquitous in the environment (Costerton 1980) and nosocomial setting (Wilson and Dowling 1998) and have been identified as the causative agent in infections of

the urinary tract, especially in patients with indwelling urinary catheters. From 1986 to 1990, this organism was the third leading cause of UTIs (11%) (Jarvis and Martone 1992).

Adherence to host protein-coated catheter surfaces is essential for biofilm formation and initiation of CAUTI. Urinary tract isolates of *P. aeruginosa* were found to attach more efficiently to Vero cells than did respiratory tract isolates, indicating differentiated attachment among *P. aeruginosa* strains based on the infection site of isolation (Izdebska-Szymona and Laziuk 1988). Several *P. aeruginosa* adhesins have been described, including pili (Comolli et al. 1999; Gupta et al. 1994; Lee et al. 1994; Schweizer et al. 1998) and nonpilin adhesins such as flagella (Feldman et al. 1998; Ramphal et al. 1996), LPS (Gupta et al. 1994; Zaidi et al. 1996), alginate, and exoenzyme S (Olson et al. 1997, 1999; Rucks et al. 2003). These adhesins recognize and attach to eukaryotic cell surface receptors to initiate adherence either on the catheter or on uroepithelial surface during CAUTIs. Attachment and colonization are further facilitated by the three mechanisms of motility possessed by *P. aeruginosa*: flagellum-mediated swimming, type IV pilus-dependent twitching, and swarming (Deziel et al. 2001; Kohler et al. 2000). Flagella also play a critical role in the development of biofilms, where they assist in initial surface adhesion as well as biofilm dispersal (O'Toole and Kolter 1998; Sauer et al. 2002).

Upon the entry of *P. aeruginosa* into the urinary tract, these organisms begin the process of biofilm formation through the coordinated expression of a number of factors to first attach to the catheter surface and subsequently accumulate and develop into a fully mature biofilm. After planktonic bacteria form initial reversible associations to a biomaterial surface, these organisms form more permanent attachments, which are essential for the formation of the monolayer. Upon the completion of monolayer formation, these surface-associated organisms develop into microcolonies through clonal growth or cell aggregation, or both. Biofilm maturation is accompanied by the encasement of microcolonies within an exopolysaccharide matrix consisting of complex aqueous channel systems that permit the transport of nutrients, oxygen, metabolic end products, and various signaling molecules. The characteristic biofilm structure of *P. aeruginosa* contains exopolysaccharides (alginate) (Leid et al. 2005) and rhamnolipids (Pamp SJ and Tolker-Neilsen T 2007) that serve to protect the biofilm. Alginate consists of a simple, high molecular weight unbranched polysaccharide composed of two uronic acids: β-D-mannuronic acid and its C_5 epimer α-L-guluronic acid. The presence of alginate serves as a virulence factor and confers several selective advantages to these organisms (Govan and Deretic 1996), including the ability to resist opsonic phagocytosis (Hatano et al. 1995), neutralize oxygen radicals produced by inflammatory immune cells (Matthee et al. 1999), stimulate inflammatory cytokine production indicative of a Th2-type immune response (Pedersen 1992; Pedersen et al. 1990), and contribute to the matrix material of the mucoid *Pseudomonas aeruginosa* biofilm (Nivens et al. 2001).

QS systems have developed in *Pseudomonas* that enable these organisms to communicate with each other and regulate numerous biofilm development,

colonization, and virulence factors in a coordinated, cell-density-dependent manner (Heydorn et al. 2002). Mutants in *P. aeruginosa* QS are severely attenuated in virulence in animal infection models (Lesprit et al. 2003; Rumbaugh et al. 1999; Zhu et al. 2004). However, a study by Schaber et al. (2004) identified five QS-deficient mutants out of 200 total isolates from patients with urinary tract, lower respiratory tract, and wound infections, suggesting that although these systems are important for virulence, other factors contribute to the establishment of infection in humans.

5 Prevention of CAUTIs

Over 5 million patients per year receive urinary catheterizations (Maki and Tambyah 2001) and all are at risk for developing CAUTIs. Current effective infection control measures against CAUTIs are ones that only delay the onset of bacteriuria since no methods have been developed that prevent these types of infections (Trautner and Darouiche 2004). This section will describe the different practices that have been proposed for reducing or eliminating the formation of biofilm on urinary catheters. These include the development of new biomaterials that reduce biofilm formation on the surface of catheters, the addition or impregnation of antimicrobial agents on catheter materials, and the use of probiotics.

5.1 Novel Surfaces

Once a uropathogen gains access to the urinary tract, adhesion to the catheter material and the uroepithelium are imperative to the initial establishment of biofilms in CAUTIs. Bacterial attachment to catheter surfaces is dependent upon the hydrophobicity of the organisms and the biocompatible surface of the catheter which immediately becomes conditioned by serum and other fluids after implantation. Following urine application in the catheter lumen, a conditioning layer is formed from the deposition of proteins, minerals, polysaccharides, and other host-derived factors in the urine (Donlan 2001a,b). The resulting attachment of host factors provides binding sites for uropathogens. Therefore, the development of surfaces resistant to organism colonization is important in the prevention of CAUTI and is going on. Although several control measures have been examined for the prevention of biofilm formation on catheters, no single biosurface, including silicone, polyurethane, composite biomaterials, or hydrogel-coated materials, or method has been shown to be effective in preventing colonization due to the development of the conditioning layer (Donlan 2001; Maki and Tambyah 2001; Tunney et al. 1999).

5.2 Catheters Containing Antimicrobial Agents

Preventive measures that have been examined but have not yielded any proven benefit in the prevention of bacteriuria in catheterized patients include the application of antimicrobial solutions and lubricants on the catheter surface prior to catheter insertion and the addition of antimicrobial agents in the collection bag (Beizer 1996). Conversely, in separate prospective, randomized trials, antimicrobial-impregnated catheters containing either nitrofurazone (Maki et al. 1997) or the combination of broad-spectrum antibiotics minocycline and rifampin (Darouiche et al. 1999) demonstrated significant reductions in bacterial CAUTIs. However, these trials were limited and the emergence of resistant strains was not resolved (Maki and Tambyah 2001).

Besides antibiotics, impregnation of catheter material with antiseptics, such as silver compounds, has been widely studied as a possible preventive measure with conflicting results. A full review of all such trials is outside the scope of this work. In one prospective clinical trial involving 482 hospitalized patients, silver-oxide-coated catheters reduced the incidence of UTI only among women not receiving antimicrobial agents (19% for control catheter vs. 0 for silver catheter, $P = 0.04$), compared with a control silicone catheter (Johnson et al. 1990). A randomized study of 1,309 patients catheterized longer than 24 h failed to demonstrate effectiveness of a silicone catheter coated externally with 5% silver oxide, compared to a standard silicone elastomer-coated latex catheter. However, these silver oxide catheters did show a significantly increased incidence of bacteriuria in male patients and a significantly increased occurrence of staphylococcal bacteriuria (Riley et al. 1995). Thus the efficacy of the various silver-based strategies remains controversial.

Besides silver, other antiseptics have been examined for their efficacy against bacterial colonization. Catheters impregnated with synergistic combinations of chlorhexidine, silver sulfadiazine, and triclosan prevented adherence of a broad spectrum of extraluminal bacteria on their outer surfaces, when compared with silver hydrogel latex and nitrofurazone-treated silicone catheters in an *in vitro* urinary tract model (Gaonkar et al. 2003).

Catheter encrustation is a problem of long-term bladder management and to prevent its development, it is necessary that any antibacterial agent coated onto or incorporated into catheters should diffuse into the urine and prevent the rise in urinary pH and the crystallization of the calcium and magnesium phosphates. Maintaining the release of effective concentrations of antibacterials for the life time of long-term catheters is a challenge. Bibby et al. (1995) suggested that the catheter balloon could be used as a reservoir for substantial quantities of antibacterial chemicals and that the membrane of the balloon might ensure its controlled release into the residual urine over extended periods. They found that mandelic acid diffused through the catheter balloon, achieving concentrations of around 0.1 mg mL^{-1} in urine. Unfortunately, mandelic acid is not very active against *P. mirabilis* or other urinary pathogens, being bactericidal in urine at concentrations of around 5 mg mL^{-1}

(Rosenheim 1935). The biocide triclosan however is extremely active against *P. mirabilis*. The MIC of this agent against *P. mirabilis* isolates from encrusted catheters was found to be 0.2 μg mL^{-1} (Jones et al. 2005). In experiments in laboratory models supplied with artificial urine and infected with *P. mirabilis*, triclosan was shown to diffuse through the balloons of all-silicone catheters into the residual urine. The rise in urinary pH and crystalline biofilm formation on the catheters was inhibited. Catheters with their retention balloons inflated with water blocked within 24 h, while catheters inflated with triclosan (10 mg mL^{-1} in 5% w/v poly(ethylene glycol)) drained freely and showed minimal encrustation at the end of a 7-day experimental period (Stickler et al. 2003). As the triclosan strategy inhibits encrustation under these severe experimental conditions, it may well extend the lifespan of catheters in patients infected with *P. mirabilis.*

5.3 Probiotics

Bacterial interference or the instillation of nonpathogenic strains into the bladder has the potential for prevention of symptomatic infection through the hindrance of uropathogen colonization (Reid et al. 2001). *In vitro* (Trautner et al. 2002, 2003) and *in vivo* (Darouiche et al. 2001) studies have shown that nonpathogenic strains of *E. coli* 83972 reduce the colonization of catheters by a variety of uropathogens. In one study with 21 patients inoculated with *E. coli* 83972, the patients experienced no symptoms of bacteriuria while this same group of patients experienced a mean of 3.1 symptomatic UTIs per year prior to colonization (Hull et al. 2000). A study conducted by Trautner et al. (2005) showed that a urinary catheter surface colonized by a colicin-producing avirulent strain of *E. coli* subsequently prevented colonization of a uropathogenic clinical isolate of *E. coli in vitro*.

5.4 Future Developments

Some approaches that offer promise in the reduction of bacterial adherence and biofilm formation to the catheter and the urinary tract include utilization of biofilm inhibitors on catheter surfaces, development of hydrophilic and nutrient-scavenging biomaterials and low-energy surface acoustic waves.

Identifying the bacterial factors involved with biofilm formation on catheters and developing and/or determining inhibitors for these factors will be critical in preventing bacteriuria. One such factor is the protein GlmU (*N*-acetyl-D-glucosamine-1-phosphate acetyltransferase), involved in the biosynthesis of the activated nucleotide sugar UDP-GlcNAc. It also has a role in the synthesis of the β-1,6-*N*-acetyl-D-glucosamine polysaccharide adhesin required for biofilm formation in *E. coli* and *S. epidermidis* (Itoh et al. 2005). These compounds have antibiofilm activity against clinical isolates of *E. coli*, *P. aeruginosa*, *K. pneumoniae*,

S. epidermidis, and *E. faecalis*, and oPDM-plus-PS-coated silicone catheters had a reduced bacterial colonization rate of *P. aeruginosa* and *S. epidermidis* than did catheters coated with silver hydrogel *in vitro* (Burton et al. 2006). However, because of the number of different pathogens that can cause CAUTIs, it may be unlikely to find a universal compound or combination that prevents colonization and biofilm formation by all microorganisms.

Determining factors that inhibit QS may be important in the prevention of biofilm formation and subsequent bacteriuria. Furanones, compounds isolated from a marine red macro alga *Delisea pulchra* that interfere with QS (Kjelleberg et al. 1997), are of limited use because of toxicity, and results from studies examining the efficacy of these compounds for clinical use were found to be variable (Trautner and Darouiche 2004). Therefore, other less toxic alternatives need to be discovered.

The development of new biomaterials for the manufacture of catheters that are not suitable for bacterial colonization and are nontoxic toward patients are a logical progression in the prevention of bacteriuria during catheterization. The use of LoFric catheters (catheters with a hydrophilic coating such as poly(vinylpyrrolidone) and salt) was associated with less hematuria and a significant decrease in the incidence of UTIs (Vapnek et al. 2003).

A promising method of bacteriuria prevention is the development of biomaterials that scavenge essential nutrients such as iron. Preliminary studies of catecholamine inotropes encourage biofilm formation by *S. epidermidis* by transferring iron to the bacteria from the host iron-binding protein transferrin (Lyte et al. 2003). The addition of exogenous lactoferrin to *P. aeruginosa* prevents biofilm formation through the stimulation of twitching motility (Singh et al. 2002). More work is needed to determine the efficacy of such materials.

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Biofilms in Hemodialysis

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Abstract During end stage renal disease the kidneys are not functioning properly and as such longer adequately remove waste from the blood stream. Hemodialysis is an invasive medical treatment were by the human body is routinely treated with large quantities of water as a mean to flush these waste from the body. As in all water systems this system is susceptible to biofilms. Advanced water purification system are used to purify the water entering these systems, however, bacteria have adapted to low nutrient niches such as this and a even a single bacteria may be sufficient to start biofilm development. These biofilms have been shown to develop throughout hemodialysis systems and have in some cases are suspected of causing out breaks for infection within dialysis centers. Additionally, biofilms produce endotoxins and other cytokines that can migrate across the dialysis membrane and cause an inflammatory response, which in time causes chronic inflammatory syndrome and further damages the body of what often very ill patients. Making the problem more challenging is the difficulty of removing biofilms from in-place water systems, as such as is used in clinical settings. For this reason a multi-step cleaning, descaling, and disinfection process in recommended for these system. With the ever-increasing numbers of patients on dialysis and other invasive medical treatment the need for understanding, treatment for and prevention of medical biofilms will only continue to expand.

For decades now the medical community has made major and commendable strides to improve and expand treatment of numerous diseases. Many of these treatments have greatly improved the quality of life for millions of people worldwide; however, several of the treatments have come with unforeseen difficulties. Biofilms have become an ever more prevalent challenge for many treatments, especially those involving artificial implants or extended-use treatment devices. One of the best examples of this is the treatment of end-stage renal disease (ESRD), where treatments increasingly rely on artificial means to purify the blood.

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A number of patients have kidney disability and failure due to diseases such as diabetes, hypertension, glomerular nephritis, and cystic kidney disease. As these diseases progress, the output of the kidneys decreases and doctors must turn to more invasive treatment options. Patients with ESRD are treated by three major forms of renal replacement therapy: hemodialysis applications (conventional dialysis, hemofiltration, or hemodiafiltration), peritoneal dialysis (continuous ambulatory peritoneal dialysis, intermittent peritoneal dialysis, and automated peritoneal dialysis), or kidney transplant. For many with ESRD, kidney replacement is the best chance for leading a normal life; however, like most organs, there is a discrepancy between the need for kidneys and the number donated. Additionally, as our society expands its ability to save and extend life, this gap between need and donor organs continues to increase, leading to more patients on dialysis treatments.

The number of patients who have ESRD has increased markedly in the past 40 years. Data from the United States Renal Data System (USRDS) suggest that by 2003 there were ~453,000 patients with ESRD. In the United States, the predominant form of renal replacement therapy is maintenance hemodialysis (~298,000). Only about 6–7% of all patients receiving dialysis therapies are treated by one form of peritoneal dialysis (USRDS 2005).

In 1967, ~1,000 patients were undergoing maintenance, or chronic hemodialysis. In 1973, when full Medicare coverage was extended to persons with ESRD, ~11,000 patients were undergoing dialysis in independent or hospital-based centers and in homes in the United States. At the end of 2002, there were ~264,000 patients undergoing maintenance hemodialysis and 58,000 staff members in 4,035 dialysis centers throughout the United States (Finelli 2005). The ESRD program is administered by the Center for Medicare and Medicaid Services of the Department of Health and Human Services. It is the only Medicare entitlement that is based on the diagnosis of a medical condition.

The technology for performing dialysis, and the potential for complications, has changed significantly over the years. In the early 1960s, hemodialysis was used almost exclusively for the treatment of acute renal failure. Subsequently, the development of the arteriovenous shunt and certain other ancillary technologic advances in dialysis equipment expanded the use of hemodialysis to maintenance therapy for ESRD. In the 1970s, the primary mode for dialysis treatment was hemodialysis performed with various types of artificial kidney machines. Subsequently, the use of peritoneal dialysis, accomplished by automated machines or by intermittent cycling, increased. By the end of 2003, there were only 25,825 (~8%) patients being treated by peritoneal dialysis applications. Continuous ambulatory peritoneal dialysis, automated peritoneal dialysis, or intermittent peritoneal dialysis modality is more popular among pediatric nephrology programs (~40% of all pediatric dialysis patients) (USRDS 2005). One must also recognize that patients may change modality because of vascular access failure, peritonitis, peritoneal transport issues, etc.

Of the various forms of ESRD treatment, hemodialysis will be the focus of this chapter. Biofilms related to peritoneal dialysis are much more unique to the

individual being treated, as the biofilms related to this treatment are very dependant on the individual's life style, cleanliness, and natural flora. Least understood is the role of biofilms in organ replacement such as the replacement of a kidney. Of the therapies for ESRD, hemodialysis has had most research associated with the formation of and the problems related to biofilms.

Hemodialysis purifies blood by passing blood across one side of a dialyzer (dialysis membrane) and dialysate (a saline like solution made with purified water) across the other side. Thus, hemodialysis is essentially a liquid-based extraction/purification system, and like all water distribution systems it is susceptible to the formation of biofilms. The increased nutrients provided by the dialysate and components of the blood that cross the membrane make this a near optimal growth environment for many biofilm-forming microorganisms. The biofilm problem in these systems is compounded by the fact that all patients with chronic kidney disease, including dialysis patients, have a compromised immune system and other comorbidities that place them at increased risk for infectious diseases. The combination of these challenges leaves dialysis facilities with the dilemma of how to deal with the hazards created by biofilms without compromising patient care. This chapter provides information on the scope of the problem and where some of the research and treatment efforts are focusing.

1 Discussion of Hemodialysis Systems

1.1 Not a Drop of Sterile Water for Hemodialysis

When hemodialysis was first developed in the early 1960s, the hemodialysis blood exchanges were performed against drinking water. The concept of additional water treatment did not emerge until the late 1970s, when numerous difficulties deriving from the use drinking water were realized. Initially the problem was associated with chemical interactions such as "hard water syndrome" and "aluminum dementia." The dilemma was not restricted to chemical intoxications, but was soon realized to include the much more challenging microbiological-related diseases such as mediated endotoxin syndromes or pyrogenic reactions. The discovery of the importance of water quality has spurred a progressive push for improved purification. Initially, the treatment was simple, utilizing particulate filtration, softeners, and deionization. These treatments made a tremendous improvement in preventing chronic toxicity diseases such as hard water syndrome, but resins and membranes offered ideal conditions for bacterial development, leading to an increase in endotoxin levels. The addition of reverse osmosis (RO) which retains greater than 99% of bacteria and endotoxins significantly decreased the microbiological risk. Nowadays, the dialysis water treatment plants are highly sophisticated with hemodialysis water system providing some of the best treatments in the water industry (Canaud et al. 2000). Figure 1 illustrates the standard water treatment in use today, and includes the following (Capelli et al. 2005):

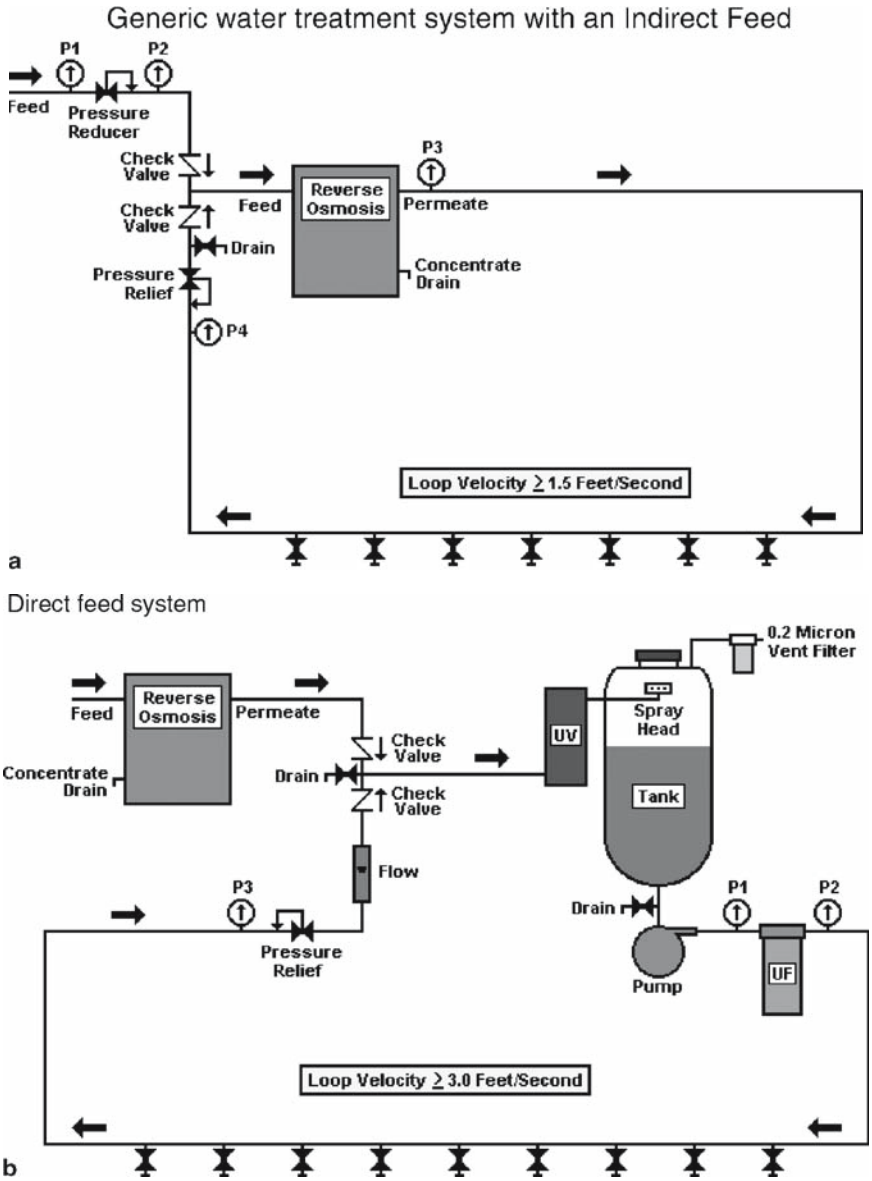


Fig. 1 Schematic representations of three common dialysis water treatment systems: (**a**) generic water treatment system with an indirect feed, (**b**) direct feed system, (**c**) direct feed system with pasteurization (continued)

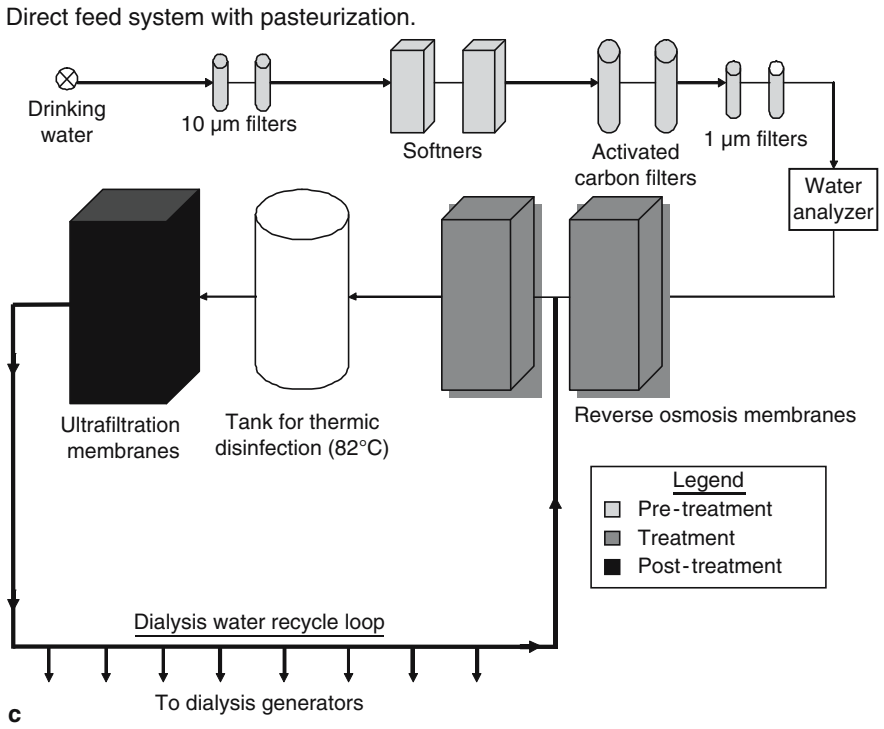


Fig. 1 (continued)

- Pretreatment, consisting of filters for the retention of suspended particles, softeners to remove calcium and magnesium, and activated carbon filters to eliminate any trace of chlorine, chloramines, and organic materials; each run in series.
- Reverse osmosis for the retention of the salts, added during softening, and the remaining mineral and organic materials that are not removed in pretreatment, including bacteria and endotoxins.
- The posttreatment, which is most effective for the removal of any remaining microorganisms, uses microfiltration (0.2-µm pore size), alone or in combination with ultrafiltration.

The complexity of the system and the advanced technology of each component lead us imagine that the final quality of hemodialysis water is highly sterile. So, how is it possible for biofilms to develop in these systems? In other words, how does water devoid of organics, minerals, and microorganisms support the growth of biofilms?

The answer has much to do with what makes biofilm unique and prolific, such as an ability to survive in inhospitable environments. To understand this, it is necessary to first look at the magnitude of water necessary for hemodialysis. About 200 L

of dialysis water is needed for an individual dialysis session. A patient treated by hemodialysis for 2 years receives roughly the same volume of water as he or she will drink during an entire lifetime. The volume of water produced daily by a single treatment system for a dialysis clinic reaches many tens of cubic meters (enough water to fill a residential swimming pool). This is important for two reasons: the number of organisms that must be filtered and the trace nutrients available in the water. Most drinking water entering a dialysis system is relatively clean and contains on average between 10,000 and 100,000 organisms per liter. However, owing to the volume of water needed for the operation of a dialysis clinic, the water treatment system must remove billions of organisms each day. The shear number of organisms passing through these systems makes one in a billion probabilities of an organism crossing the membrane or leaking around the RO seals possible. Many of the current membranes can handle this, but unless they are regularly treated and replaced, they will eventually allow organisms through. Despite the possibility of bacteria crossing the membrane, the most likely place for contamination to enter is during setup and maintenance of the water system. Because no matter how good a membrane, it cannot prevent contamination of bacteria which get behind it during installation or maintenance, operations which are usually not performed using sterile technique, and commonly rely on antimicrobial treatment to remove the contamination they cause.

The microbial control is further confounded by the fact that most microbial treatments for drinking water such as chlorine compounds are toxic to humans, if they are introduced into the bloodstream. Therefore such compounds, used to control the microbial contamination, must be completely removed from dialysis water to prevent them from affecting patients.

This is where biofilms become a problem for hemodialysis. Any bacteria that are able to evade the treatment system (during maintenance or operation) and enter the water distribution system find high purity water free of antimicrobials. Here again the large volumes play a role because although few trace impurities make it past the treatment system, much of what does will accumulate on the piping of the system. The high purity of the water selects for specific organisms (primarily bacteria) that are accustomed to grow under oligotrophic conditions and these conditions of starvation improve their natural attraction to surfaces as a way of survival. At these surfaces in the hemodialysis systems the bacteria find little competition and an increased food source due to adsorption of trace nutrients. This adsorption concentrates the nutrients, providing a food supply for microorganisms. Once adsorbed to the surface, these organisms will form biofilms as a means to remain on the surface. In this way an organism has the potential to form a biofilm and multiply in large numbers in locations previously thought to be devoid of life. Sampling of hemodialysis water has shown that these systems mainly contain Gram-negative rods, yeasts, fungi, and *Mycobacteria*. The main microorganisms currently found inside dialysis water are listed in Table 1.

The current technology based on RO is unable to continuously produce high volumes of water without some level microbiological contamination (Lonneman 2004). So why are not sterile water systems used instead of the current hemodialysis

Table 1 The microbial genera most commonly found in hemodialysis water (Comila et al. 2005; Morin 2000; Alter et al. 2004; CDC Unpublished data 2005)

Gram-negative rods	Yeast and fungi	Others
Pseudomonas	*Candida*	*Mycobacterium*
Flavobacterium	*Rhodoturula*	*Staphylococcus*
Acinetobacter	*Cryptococcus*	
Serracia	*Aspergillus*	
Aeromonas	*Streptomyces*	
Alcaligenes	*Cladosporium*	
Burkholderia	*Penicillium*	
Enterobacter	*Phialemonium*	
Moraxella	*Fusarium*	
Stenotrophomonas	*Acremonium*	
Sphingomonas		
Variovax		
Afipia		
Achromobacter		
Agrobacterium		
Ralstonia		

water systems? That is because there are only two systems available for the production of sterile water: packaged sterile water (treated by autoclaving) and pyrodistillation (in which the water is heated to more than 600°C), but these systems allow the production of only small volumes of sterile water and therefore it is not possible to produce the volumes of water needed for dialysis in the normal clinical setting.

As previously stated, the usual contamination level of the drinking water entering the dialysis water treatment plant is ~100 colony forming units (cfu) mL^{-1} (i.e., 100,000 cfu L^{-1}), and depending on national drinking water standards, can contain as much as 500 cfu mL^{-1} of total heterotrophs. But after treatment, the bacterial and endotoxin levels measured in hemodialysis water can attain concentrations more than 1,000 cfu mL^{-1} and 5 Endotoxin Units (EU) mL^{-1} if not appropriately maintained. This difference is due to biofilms that develop inside the dialysis water treatment plants that release living cells and increase the contamination level of the water.

Because of the hazards caused by microbiological contamination, European authorities and the American Association for the Advancement of Medical Instrumentation (AAMI) have established standards for the bacterial concentrations and endotoxin levels in dialysis fluids (water, dialysate, substitution fluid). These standards (at the time of this publication) are given in Table 2. Many dialysis proponents argue that the AAMI standards are too lenient, which has facilitated AAMI to raise the standards, but on a global scale the real problem is that even these standards are not followed in many parts of the world at the present time. The actual compliance to AAMI standards ranges from 70 to 94% for dialysis water and from 57 to 88% for the dialysate.

Table 2 European and American (AAMI) standards for hemodialysis fluids (Anonymous 2000; European Pharmacopoeia 2002; AAMI 2004)

	European standards		AAMI standards	
	Culturable bacteria	Endotoxins (EU mL^{-1})	Culturable bacteria	Endotoxins
Water for conventional hemodialysis	100 cfu mL^{-1}	0.25	200 cfu mL^{-1}	2 EU mL^{-1}
Dialysate for conventional hemodialysis	–	0.5	50 cfu mL^{-1}	1 EU mL^{-1}
Water for on-line hemodiafiltration (HDF)	10 cfu L^{-1}	0.25	50 cfu mL^{-1}	1 EU mL^{-1}
Dialysate for on-line HDF	10 cfu L^{-1}	0.25	1cfu 10 mL^{-1}	0.03
Reinjection solution for on-line HDF	No cfu in 20 L	0.05	<1/1,000 L	0.03

1.2 Nonsterile Water and Nonsterile Dialysis Concentrates Produce Nonsterile Dialysate

The control of contamination of dialysis water upstream from the dialysis machine is essential because it is used for the preparation of the dialysate, which will interact with the patient's blood across the dialysis membrane (Hoenich and Levin 2003; Hoenich et al. 2006; Ledebo and Nystrand 1999; Lonneman 2000 a,b). Dialysate, an enriched buffer solution (pH 7.3) containing minerals, bicarbonate, and glucose heated to 37°C, is the purification solution passed counter current to the blood flow for purification. The saline and nutrients present in this solution provide nearly optimal growth conditions for many opportunistic pathogens. Microbial contamination, unlike chemical contamination, has the ability to proliferate if not completely removed. It is for these reasons that microbial contamination, especially biofilms, which serve as microbial reservoirs, are so important and why it is necessary to examine the entire distribution system and not just the purification system.

In addition to the purification system, hemodialysis water systems consist of a water distribution line leading to the dialysis machines, with many of the systems having a recycling loop back to the RO membrane. The water systems are usually designed to deliver purified water to the machines at a set pressure. This water is used for the dilution of dialysis concentrates forming what is know as dialysate, as previously discussed. Excess water is produced to assure constant pressure and flow rates, so that each patient receives a consistent treatment despite variations such as the clinics current patient number. The unused water is returned to the distribution system, upstream the RO system to avoid wastage of purified water. Inside the machine, the dialysis water is heated to 37°C prior to addition of bicarbonate and acid concentrates. The dilution rate is between 1:25 and 1:35 depending on the brand of the concentrate. The dialysate must be produced continuously and consistently during the dialysis session to provide the patient optimal treatment conditions.

A dialysis session lasts 3 or 4 h, a sufficient duration to allow significant growth of microbial contamination. Live organism have been found in the water, the bicarbonate concentrate, and even the acid concentrate (*Sphingomonas paucimobilis* is an example of an oppotunistic waterborne pathogen that has been isolated [unpublished work]). For this reason it is necessary to expand the search for biofilms to all of the various parts of the dialysis system. Bicarbonate concentrates need to be closely scrutinized, as these offer the most favorable conditions for bacterial growth because of their chemical composition and pH (7.3). As a matter of fact, many troubles related to the bacterial contamination of bicarbonate concentrates have been referenced in the literature, especially at the beginning of the marketing of this product (between 1980 and 1990), when the live bacterial concentrations were found to have reached 10^8 cfu mL^{-1} in samples take from commercial concentrate. Currently, the bicarbonate concentrates are in the form of solutions. However, solutions are progressively being replaced by powder, which minimizes the chance for bacterial contamination. From a microbial contamination standpoint the best concentrates come as presterilized bags or cartridges, which minimize the need for handling. Nevertheless, care should be taken in manufacture, storage, and handling of concentrate, because bacterial contamination, even in low numbers, will contribute to an increase in the bacterial level in the dialysate during the dialysis session.

1.3 Sites for Biofilm Development Inside Hemodialysis Fluid Pathways

All the surfaces within the hemodialysis fluid pathways are susceptible to biofilm invasion (Phillips et al. 1994; Man et al. 1998; Capelli et al. 2000; Smeets et al. 2003). The importance and speed of biofilm growth will depend on three major factors: the types of surface materials; the design of the distribution system, including flow rates and dead zones; and the frequency and efficacy of disinfection treatments. The drinking-water lines feeding the dialysis centers are known to be colonized with biofilm. These organisms rapidly colonize the water treatment system with adherent cells up to the point of the RO membrane. As discussed earlier, there are routes by which microorganisms can get past the RO membranes. In this way most dialysis facilities develop at least some biofilm throughout the entire water system. Some specific spots in the system are more susceptible to contamination than others: membranes and resins, sharp bends, rough joints (including glued and soldered joints), storage tanks, and dead spaces (also known as dead legs). The current trend is to systematically eliminate dead spaces from the water treatment plants, to reduce as much as possible the sharp bends and soldering, and to either eliminate or regularly treat storage tanks. But membranes and resins still remain, thus the pretreatment is the most prolific site to biofilm growth and therefore discussions are going on for pretreatment such as UV irradiation to reduce bioburden. This does not mean that other components of the treatment system remain clean, but that the thickest and fastest biofilm development will be found in the

pretreatment system. Dialysis water distribution loops are not spared from microbial contamination, nor are dialysis machines. For this reason, it is advisable that dialysis systems use material that either minimize colonization (very smooth surface, and minimal solders/welds) or are easily treated (smooth and chemically stable to repeated treatment). Some of the preferable materials currently include 316L stainless steel, PPVDF, and PEX.

The preferential spot for biofilm growth is the connection between the water distribution loop and the machines. The connecter is usually a 1–3-m section of reinforced silicone tubing (2 cm ID) and is seldom if ever disinfected. It represents the most commonly contaminated part of the system.

Similar to the water system, the dialysis machine is also susceptible to biofilm formation. Some of the most susceptible components include detection probes, connections, pumps, and especially the components that disinfectant does not reach. The main component of the dialysis machine fluid pathway is silicone tubing, and this tubing has been repeatedly shown to produce biofilms (Man et al. 1998; Phillips et al. 1994). This material was chosen because of its relatively smooth surface, but when examined on the microscopic level of a bacterium, this material shows an irregular grooved surface, highly susceptible to bacterial attachment. Biofilms containing up to 10^6 cfu cm^{-2} and 10 EU cm^{-2} have been found on silicone tubing of dialysis generate.

The dialysis membrane is the least likely to be contaminated by biofilms; however, it is still the cause for the greatest concern because of the intimacy of blood contact. As an attempt to alleviate the chance of a biofilm problem in the dialyzer, many countries have passed requirements that dialysis membranes be single use. One notable exception is the USA, which allows membranes to be reused on the same patient. In a number of countries (e.g., USA, Canada, China, and Latin America), the dialyzer is designated to one specific patient and that patient reuses the dialyzer on each visit until it loses 20% of its original fiber bundle volume, develops membrane or housing leaks, or no longer has an aesthetic appearance (AAMI 2003). In many cases a dialyzer may be used for an average of 15 times (range, 1–156). After each dialysis session, the membrane is rinsed, pressure- and volume-tested, then filled with a disinfectant solution and stored until the next dialysis session. This raises two potential problems: first of all, the potential retention of the bacteria and endotoxins, and second, the disinfectant solution may alter the surface of the membrane. If treatment practices are not properly followed, there is a chance that resistant bacteria could attach to the surface and survive the disinfection process, and then subsequently outgrow during the next dialysis session to form a biofilm, making them further resistant. Additionally, continuous exposure of the membrane to certain disinfectants could increase the porosity and lead to the release of bacteria and endotoxins into the patient's blood. This reuse strategy has the potential to alter the dialysis membrane from a medical treatment tool to a reservoir for bacteria and toxins. Prominent biofilms have been observed on the inner surface of hollow fibers of filters used for on-line hemodiafiltration (Man 2004). For this specific dialysis method, machines are equipped with two filters mounted in series at the outlet of

the machine, to filter the dialysate to be reinjected into the patient's blood (called "reinjection solution"). These filters are similar to a dialysis membrane. Biofilm growth inside these filters is due to both the retention of bacteria, salts, and crystals, and the bacterial growth on the fibers. The contamination has been observed on both the filters, suggesting the release of bacteria from the first filter. For these reasons it is not possible to exclude the potential for the release of bacteria and endotoxins from those filters into the blood.

1.4 Composition and Examples

Studies have shown that biofilm composition and properties vary markedly with environment. Likewise, biofilms inside hemodialysis systems have a specific composition depending on the specific location in which they grow. Biofilms found on the first part of the system, from the water treatment system to the inlet of the dialysis machine, are composed of oligotrophic bacteria and slime but contain little or no minerals, in other words no scale. Conversely, the dialysis machine is likely to have significant scale. This scaling reduces flow, and more importantly, the presence of scale increases adherence of biofilms, making them more difficult to remove.

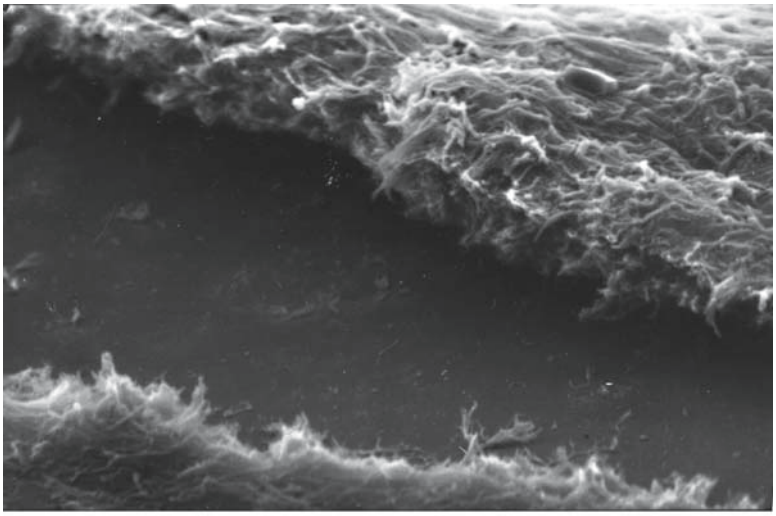
Another factor indirectly responsible for creating difficult biofilms is the variation of flow rates with some dead zones and most of the system at a flow rate of at least 500 mL min^{-1}. The dead zones create bacterial reservoirs, while the high flow rate areas select for highly adherent biofilms. Examples of biofilms isolated from various parts of the dialysis system are shown in Fig. 2.

2 Consequences of Hemodialysis Biofilms

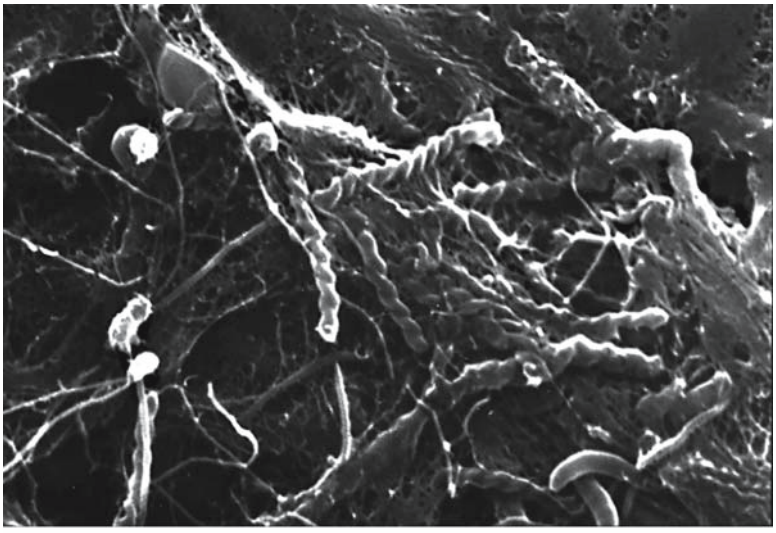
Biofilms in hemodialysis systems may impair the operation of the machine, reducing fluid dynamic aspects, heat transfer rates, and accuracy of the sensors, as well as causing partial blockage of the drains; however, the real problem remains the clinical consequences of the biofilms, which cause an elevated health risk for the patient.

2.1 The Impact of Live Bacteria

Infection is a major cause of morbidity and mortality in patients on chronic hemodialysis, accounting for 12–38% of the mortality of these patients (Roth and Jarvis 2000). Some microorganisms found in hemodialysis fluids have the potential to cause nosocomial infections, and therefore represent a health risk to patient. The following genera have been described as causative agents of bloodstream infections (BSI) during chronic hemodialysis: *Acinetobacter*, *Afipia*, *Burkholderia*, *Bacillus*,

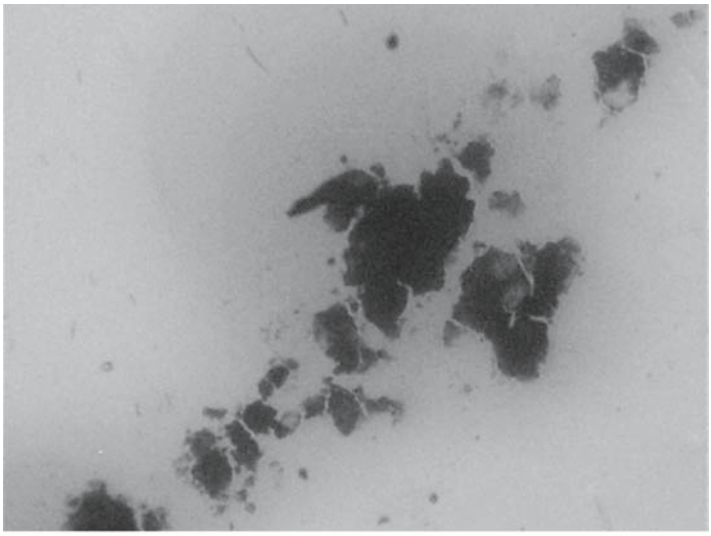


a

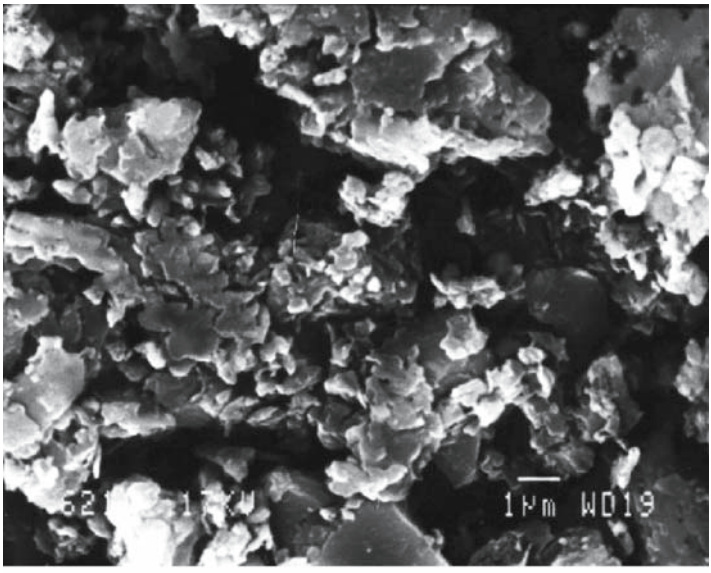


b

Fig. 2 (**a**) Biofilm isolated from the connection between the water distribution loop and the machine (scanning electron microscopy (SEM), ×1,000). (**b**) The biofilm in Fig. 2a, at a higher magnification (SEM, ×8,000). (**c**) Biofilm isolated from the inner surface of silicone tubing inside the machine, downstream the production of dialysate (crystal violet staining and optical microscopy observation, ×400). (**d**) Biofilm isolated from the inner surface of hollow fibers of the first filter on dialysis machines for on-line hemodiafiltration (SEM, ×5,000). (**e**) Biofilm isolated from the inner surface of hollow fibers of the second filter on dialysis machines from an on-line hemodiafiltration system (SEM, ×10,000)

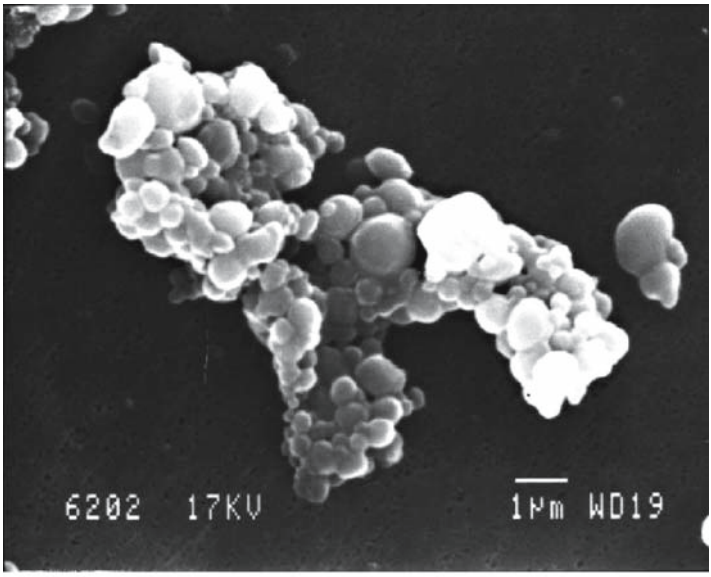


c



d

Fig. 2 (continued)



e

Fig. 2 (continued)

Candida,*Enterobacter*,*Klebsiella*,*Mycobacterium*,*Pseudomonas*,*Stenotrophomonas*, and *Ralstonia* (Roth and Jarvis 2000; Alter et al. 2004). BSI has been identified as the cause of death of 6–18% of patients on dialysis. Outbreaks of BSIs usually have been caused by maintenance failures or dysfunctions of the system. For example, three clusters of Gram-negative bacterial bloodstream infections occurred in Canada (1995), USA (1997), and Israel (1997) (Block et al. 1999), resulting from contamination of the waste drain ports in the same model of hemodialysis machine (CDC 1998; Jochimsen et al. 1998). More recently, an outbreak of *Burkholderia cepacia* complex bacteraemia occurred in a dialysis clinic in Brazil (Magalhaes et al. 2003). This microorganism colonized the RO membrane, leading to extreme contamination of postosmosis water (up to 8,000 cfu mL^{-1}). In this case the contamination level of the dialysate was so high that a huge amount of bacteria and endotoxins were adsorbed on the surface of the dialysis membrane; consequently, the retention capacity of the membrane was progressively reduced and finally, live bacteria were able to transfer across the membrane into the patient's bloodstream.

It is well known that the size of live bacteria is too large to allow their crossing through the dialysis membrane (the cut off of a dialysis membrane is about 5 kDa), therefore BSIs should be minimized in countries where the dialysis membrane are single use. Unfortunately, despite the modern technology and quality present in the membrane industry, bacteria have still been shown to pass low molecular weight membranes. This is especially true if there are defects present in the membrane and/or the level of bacterial contamination is high, often because of inadequate dialyzer reprocessing.

Major incidents have been linked to dialyzer reuse, either because the reused dialysis membrane had been altered during reprocessing or because a biofilm that had developed despite the regular disinfections of the membrane had released live bacteria. One example is an outbreak of Gram-negative bacteremia caused by *B.* (*Pseudomonas) cepacia*, *Stenotrophomas (Xanthomonas) maltophilia*, *Citrobacter freundii*, *Acinetobacter calcoaceticus*, and *Enterobacter cloacae* that occurred in 11 patients because of biofilm development on the O-rings of reprocessed dialysis membranes (Flaherty et al. 1993). A second example involving rapidly growing *Mycobacterium chelonei* was the cause of infections (bacteremia and soft-tissue infections) in 27 patients receiving dialysis through reprocessed dialyzers (Bola 1994). The disinfection procedure used for reprocessing has had a number of failures. From 1980 to 1999, the CDC investigated seven outbreaks of bacteremia, seven outbreaks of pyrogenic reactions, and one outbreak of toxin-induced acute liver failure (Roth and Jarvis 2000). More than 50% of these outbreaks were caused by failures during dialyzer reprocessing (suboptimal concentrations of the disinfectant or alteration of membrane integrity), 33% resulted from contamination of dialysis fluids (water or dialysate), and 13% were related to the contamination of dialysis machines.

2.2 Effects of Bacterial By-products

Bacterial compounds represent a second highly important risk category for hemodialysis fluids. The most dangerous of these substances are collectively termed pyrogens or cytokine-inducing substances. These substances are released by the bacteria particularly during the lysis of the dead cells. Many of these compounds have the potential to transfer across the dialysis membrane because of their small molecular size; the extent of transfer is likely to be dependant on the type of compound, dialysis system, and dialyzer (Weber et al. 2004). Pyrogenic compounds or pyrogens are substances that are able to induce fever. The best-known group of pyrogens is lipopolysaccharides (LPS) from Gram-negative bacteria, also known as endotoxins. Endotoxins are components of Gram-negative bacterial cell walls, which can remain after cell death. These compounds are chemically stable, and hardly affected by the conventional techniques and products used to treat bacteria. The only proven treatment for the destruction of endotoxins is heating for 4 h at temperatures higher than 180°C. A further complication for hemodialysis systems is the affinity LPS has for surfaces where they strongly attach by hydrophobic interactions.

For a long time endotoxins were considered as the only pyrogenic compounds; however, the improvement of the detection of pyrogenicity has led to highlight many other components with pyrogenic properties (Sparswasser 1997). These products are listed in the Table 3.

The toxicity of pyrogens is expressed through an inflammatory cascade. Pyrogens trigger the specific receptors of peripheral blood mononuclear cells (MNC) which induce enzymatic reactions activating transcription factors (NF-κB,

Table 3 Bacterium-derived pyrogenic substances (kDa) in dialysis fluids (Berland, 1998)

Substances with molecular weight >5 kDa	Substances with molecular weight <5 kDa
Lipopolysaccharide (>100)	Lipid A (2–4)
Peptidoglycans (1–20)	Peptidoglycans (1–20)
Exotoxins (20–70)	Muramyldipeptides (0.4–1)
Bacterial DNA (>5)	Exotoxin fragments (<5)

NF-IL-6, etc.), leading to the production of different substances such as nitrogen monoxide, coagulating factors, prostaglandins, and mainly proinflammatory cytokines (interleukin-1β and tumor necrosis factor). These substances are mediators for nonspecific inflammatory responses. With the exception of endotoxin, the toxicity of pyrogens varies according to the bacterial strain from which they come. Consequently, it is difficult to establish accurate limits for their concentration in human blood (endotoxin limits are 5 EU $kg^{-1}h^{-1}$). However, efforts have been made that have characterized pyrogen production during hemodialysis by employing a whole blood approach (Marion-Ferey et al. 2005).

The dialysis membrane retains the complete LPS molecule, but LPS fragments and some of the other pyrogenic compounds can penetrate and enter the bloodstream. Dialysis membranes differ vastly with respect to their endotoxin permeability characteristics. The pyrogen retention properties of membranes are essentially determined by the chemical characteristics, most importantly polymeric composition. Adsorption is considered to be the more important mechanism of pyrogen retention. It is, however, currently impossible to accurately predict the percentage of pyrogens transferred through the dialysis membrane during a dialysis session. This is largely owing to the fact that transfer depends on numerous factors, mainly the nature of the dialysis membrane and the nature of the pyrogens, which vary with organism and environmental conditions. The percentage of pyrogens passing the membrane has been estimated to range between 0.1 and 1.9% of the compound to which the membrane is exposed. One example is the synthetic high flux membranes (i.e., polysulfone), which only retain negatively charged molecules that can be adsorbed by electrostatic interactions.

Pyrogen penetration is elevated during backfiltration, which is a common part of the dialysis sessions. Backfiltration is a convective force, caused by the relative pressure gradient along the length of the dialyzer in the blood and dialysate compartments, enhancing the pyrogen permeability and leading to the transfer of contaminants into the blood compartment of the membrane unit (Ofsthun and Leypoldt 1995). This phenomenon is inevitable with the high flux dialysis membranes because of their porous structure. Moreover, it is hypothesized that the pyrogen might be able to stimulate the MNCs without crossing the dialysis membrane. The simple adherence of the pyrogens and the MNC, on both sides of the dialysis membrane may be sufficient to activate the MNCs and release inflammatory modulators (Ismail et al. 1996).

The transfer of pyrogenic compounds into the patient's blood may have tragic consequences, including death. On one hand, a massive flush of pyrogens into the

bloodstream can lead to acute effects called pyrogenic reactions, which may be expressed as minor symptoms such as fever, headache, nausea, diarrhea, hypotension, or septic syndrome (also known as endotoxemia, endotoxic shock), which in extreme cases, may lead to death.

A dose–effect relationship has been established for acute effects of pyrogens and the significance of the clinical symptoms has been related to the immune status of the patient. And for years, acute effects have been believed to be the only importance of pyrogens; however, there is increasing evidence of damage resulting from recurrent exposure of low to moderate levels of pyrogens during hemodialysis (Lonneman 2000a,b; Jofre et al. 2006). Dialysis-related cytokine induction has recently been incriminated in causing a number of chronic diseases, including amyloidosis (β2-microglobulin deposition involving carpal tunnel syndrome, periarthritis, spontaneous fractures), cachexia, metabolism dysfunctions, cardiovascular syndrome, immunodeficiency, and resistance to erythropoietin therapy. In parallel with the onset of these diseases, clinicians and researchers have also observed an increase in the levels of chronic inflammation indicators such as cytokines, interleukin-1 receptor antagonist, and LPS antibodies in the blood (Canaud et al. 2001; Jofre et al. 2006). These inflammatory response markers are predictive of hospitalization and mortality in chronic hemodialysis patients.

Acute pyrogenic effects are becoming less frequent because of the improvement of the dialysis fluid quality and the development of standards, but the problem of silent chronic inflammation still remains (Lonneman 2000a,b). This is due in part to biofilms, which act as a source of bacterial products released into the dialysis fluids. These bacterial reservoirs represent a major factor for chronic inflammation due to their ubiquitous occurrence, resistance to treatment, and long-term persistence in water systems. This problem is exacerbated by the fact that treatment of the biofilms may increase the amount of pyrogens in the water supply. This is especially true when biofilms are treated by disinfectants which leave the dead cells and cellular debris stuck to surfaces within the system. For this reason, dialyzer reuse is also associated with increased incidence of pyrogenic reactions (Rudnick et al. 1995; Archibald et al. 2006).

These findings have not been overlooked by the medical standard groups and government agencies which have worked to improve water quality. The beneficial role of ultrapure dialysis fluids, in locations where these have been used, has been clearly demonstrated by a reduction in the incidence of dialysis-related inflammatory syndromes (Bommer and Jaber 2006). Patients treated for a long period on a dialysis machine filled with ultrapure fluids have less severe complications such as carpal tunnel syndrome and their levels of chronic inflammation indicators in the blood are on average lower than those when more traditional water sources are used.

Chronic inflammation in patients on dialysis is not only due to pyrogens but also due to other factors such as secondary infections, and in some cases the dialysis systems themselves. The materials constituting the dialysis systems, especially tubing and dialysis membranes, are able to stimulate the inflammatory system of the patient (Banche et al. 2006). This phenomenon is known as bioincompatibility and although medical products are thoroughly tested to minimize this problem, there

is still some evidence of the presence of incompatible compounds in dialysis machines. We can say that biofilms, acting as pyrogens reservoirs, increase the bioincompatibility of hemodialysis systems. This problem is a real challenge for physicians and clinics who must distinguish the effects of the hemodialysis-transferred pyrogens from the other disease factors, in what are often very ill patients.

3 Treatment of the System

Regular disinfection of the water treatment system and the dialysis machines is essential to maintain the quality of dialysis fluids (Pizzarelli et al. 2004). The cleaning and disinfection of dialysis systems can be broken into three parts: cleaning (removal of organic materials), descaling (removal of inorganic substances), and disinfection (removal of microorganisms). This process is further complicated by different water sources each having its own chemical composition and bacterial load, and therefore each dialysis center must have its own specific disinfection protocols. Disinfection protocols are part of the quality insurance of the center and must be thoroughly followed by the dialysis nurses and technicians. In Europe, this is taken a step further as the European Pharmacopoeia recognizes dialysate as a drug and places the final responsibility with the pharmacist of the clinic or the hospital. Therefore European pharmacist must play an active role in the disinfection of the system. However it is implemented, if appropriate regular treatment is not performed the system will be colonized by biofilms creating the potential for pyrogens to enter the blood as well as bacterial infection.

3.1 Current Disinfectants and Their Conditions of Use

The current practices for the disinfection of dialysis systems (water treatment and dialysate generation) vary markedly (Ghezzi et al. 2007). In addition to the variations that occur in water quality, there are also multiple dialysate machine companies, many vendors of water treatment equipment, and many companies producing cleaning/disinfection products. This can be illustrated by examining the different compounds currently used for the disinfection of dialysis systems. They are included in various formulations, leading to an important choice of products. Some of the most common compounds are listed in Table 4 in the order of their frequencies of use (ozone is notably absent because of insufficient data).

Most of these compounds are very good biocides, with steam being the most efficient. Unfortunately only one dialysis machine, called Miroclav® (Baxter), uses steam (water heated at 121°C under1.5 atm) disinfection, but it is no longer manufactured. The main disadvantage of hot water and water steam is the limitation in construction materials compared to those which are compatible at high temperatures. Another disadvantage is the lack of descaling and cleaning activities. Finally, the temperatures within

Table 4 Disinfection compounds currently used in dialysis systems (Allard 1999; Holmes et al. 2004; Marion-Ferey et al. 2003)

Compound	Biocide efficacy	Descaling efficacy	Cleaning efficacy
Peracetic acid[a] (CH_3COOOH)	+++	+	+
Sodium hypochlorite (HClO)	+++	0	++
Hot water (85–90°C)	+++	0	0
Citric acid at 85–95°C $C_6H_8O_7$	+++	+++	+
Glycolic acid ($C_2H_4O_3$) at 85–95°C	+++	++	+
Water steam (125°C, 1.5 atm)	+++(+)	0	0
Hydrogen peroxide (H_2O_2)	++	0	+
Chlorine dioxide (ClO_2)	+++	0	++
Aldehydes (RCHO)	+++	0	0
Acetic acid (CH_3COOH)	0	++	+
Lactic acid ($C_3H_6O_3$)	0	+++	+
Oxalic acid ($C_2H_2O_5$)	0	++	+

+++, high efficacy; ++, intermediate efficacy; +, low efficacy; 0, no efficacy
[a] Mixture with acetic acid and hydrogen peroxide

these systems during pasteurization are not homogeneous along the hydraulic pathway, thus the efficacy will be diminished in low temperature zones. These problems have influenced dialysis centers, creating a preference for chemical treatments, such as mixtures of peracetic acid (formulations containing the equilibrium of acetic acid + hydrogen peroxide ↔ peroxyacetic acid), which is currently the most commonly used product worldwide. This is due to its toxigenic safety relative to such treatments as sodium hypochlorite. The main disadvantage of this product is its irritant odor and its corrosive power. There is some trade off between peracetic acid and other treatments disinfection properties in comparison with sodium hypochlorite, depending on the concentrations used. It is cheap and easy to use; it is the only compound that has a little cleaning action associated to a very good biocide activity. Its main disadvantages are its toxicity and high oxidative power leading to a highly corrosive action.

Citric, glycolic, and acetic acids are good descaling agents. They are recommended for the removal of minerals such as calcium and magnesium, which are abundant inside dialysis machines. They get their biocide properties by combining with biocides (peracetic acid) or with hot water pasteurization (85°C): this is called thermochemical disinfection and it is more and more common today inside new dialysis machines which are thermo-resistant.

The use of aldehydes (formaldehyde, glutaraldehyde) is diminishing because of their toxicity and environmental regulations. In the early years of dialysis, formaldehyde was the main disinfectant used. Accidents related to the persistence of traces of product after rinsing have been reported, mainly because of the adsorption of the product on the plastic components of the machine. This product has a pungent odor, irritant properties, and carcinogenic potential, which have affected both patients and staff.

Regulations on the disinfection of dialysis machines vary with country, most of which do not require cleaning of the internal water system between patients. The general recommendation is that facilities treat the internal fluid paths at least once a week; however, many facilities still treat only once a month and some even less. The connection between the water distribution loop and the dialysis machines is disinfected only if a total on-line disinfection is possible, or if a specific protocol has be established. Usually, they are not disinfected and at best they are replaced once a year. As previously described, dialyzer reprocessing is performed between each dialysis session, and the disinfectant remains static inside the dialyzer until the next session.

3.2 Efficacy of Disinfectants on Hemodialysis Biofilms

Very few studies address biofilms in hemodialysis systems; however, the studies that have been performed agree that biofilms are a major problem for which the current treatments are ineffective (Holmes et al. 2004; Marion-Ferey et al. 2003). Biofilms studied in these systems are highly resistant to treatment and it is therefore postulated that only the outermost layers of the biofilms are affected by disinfectants, their diffusion into the biomass being the major limiting factor. Most of the disinfectant is consumed in the interaction with the exopolymeric matrix before reaching the bacteria. Moreover, biofilm bacteria that exhibit stationary-like behavior are known to be more resistant to biocides than are their planktonic counterparts. For these reasons, the concentrations of disinfectants usually have to be significantly increased in order to be effective against biofilms. (For example, the concentration of bleach has to be 150–3,000 times that normally used for treating planktonic organisms.)

At the time of this publication only a few products have been tested for their activity on biofilms. We commend the researchers in the dialysis community who have made the effort to perform biofilm studies; however, standards have only recently become available so when most of the existing studies were performed there was no uniformity in the assessment of disinfectant efficacy. So, usually, a disinfectant is said to be "efficient on biofilms" when it is able to reduce significantly the number of living cells on the surface of a laboratory-modeled biofilm. Laboratory models are usually performed under operating conditions, which are often separated from the clinical reality and in which the relationship between the bacteria/media/material is not always taken into account. Moreover, the remaining endotoxin level on the treated surface is not always measured. The problem with this is that good bactericides commonly produce a large amount of endotoxins during the treatment of bacteria. In other words, limiting the assessment of the treatment to organism viability (kill) is overly simplistic and not sufficient for complete evaluation of product efficacy. This assessment should be completed by at least measuring the residual surface coverage which is well correlated to the level of residual total bacteria and pyrogens. An example of this, taken from Marion-Ferey et al. (2003), and shown in Table 5 and Fig. 3, illustrates how it is possible to examine the multiple aspects of biofilm treatment.

Table 5 Assessment of the antibiofilm efficacy of some of the most commonly used disinfection protocols as tested in an in vitro model

Disinfection protocol	Kills (%)	Coverage reduction (%)	Endotoxin level reduction (%)
Citric acid 3%, 20 min, then bleach 0.3%, 20 min	>99.999	65	99.63
Bleach 0.3%, 40 min	>99.999	58	94.13
Mixture PAA 3%, 40 min	>99.999	15	92.20
Citric acid 3%, 5 min, then autoclave at 121°C, 30 min	>99.999	62	99.91
Citric acid 3%, 90°C, 40 min	99.982	7	56.57
Glycolic acid 0.6%, 85°C, 30 min	>99.999	12	98.67
Hot water 90°C, 40 min	99.995	7	76.70
Citric acid 3%, 5 min, then mixture PAA 3%, 40 min	>99.999	68	98.83

PAA peracetic acid + hydrogen peroxide + acetic acid

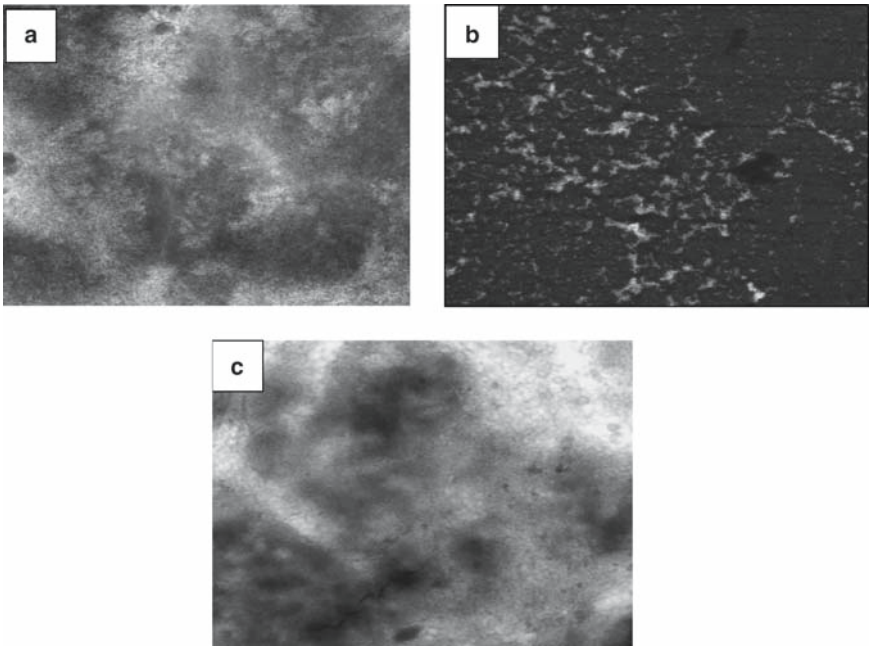


Fig. 3 Representative images of hemodialysis biofilms taken with a confocal scanning laser microscope after staining with BacLight bacterial viability kit (Invitrogen, Carlsbad, CA). (**a**) Untreated mature biofilm; (**b**) biofilm after treatment with high removal efficacy (reductions of greater than 50%); (**c**) residual biofilm after treatments with low removal efficacy (reductions of less than 25%)

When considering only killing action, these disinfection protocols all appear to be highly efficient on biofilms. But things are very different as far as coverage reduction is concerned. No treatment thus far has shown complete biofilm removal (and consequently endotoxins) from silicone surfaces. Among all the protocols tested, an acid

descaling pretreatment, followed by autoclaving and peracetic acid or bleach wash, appears to be the best way to both kill bacteria and reach a low level of endotoxin (Holmes et al. 2004). Descaling alone is not sufficient, even at high temperature. Conversely, treatments performed at high temperature are not effective in removing biofilm. Increasing the temperature is believed to lead to "baking" of the biofilm, which becomes highly adherent to the surface of the tubing. This environment promotes subsequent biofilm growth by providing nutrients to the bacteria, increasing the adherence to the surface, and also limiting removal of endotoxins. Bleach, even when used alone, appears to be a relatively good agent for biofilm removal. This may be due to its cleaning power allowing the detachment of part of the biomass.

A very few tests have been performed *in situ* and one example is the comparison between chlorine and peracetic acid on a biofilm that had developed on granular activated carbon. Sloughing was shown to increase after both treatments. Peracetic acid detached about 40% of the fixed biomass within 10 min, although chlorine needed a 24-h-contact time to detach 75% of the biomass. Peracetic acid appeared to have a faster action, which might be related to a better diffusion within the biofilm because of a lower redox potential inducing a slower oxidation kinetic and disturbing the organization of the biomass. However, these two disinfectants, like many antimicrobials, have a limited reaction time because of their reactive nature. They must additionally be removed from the system prior to operation because of their incompatibility with blood, and therefore these cannot be used as a continuous treatment. Thus any surviving biofilms rapidly return to their original thickness.

3.3 Alternatives Using New Disinfection Methods: UV or Ozone

Two traditional water treatment methods, UV irradiation (Elasri and Miller 1999; Pozos et al. 2004; Stagier 2005; Smeets et al. 2003) and ozone treatment (Nagayoshi et al. 2004; Smeets et al. 2003), were tested for their efficacy in preventing biofilm development. Howerver, these two methods have limited use in dialysis systems today. Ultraviolet light was shown not to have a consistent impact on biofilms because of very low penetration inside the biomass. The biofilm showed a protective property by physical shielding against UV A, B, and C. The efficacy of UV A might be slightly increased by the presence of photosensitizers (for example psoralen). In some cases UV radiation was shown to increase the concentration of easily assimilated organic carbon, providing increased nutrients for bacterial growth.

Ozone, on the other hand, has a higher removal efficacy, but limited biofilm kill. In routine application as a preventive treatment, ozone limits biofilm accumulation downstream from the treatment. The optimal concentration of ozone needs more thorough study because oxidative levels that are effective against bacteria can produce endotoxins, while high ozone level can reduce the lifetime of some materials. Destruction of both the bacteria and endotoxins may be possible if super-oxidative concentrations can be achieved.

The challenge with these two treatments, like so many of the treatments tested on biofilms, is that they will kill the bacteria from the outermost layers, and cause some damage to the structure of biofilm; however, they will commonly leave viable cells within the biofilm and much of the structure intact. This is a real problem for hemodialysis systems, as it causes sloughing of biofilm clumps containing a mixture of living cells, dead cells, and endotoxins. And because of the poor detachment efficacy of disinfectants, sloughing can continue after treatment for hours or days, increasing the microbial and endotoxin contamination of the water. With the improvement made to hemodialysis water quality, mentioned in the previous section, endotoxin contamination of hemodialysis fluids is much more likely to be due to the residual dead cells left on the surfaces by disinfectants, than due to the living planktonic cells themselves.

3.4 "Detachment-Promoting Agents"

The study of biofilms has lead to a change in thinking on bacterial treatment. In most medical applications such as hemodialysis systems, it is not sufficient to kill the bacteria, as the dead biofilm provides both a ready environment for the reestablishment of the biofilm and a source of pyrogenic compounds. This issue has led to the concept of detachment-promoting agents. These agents include enzymes, surfactants, chelating agents as well as signaling compounds and have the properties to remove bacteria, adherent organic and mineral materials from surfaces. Studies have shown that these agents have some limited efficacy in biofilm removal (Johansen et al. 1997; Gibson et al. 1999; Xavier et al. 2005). However, the optimal detachment efficacy appears not by the use of a single agent, but by the rigorous combination of multiple agents. Thus an optimal treatment strategy may be obtained by including these products in the protocol in combination with a biocide agent. One such product is Pronetron®, which implements a detachment–disinfectant procedure based on the combination of synergetic products for an optimal efficacy (Marion et al. 2005; Williams 2005). In its hemodialysis application, a regular preventive treatment performed between each dialysis session markedly reduced biofilm coverage as well as culturable bacterial cells and endotoxin levels. A complete detachment of more than 10^8 cfu cm^{-2} corresponding to a 99% surface coverage was possible.

Clearly there is no silver bullet for the eradication of biofilms from medical field. Therefore the best strategy currently available is a total and regular treatment of all components of the dialysis system; i.e., make sure there is good quality water, that the water system is designed to minimize biofilm-enhancing environments, and that there is regular treatment of all aspects of the biofilm (scaling, structure, bacteria, and endotoxins) in both the water system and the dialyzer.

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Bacterial Endophthalmitis Following Cataract Surgery

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Abstract Cataracts are the most common cause of blindness in the world and cataract surgery is one of the most common surgical procedures performed. During surgery the opacified crystalline lens is removed and replaced with a lens implant. Bacterial infection of the internal eye (bacterial endophthalmitis) following cataract surgery appears to have increased since the 1990s. The infection is the result of inoculation of the patient's bacterial flora into the eye during surgery. Under normal circumstances a small inoculum of bacteria can be cleared from the eye without clinical infection. In this chapter, we discuss many factors that can predispose a patient to developing a clinical endophthalmitis, including those related to the lens implant and host response. There is evidence to suggest that the intraocular lens may provide a niche where bacteria are protected from the host mechanisms that normally clear bacteria from the eye.

1 Introduction

Cataracts are the leading cause of blindness in the world and this has likely been true throughout human history. A cataract is a term that describes opacity of the crystalline lens of the eye causing decreased vision. It is most commonly associated with aging and sun exposure, but can also be linked to severe diarrhea, nutritional deficiencies, genetic disorders, corticosteroids, and trauma (Javitt et al. 1996; West et al. 1998). The onset of impaired vision related to cataracts can vary widely between societies. Agricultural workers in equatorial regions often develop symptomatic cataracts in the fifth decade of life, whereas this is delayed by 10 or 20 years among people working indoors in developed countries (Javitt et al. 1996; West 1999).

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The history of surgical attempts to treat cataracts stretches back to antiquity. Couching is an ancient treatment for cataracts still practiced in some medically underserved regions today. In couching, the opaque crystalline lens is dislocated into the posterior segment of the eye, or vitreous chamber. This is accomplished by passing a sharp instrument, such as a wire or thorn, through the cornea, or by blunt pressure applied to the eye. While this procedure can restore some vision in profoundly blind patients, it is associated with a high risk of inflammation, infection and other ocular complications (Goyal and Hogeweg 1997). Furthermore, no lens replaces the dislocated lens, typically leaving the patient with ~20 diopters of uncorrected refractive error. With the development of spectacles, the situation improved significantly. However, even modern glasses, which attempt to correct refractive discrepancy of a patient lacking a crystalline lens (an aphakic patient), are less than satisfactory. This is due to a host of optical aberrations associated with spectacles lenses of the required power. As the problems with couching illustrate, an ideal cataract surgery would combine both the safe removal of the crystalline lens from the eye, thereby eliminating the inflammatory potential of retained lens material, and the implantation of an artificial lens with refractive properties similar or better than the healthy crystalline lens.

Progress toward this goal of removing a cataract and replacing it with an artificial lens has been pursued for more than 200 years. Attempts at removing the cataract through a corneal incision began in the mid-eighteenth century. Interestingly, in his diaries Casanova recorded discussions regarding the possibility of implanting glass lenses after cataract surgery. Apparently inspired by such proposals, the Dresden court ophthalmologist, Cassamata, unsuccessfully attempted to implant a glass lens in 1795 (Jaffe 1996). It was not until 1949 when Harold Ridley implanted the first modern intraocular lens (IOL) (Fig. 1). Ridley had been encouraged by the observation that fragments of poly(methyl methacrylate) (PMMA) from cockpit canopies were well tolerated in the eyes of British fighter pilots, suggesting that synthetic medical implants could be tolerated inside the eye (Apple and Trivedi 2002). At the time, this represented one of the first attempts to implant a prosthetic device in the human body and was received with a great deal of skepticism by the medical community. However, over the ensuing three decades refinements in the design and manufacture made implantation of IOLs routine in the developed world (Jaffe 1999). This innovation was critical not only to ophthalmology, but to the acceptance of abiotic medical implants in general.

Modern cataract surgery involves the removal of the crystalline lens through a 3-mm incision in the eye (see Fig. 2a for a schematic representation of the eye). A 4–5-mm hole is then created in the lens capsule and the cataract is removed while maintaining the integrity of the remaining capsule. Finally, an IOL is placed in the capsular bag and the incision is closed (Fig. 2b). In most cases, there is prompt recovery of normal vision and postoperative care is minimal. Though cataract surgery can be effectively practiced in both developed and developing countries, there remains a backlog of patients waiting for cataract surgery to cure their blindness that numbers in the tens of millions. Most of these patients are in developing countries, where the resources do not always exist to provide eye surgery to the entire

Fig. 1 An acrylic intraocular lens

population. Thus, there is a large unmet need for efficient and effective surgery to address this treatable cause of blindness in many parts of the world.

Endophthalmitis refers to inflammation, infectious or noninfectious, within the cavity of the eye. Clinically, endophthalmitis is used most often to describe inflammation associated with bacterial infection that occurs most commonly after cataract surgery, or after other globe-penetrating trauma (Fig. 3). Patients usually present within 2 weeks of cataract surgery with an inflamed and painful eye and a striking decrease in vision. White blood cells are visible in the interior of the eye often layering out in front of the inferior iris to form a characteristic white or yellow band known as a hypopyon. Currently, endophthalmitis occurs in 2.5 per 1,000 cases of cataract surgery in the United States (Taban et al. 2005; West et al. 2005). Recent data indicate that the rate of bacterial endophthalmitis has been increasing (Taban et al. 2005; West et al. 2005). A large study reported that one third of endophthalmitis patients had vision of less than 20/60 6 months after treatment (Ng et al. 2005). Because millions of cataract surgeries are performed each year, endophthalmitis affects the vision of thousands of people. *Acute* endophthalmitis is most common following cataract surgery; however, a *chronic* form of bacterial endophthalmitis can also occur. These patients present months to years following cataract surgery with mild, often steroid-responsive inflammation (Ciulla 1999).

An understanding of the pathogenesis of postcataract endophthalmitis is vital to developing preventive and therapeutic strategies. In recent years more attention has been paid to the role that bacterial biofilm formation may play in this infection. Particular interest has been focused on the ability of bacteria to attach to the IOLs (Fig. 1), which are implanted during cataract surgery. In this chapter we will

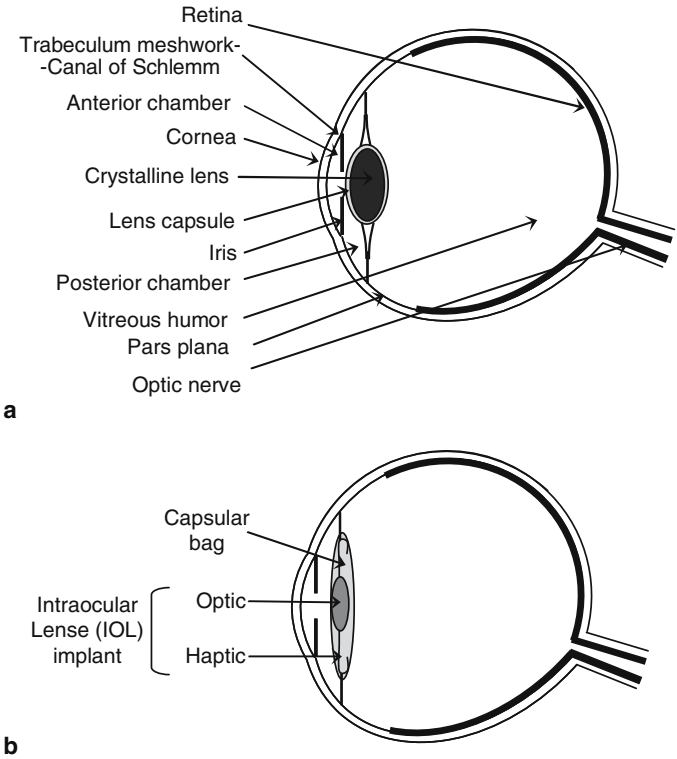

Fig. 2 (**a**) Schematic vertical section of a human eye (not in scale). (**b**) The crystalline lens has been replaced by an intraocular lens (IOL) implant after a cataract surgery

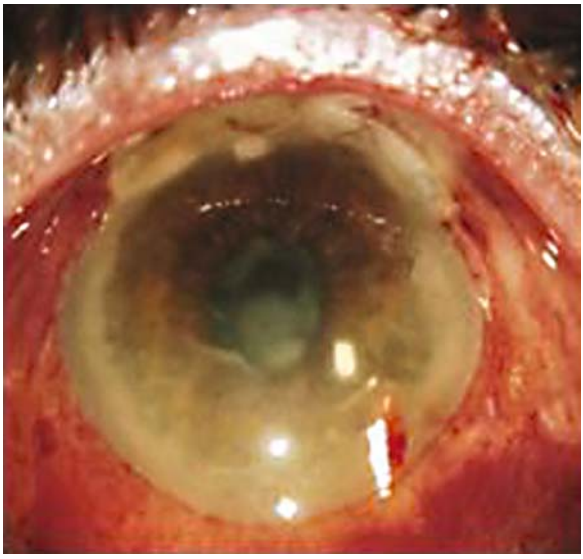

Fig. 3 Example of an eye with acute endophthalmitis (courtesy of M. Gilmore)

explore the role of IOLs as a niche for colonization by bacteria and of biofilm formation in the pathogenesis of postcataract endophthalmitis.

2 Epidemiology

Endophthalmitis is now a rare event after cataract surgery but this was not always the case. In the early twentieth century, endophthalmitis following cataract extraction was estimated to occur in 5–10% of cases (Taban et al. 2005). As recently as the 1950s, the endophthalmitis rate following cataract surgery was reported to be 3% in certain medical centers (Jampel 1999). Since the 1950s the incidence has steadily declined. This decline was likely the result of multiple factors, including improved sterile technique, decreasing incision size, decreasing time of surgery, the introduction of ocular antibiotic preparations, and the refinement of sterile irrigating solutions for the eye. By the early 1990s the rate of postcataract endophthalmitis had fallen to 1 case per 1,000 surgeries or less.

However, after decades of decline, two recent, large studies suggest that the prevalence of endophthalmitis following cataract surgery appears to have increased over the last 10 years. West et al. (2005) conducted their analysis using Medicare data from the period of 1994 to 2001, whereas Taban et al. (2005) performed a metaanalysis of published studies from 1963 to 2003. Both reached the conclusion that the prevalence of endophthalmitis following cataract extraction is increasing. West's analysis indicated that between 1994 and 1997 endophthalmitis following cataract surgery occurred at a rate of 1.8 per 1,000 cataract surgeries, but increased between 1998 and 2001 to 2.5 per 1,000 (West et al. 2005). Likewise, Taban's literature-based study suggested that postcataract endophthalmitis occurred in 2.65 per 1,000 surgeries in the years 2000–2003, compared with 0.87 per 1,000 surgeries in the 1990s (Taban et al. 2005). Both of these reports suggested that a change in wound construction techniques might be responsible for this increase.

A style of wound construction known as "sutureless clear corneal incision" became popular in the early 1990s (Cooper et al. 2003). This type of wound was attractive to surgeons because it reduced surgical time, and the lack of a suture was felt to be advantageous because it reduced the possibility of suture-related irritation to patients, and did not leave a foreign body on the ocular surface as a potential focus of infection and inflammation. However, several reports now suggest that in general sutureless clear cornea incisions may not seal as effectively as other incision techniques, and therefore may allow bacterial entry to the cavity of the eye after cataract surgery has been completed (Cooper et al. 2003; Taban et al. 2005). Other possible explanations for the increasing rate of endophthalmitis include increased antimicrobial resistance of bacteria, possible decreased attention to aseptic technique in cataract surgery, and decreased use of postoperative antimicrobial strategies such as subconjunctival antibiotic injections.

Given the increased rate of endophthalmitis, there has been a renewed focus on preventive strategies. In a 2002 metaanalysis of strategies for prophylaxis against

bacterial endophthalmitis, Ciulla et al. (2002) concluded that preoperative topical povidone-iodine had the best efficacy among the various strategies evaluated. A recent prospective, randomized trial conducted by the European Society of Cataract and Refractive Surgeons (Barry et al. 2006) reported a fivefold reduction in the rate of endophthalmitis among patients who received intraocular cefuroxime at the end of surgery. A reduction in the rate of endophthalmitis was also observed in patients who received perioperative levofloxacin eyedrops, but this result did not reach statistical significance (Barry et al. 2006).

3 Bacterial Skin Flora is Generally the Source of the Inoculum in Postcataract Endophthalmitis

The first event required in the pathogenesis of bacterial endophthalmitis is the inoculation of bacteria into the cavity of the eye. Although these microbes may be derived from the operating room environment or contaminated surgical materials in individual cases, there is a large body of evidence demonstrating that in most cases the causative organism originates from the patients' own bacterial flora. Speaker et al. (1991) isolated bacteria from the eyelid, conjunctiva, or nose of endopthalmitis patients that were genetically identical to that patient's intraocular isolate in 82% of cases. In the Endophthalmitis Vitrectomy Study (EVS), intraocular and eyelid isolates were compared with endophthalmitis isolates from 105 cases (Bannerman et al. 1997). Of these, 67.7% were found to be identical to isolates from the patient's eyelids, using pulsed-field electrophoresis.

Intraocular bacterial contamination after cataract surgery appears to be quite common. When the intraocular fluids are sampled at the completion of routine cataract surgery in which endophthalmitis did not occur, microbial contamination has been reported to range from almost 0 (Leong et al. 2002) to 43–46% (Dickey et al. 1991; Srinivasan et al. 2002) of cases, depending on the study. This suggests that the eye routinely clears a small inoculum of bacteria following intraocular surgery without progression to endophthalmitis (Ciulla et al. 2002; Speaker et al. 1991; Srinivasan et al. 2002; Tervo et al. 1999). Experimental models of endophthalmitis confirm that eyes can tolerate a small inoculum of bacteria without progression to clinically evident disease. A rabbit model of *Staphylococcus epidermidis* endophthalmitis showed that an inoculum lower than 3×10^5 bacteria does not lead to an infection and can be cleared from the eye within 24–64 h postinoculation (Maxwell et al. 1993).

4 Microbiology

Since the causative microbes in endophthalmitis are usually derived from the bacterial flora of the face, it is not surprising that Gram-positive bacteria are most commonly cultured in cases of postcataract endophthalmitis. In the EVS, of the 291 positive

cultures, 94.2% were Gram-positive bacteria, and among those 70% were coagulase-negative staphylococci (Han et al. 1996). *Staphylococcus aureus*, *Streptococcus* species, and *Enterococcus* species were the next most common in descending order (Han et al. 1996). Only 5.9% of the isolates were Gram-negative species. Other studies have reported a similar predominance of *Staphylococcus* as the causative organism in endophthalmitis (Recchia et al. 2005; Speaker et al. 1991).

A recent extensive study analyzed the microbiological spectrum of postcataract endophthalmitis over 11 years (from 1989 to 2000) within one institution and showed that the rate of infection by Gram-positive organisms has increased since 1995, reaching 95% (Recchia et al. 2005). Among those, the largest subgroup was coagulase-negative staphylococci, including *S. epidermidis*, *S. warneri*, *S. haemolyticus*, *S. cohnii*, *S. capitis*, *S. saccharolyticus*, and *S. homidis*. As with the EVS, the other major subgroups identified included *S. aureus*, *Enterococcus* species, *Streptococcus* species as well as isolates of *Clostridium diphteriae*. The EVS found that the visual prognosis varied significantly depending on the infecting organism. Eighty-four percent of patients infected with Gram-positive, coagulase-negative micrococci achieved a vision of 20/100 or better, while only 56% of patients with a Gram-negative infection and 14% of patients infected with enterococci achieved this level of vision (Anonymous 1996).

Antibiotic resistance of bacteria causing endophthalmitis has increased during the past decade (Recchia et al. 2005; Soriano and Nishi 2005). In particular, bacterial isolates from endophthalmitis are significantly more resistant to ciprofloxacin than in the early 1990s. A more recent study demonstrated that resistance to fourth generation fluoroquinolones such as moxifloxacin and gatifloxacin is also significantly increasing (Miller et al. 2006). This is a matter of concern because topical fluoroquinolones are commonly given as a prophylactic antibiotic in the perioperative period. Resistance to vancomycin was unchanged and it remains the intravitreal antibiotic of choice with consistent efficacy against Gram-positive bacteria (Recchia et al. 2005).

While acute endophthalmitis is most common following cataract surgery, there is also a chronic and more indolent type that occurs. In delayed-onset or chronic endophthalmitis, patients typically present months to years following cataract surgery with a mild, often steroid-responsive inflammation in the anterior chamber of the eye. Vision is usually only mildly reduced. In these patients the microbiology is somewhat different than in patients with acute endophthalmitis. *Propionibacterium acnes*, a fastidious, anaerobic Gram-positive bacillus, and coagulase-negative staphylococci are the most commonly reported agents associated with chronic postoperative endophthalmitis (Hanscom 2004; Mandelbaum and Meisler 1993; Ormerod et al. 1993; Recchia et al. 2005). Both of these microbes form colonies sequestered in between the IOL and the patient's lens capsule. In some cases, intraocular antibiotic treatment is insufficient to eradicate these organisms, particularly *P. acnes*. Resolution of endophthalmitis is often only seen after the IOL and lens capsule have been surgically removed. Uncommonly, filamentous fungi or yeast, such as *Bipolaris australiensis* (Newell et al. 2006) or *Bacidiomycete* species (Bartz-Schmidt et al. 1996), have also been isolated in patients with chronic endophthalmitis.

5 Role of Biofilms in the Progression of the Infection

5.1 IOL Materials and Adhesion

Is bacterial endophthalmitis after cataract surgery an "implant infection"? Stated differently, does the IOL play an active role in the pathogenesis of infection, or is it simply a bystander? A model for the IOL as an important participant in bacterial endophthalmitis can be constructed. Since most eukaryotic cells have evolved defenses to resist and respond to bacterial colonization, the IOL likely provides a more susceptible niche for bacterial colonization than the other surfaces in the intraocular space. For instance, gene expression of the antibacterial peptide human β-defensin 1 has been demonstrated in the human iris and lens capsule, indicating that there is production of antibacterial compounds by the tissues in the anterior chamber of the eye (Lehmann et al. 2000). Toll-like receptors (TLRs) are key players in inducing innate immune response after a bacterial infection. TRLs are expressed by a variety of tissues in the eye, but especially the cornea, where they could be involved in the production of β-defensins (Kumar and Yu 2006). Infections in a primate pseudophakia model showed that the posterior capsule acts as a barrier to spread of infection between the anterior chamber and the vitreous cavity (Beyer et al. 1983). Thus, the biotic structures within the eye are likely more resistant to bacteria than the current abiotic materials used in the manufacture of IOLs. Attachment to an IOL may provide bacteria a sanctuary in which to avoid the mechanisms that can normally clear low titers of bacteria from the intraocular space. In this way IOLs could provide a haven for bacteria to reproduce and secrete toxins until they reach a level at which they produced clinical disease. What is the evidence to support this hypothesis?

There is in vitro evidence that the most important microbes associated with both acute and chronic endopthalmitis following cataract surgery can form biofilms (see Fig. 4 for an example of a biofilm formed by *S. aureus* on silicone). *Staphylococcus* is the predominant organism in acute endopthalmitis following cataract surgery, while *P. acnes* is the most common cause of chronic endophthalmitis. In the case of *S. epidermidis*, biofilm formation is believed to be a two-step process, where the cells first attach to the surface and then to each other (Mack 1999). The first step of biofilm development consists of the attachment on a surface and is mediated by nonspecific physicochemical forces as well as the capsular polysaccharide/adhesin (PS/A) and surface proteins. The archetype of these surface proteins is the autolysin AtlE (AtlA in *S. aureus* and AtlC in *S. caprae*) (Heilmann et al. 1997). The second step consists of cell–cell interactions, and is mediated by the production of an extracellular polysaccharide matrix or slime, that includes the polysaccharide intercellular adhesin (PIA). Both PS/A and PIA are encoded by the *ica* locus present in several staphylococci (Gerke et al. 1998; McKenney et al. 1998). A study comparing slime-positive (producing) strains and slime-negative strains of *S. epidermidis* showed that the slime-negative strains can adhere to all IOL materials tested but less effectively than the slime-positive strains (Garcia-

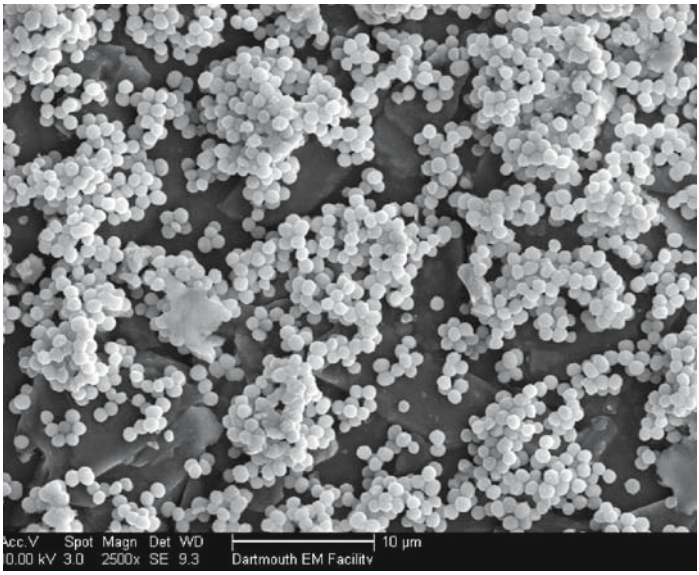

Fig. 4 Scanning electron microscopy (SEM) image of a *Staphylococcus aureus* (MZ100) biofilm on silicone elastomer (prepared by Robert Shanks (2005)). SEM by Charles P. Daghlian, Dartmouth EM facility

Saenz et al. 2000). These results also confirm previous observations showing that strains containing the *ica* locus adhere better to catheters.

The literature on *P. acnes* is less extensive. This is not surprising because *P. acnes* is a fastidious, slow-growing organism and therefore more difficult to study. Since *P. acnes* is a constituent of skin flora, it was dismissed for many years as a contaminant when isolated in cases of infection. In fact it is now recognized as being associated with chronic low-grade infections of implants at rates similar to those of *Staphylococcus* spp. *P. acnes* is mainly studied in dermatology because of its major role in acne vulgaris. This bacterium lives anaerobically in the infra infundibulum of the piolosebaceous unit where it is the predominant organism with up to 10 million microbes per unit. *P. acnes* is believed to be able to form biofilms in these pilosebaceous units, because of its tolerance to high antibiotic concentrations and its ability to produce and secrete a variety of extracellular products leading to a dermal inflammation. It colonizes and form biofilms on orthopedic implants (Ramage et al. 2003). Most endophthalmitis cases caused by *P. acnes* are believed to occur when the organisms grow anaerobically, sequestered between the posterior capsule and the IOL where it is less exposed to host defenses and antibiotics circulating in the intraocular fluids (Clark et al. 1999). Endophthalmitis caused by *P. acnes* infection is not consistently resolved by intraocular antibiotic treatment and it is often associated with recurrent inflammation after antibiotics are discontinued. Resolution often requires explantation of the IOL (Aldave et al. 1999; Ciulla 1999). All of these attributes are consistent with the classical features of a “biofilm infection.”

IOLs are formed from two different parts, the optic or actual lens, and the haptic used to secure the lens in place inside the lens capsule. In some cases, the haptics and the optics are made of different materials. IOLs can be made of silicone, PMMA, acrylic, polypropylene, or hydrogel and can be rigid or foldable. A foldable lens is desirable since it allows insertion through a smaller incision. PMMA or poly(methyl 2-methylpropenoate) is the synthetic polymer of methyl methacrylate. It is a thermoplastic and transparent plastic often used as an alternative to glass. The lenses made with this material are rigid. Acrylic lenses are foldable because they are made of a slightly different polymer called phenylethyl acrylate–phenylethyl methacrylate. This copolymer acrylic elastomer was created to meet the requirements of small-incision cataract surgery. IOLs made of silicone and hydrogel are also foldable. The haptic of the IOL can be made of polypropylene, PMMA, silicone, or a hydrogel polymer called hydroxyethyl methacrylate.

The biomaterials used in IOL manufacture have different physicochemical properties such as hydrophobicity, hydrophilicity, and surface tension (Dick et al. 2001). Such differences can effect interaction with host cells and molecules as well as bacteria and other microbes. Silicone, PMMA, and polypropylene are inherently hydrophobic polymers whereas hydrogel is a hydrophilic polymer. The case of silicone and PMMA/acrylic is interesting because these materials can be coated with various chemicals, most commonly heparin, which render them a less hydrophobic surface.

There is evidence suggesting that biofilms do form on IOLs during clinical infection, and several clinical observations demonstrated that materials used in the IOLs fabrication have an influence on bacterial colonization of the IOL and the anterior chamber. An electron microscopy study (Busin et al. 1995) analyzed 11 IOLs removed because of chronic postoperative inflammation. The authors demonstrated that bacteria were present on all removed IOLs and capsular bags they examined (Fig. 5), whereas bacteria could be cultured from aqueous fluid in only five patients. In other studies (Dilly and Sellors 1989; Menikoff et al. 1991), the authors observed that bacterial attachment on implants removed from endophthalmitis patients seemed to occur primarily on the haptics made of polypropylene, with very little on the optic made of PMMA. More recently, the risk of developing endophthalmitis, after cataract surgery, was accessed by comparing patients implanted with a silicone–polypropylene IOL and patients implanted with an all-PMMA lens. The rate of endophthalmitis was 0.16% for PMMA implants and 3.33% for silicone–polypropylene ones. This study did not examine adhesion of contaminants, but reinforced the idea that polypropylene alone, or in association with silicone, led to more postoperative endophthalmitis (Bainbridge et al. 1998). Finally, the sterility of the anterior chamber of the eye can be analyzed by culturing the aqueous fluids at the conclusion of cataract surgery. In such an experiment, Agrawal et al. (1997) reported that they could isolate bacteria from the anterior chamber of 9.5% of eyes with all-PMMA implants, whereas they identified anterior chamber contamination in 25.3% of cases in which composite lenses with polypropylene haptics and PMMA optics were inserted. These clinical data are all consistent with the suggestion the polypropylene haptics increase the risk of bacterial colonization of the IOL and

endophthalmitis. More generally they support the contention that the IOL plays a role in the pathogenesis of endophthalmitis following cataract surgery (Fig. 5).

Only a few animal models have been used so far to study bacterial endophthalmitis. Most of the reports are based on primate or rabbit models, and only recently have we seen the domestic pig model developed (Kodjikian et al. 2002). In a rabbit model of chronic *P. acnes* endophthalmitis, where the same number of bacteria was inoculated (2.5×10^6), the presence of an IOL led to a more intense and prolonged inflammation than an eye operated without implant of an IOL (Nobe et al. 1987). This animal study supports the hypothesis that the presence of an IOL favors the development of a chronic *P.-acnes*-related inflammation.

The pig is believed to be a good model to study endophthalmitis, because the eye volume and aqueous/vitreous composition are similar in pigs and humans. In a study published in 2002 (Kodjikian et al. 2002), researchers showed that adhesion of *S. epidermidis* on IOLs ranges in increasing order from hydrogel, fluorine PMMA, hydrophobic acrylic, heparinized PMMA to silicone. At 24 h after implantation, the heparin-surface-modified PMMA and silicone lenses contaminated with *S. epidermidis* showed a significant level of bacterial adhesion, which increased until the end of the study (1 week after implantation). The acrylic lenses showed a similar attachment early in the experiment, but through time this colonization decreased. On the contrary, hydrogel and fluorine PMMA lenses similarly showed a very weak degree of attachment, even after 1 week. This interesting study suggests that *S. epidermidis* are less likely to adhere to hydrophilic materials such as hydrogel or to very hydrophobic material such as fluorine PMMA (Kodjikian et al. 2002).

As described earlier, some clinical and animal studies have provided data describing the relationship between IOL biomaterial and biofilm formation; however, the bulk of our knowledge on this subject has been derived from in vitro studies. In such studies, *S. epidermidis* is more likely to attach to moderately hydrophobic surfaces, such as silicone or PMMA (Kodjikian et al. 2003; Ng et al. 1996; Schmidt et al. 1998). In 1989, a comparison between the haptic of an IOL made of polypropylene and the optic made of PMMA (Dilly and Sellors 1989) showed that *S. epidermidis* adheres more to polypropylene than to PMMA, reinforcing many of the already mentioned data. On the contrary, hydrophilic surfaces such as hydrogel allow very little attachment in vitro. Electron microscopy observations showed that the type of attachment on hydrophobic surfaces differs from that on hydrophilic ones. On hydrophobic surfaces, the bacteria form cell clusters or microcolonies, whereas the few visible cells on hydrophilic surfaces were all individually attached (Kodjikian et al. 2003). The attachment in cells clusters is typical to biofilm formation.

Gabriel et al. (Gabriel et al. 1998) showed that Gram-negative *P. aeruginosa* behaves in a way similar to *S. epidermidis*, in that it attaches better to silicone lenses than to PMMA or hydrophobic acrylic IOLs. Nevertheless, there does not seem to be a perfect lens material to avoid infections by Gram-positive bacteria. Indeed, Kobayakawa et al. (2005) showed that *E. faecalis* forms more biofilm on PMMA and acrylic lenses than on silicone lenses. Work continues toward development of IOL materials that reduce the incidence of infection by multiple bacteria, while also maintaining desirable optical and surgical properties. Such infection-resistant IOLs might

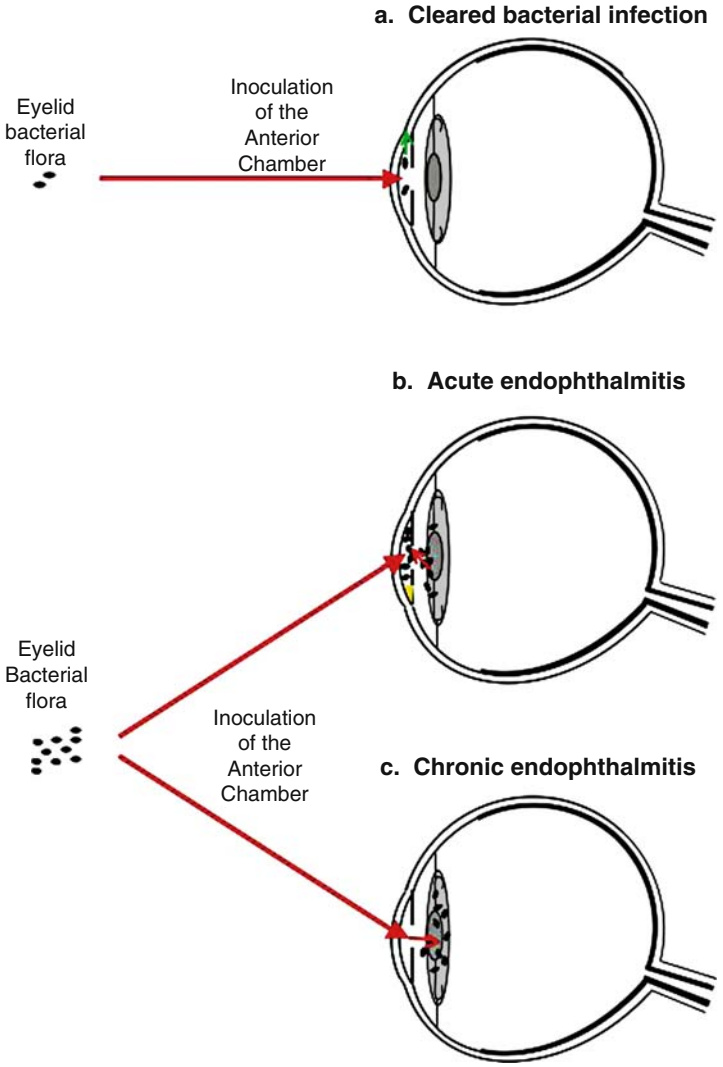

Fig. 5 Model for bacterial endophthalmitis. (**a**) Cleared bacterial infection. The skin flora (small black ovals) enters the anterior chamber during surgery (thick red arrow). The microorganisms are cleared via the trabeculum meshwork and canal of Schlemm (green arrow). No clinical infection is noted. (**b**) Acute endophthalmitis. The skin flora enters the anterior chamber in large numbers (thick red arrow), colonizes the posterior chamber and the capsular bag. Bacteria attach to the IOL. Some detach, return to the anterior chamber (thin red arrow) and are too numerous to be cleared by the trabeculum meshwork. This infection leads to inflammation causing an accumulation of white blood cells in the anterior chamber known as a hypopyon (yellow) and is clincially apparent. (**c**) Chronic endophthalmitis. The skin flora enters the anterior chamber (thick red arrow). Bacteria colonize the posterior chamber and capsular bag and then attach to IOL (thin red arrow). The bacteria are sequestered in the capsular bag and IOL. Little detachment from the IOL occurs leading to mild inflammation persisting months to years after the cataract surgery

be of particular importance in settings where infection rates are known to be higher, such as surgery in developing countries and elderly patients (West et al. 2005).

5.2 Bacterial Adhesion Factors

The initial attachment of most described bacteria in this text to a biomaterial is not fully understood, but it is believed that the physicochemical properties of both the material and the bacterial cell surface have an important role. The bacterial cell surfaces most frequently carry a net negative surface charge and are hydrophobic, which makes them more likely to adhere to hydrophobic surfaces.

In *S. aureus*, the teichoic acids present in the cell wall are essential for the initial steps of biofilm formation. A bacterium lacking these polymers has a higher negative charge, which renders the attachment to most materials extremely difficult (Gross et al. 2001). In several staphylococci, the cell wall contains the autolysins able to affect the hydrophobicity of the cell surface (Allignet et al. 2001; Heilmann et al. 1997, 2003). In mutants lacking these autolysins, the bacterial cells are less hydrophobic, do not attach well on hydrophobic materials, but attach better than the wild type on hydrophilic materials such as glass. Finally, complex surface polymers and modified membrane lipids, such as lysylphosphatidylglycerol, play an important function in the attachment process of *S. aureus* on biomaterials (Fedtke et al. 2004). These findings suggest that the ability of a bacterial cell to attach on a biomaterial depends on a sensitive equilibrium between the physicochemical properties of the material and the cell surface and especially the degree of hydrophobicity of both.

5.3 Toxins

Endophthalmitis caused by *S. aureus* often leads to a massive inflammation. This bacterium can secrete various extracellular enzymes and pore-forming toxins considered as virulence factors (Booth et al. 1995, 1997). This bacterium also secretes inflammatory factors that play a role in pathogenesis and the host inflammatory response (Coia et al. 1992). These virulence factors are controlled by two regulatory systems that function at the transcriptional level, the accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sar*) (Balaban and Novick 1995; Cheung et al. 1992; Recsei et al. 1986). It is interesting to note that these two regulatory systems interact with each other. An eye infected with a wild-type strain of *S. aureus* shows a stronger inflammation and retinal decline than does an eye infected with a strain containing a mutation in *agr* (Booth et al. 1995). Eyes infected with a *sar* mutant present no difference when compared with those infected with a wild-type strain, but when the eye is infected with a double mutant *sar–agr*, one can observe an almost complete attenuation of virulence (Booth et al. 1997).

Homologues of *agr* and *sar* can be found in *S. epidermidis*, and likely serve a similar role in regulating the virulence of this bacterium.

S. aureus produces three hemolysins: α-toxin, β-toxin, and γ-hemolysin. α-Toxin has been shown to be the main virulence factor in corneal epithelial tissue destruction during keratitis (Callegan et al. 1994). Alpha- and β-toxins are believed to play an active role in the loss of retinal function and destruction of the retinal tissue during endophthalmitis. γ-Hemolysin does not seem to play an active role by itself (Supersac et al. 1998), but a cumulative effect of the lack of all three hemolysins can be observed experimentally (Callegan et al. 2002). Finally, the Gram-positive cell wall can be considered as an unusual toxin. The cell wall and its components can lead to profound inflammation. Peptidoglycan is highly inflammatory in the vitreous, leading to a reaction similar to that triggered by the Gram-negative lipopolysaccharide (Howes et al. 1994), i.e., a rapid influx of inflammatory cells into the posterior segment.

Studies of the pathogenesis of *S. epidermidis* endophthalmitis are largely limited to the examination of the importance of various adhesins to biofilm formation on IOL material, and in the absence of significant toxin production, biofilm formation may be a main virulence trait for this organism (Baillif et al. 2006). *S. epidermidis* biofilm formation involves an autolysin, polysaccharide intercellular adhesin, fibrinogen-binding proteins, and accumulation-associated protein (Foster 2005). Its ability to form biofilms on IOLs allows it to evade immune clearance and limits the effectiveness of antibiotics (Baillif et al. 2006).

Although not the most common cause of endophthalmitis, *Enterococcus faecalis* is responsible for some of the most difficult to treat cases of postoperative endophthalmitis (Anonymous 1996), and because it produces relatively fewer toxins, it has proven to be an important model organism for studying the pathogenesis of endophthalmitis. This bacterium often contains in its genome a conjugative plasmid or pathogenicity island which encodes a cytolysin able to lyse eukaryotic as well as prokaryotic cells (Gilmore et al. 1999). *E. faecalis* isolates from endophthalmitis often show an enrichment for the cytolysin (Booth et al. 1998). Cytolysin-producing *E. faecalis* experimentally cause faster destructive changes in retinal structure and function (Jett et al. 1992). These bacteria are also more likely to lead to treatment failures (Jett et al. 1995). Animal experiments have indicated that concurrent steroid treatment can significantly improve the visual outcome in these models of endophthalmitis, suggesting that immune response to enterococcal infection plays a significant role in its pathogenesis (Jett et al. 1995). *E. faecalis* also possesses a quorum sensing system, *fsr*, which regulates production of gelatinase (GelE) and a serine protease (SprE) in a density-dependent manner, and potentially additional factors that contribute to the severity of endophthalmitis (Mylonakis et al. 2002). Deletions of *gelE*, *sprE*, or both, attenuated the severity of endophthalmitis, with the greatest contribution being provided by the *gelE* protease. However, an *fsr*-deficient mutant was found to be slightly more attenuated, suggesting that other factors may be controlled by this system as well (Mylonakis et al. 2002). As noted earlier, enterococci produce biofilms on IOL material (Kobayakawa et al. 2005). Auxiliary factors, including a surface protein, Esp, have been found to enhance biofilm formation by this organism (Tendolkar et al. 2004).

Streptococcus pneumoniae has been isolated in association with postoperative endophthalmitis. Two virulence traits of this organism, pneumolysin and autolysin, have been examined for their role in the pathogenesis of intraocular infection (Ng et al. 2002). In a rat model of endophthalmitis, infection with a pneumolysin-deficient strain resulted in a transient, but not lasting, reduction in intraocular inflammation. However, a mutant defective in production of the cell-wall-degrading autolysin was highly attenuated, highlighting the role of released cell wall debris in the pathogenesis of this infection (Ng et al. 2002).

6 Host Response in Endophthalmitis

The immune response of the eye is delicately balanced to limit inflammation and maintain a clear visual path, while affording some protection to infection. As a result, it is known as an immune-privileged tissue. This balance evolved well before the era of surgical interventions that routinely breach the integrity of the globe. The immune privilege limits the ability of the eye to respond to bacterial challenge. About 5×10^5 cfu of *S. aureus* are rapidly cleared in mice when injected subcutaneously (Hoebe et al. 2005), whereas injection into the vitreous of as few as 5×10^3 cfu routinely expand to 2×10^9 cfu within 72 h, resulting in complete destruction of the eye (Engelbert and Gilmore 2005; reviewed in Leid et al. 2002).

Cell surface (e.g., Fas ligand) and soluble factors (TGF-β, α-melanocyte-stimulating hormone, vasoactive intestinal peptide, calcitonin gene-related peptide) contribute to immune privilege (Streilein 2003). However, despite this privilege, endophthalmitis is characterized by inflammation to various degrees, depending upon the pathogen involved. As noted earlier, endophthalmitis may be acute or chronic, and clinical distinction is typically based on the extent of inflammation. Acute postsurgical endophthalmitis is usually associated with organisms capable of rapid growth and production of extracellular virulence traits, including *S. aureus*, *E. faecalis*, streptococci, and Gram-negative organisms, and is typically associated with poor visual outcome (Callegan et al. 2002). As quickly as 48 h following surgery, both the anterior and posterior segments of the eye may be involved, resulting in corneal edema, neutrophil infiltration into the aqueous humor, and vitritis (Mandelbaum 1996). Chronic inflammation is associated with slower growing, less virulent organisms, including *Proprionibacterium acnes* and *S. epidermidis,* and is often associated with a better visual outcome (Mandelbaum 1996).

In acute, postsurgical endophthalmitis, the innate arm of the immune system is mainly responsible for mounting a response. The relative roles of innate vs. adaptive arms of the immune response in delayed onset or chronic endophthalmitis have not been explored. It is now understood that a main pathway for triggering the innate response is through a series of TLRs on the surface of host cells of many types (Tosi 2005). TLRs recognize specific repeated bacterial molecular patterns, such as those contained in LPS or peptidoglycan. At least ten human TLRs have been identified, and several are specific for bacterial ligands (Tosi 2005).

Peptidoglycan and lipoteichoic acid (mainly from Gram-positive bacteria) are ligands for TLR-2; LPS from Gram-negative bacteria is a ligand for TLR-4; bacterial flagellin stimulates TLR-5; and CpG in bacterial DNA signals through TLR-9. TLR-2, TLR-4, and TLR-9 are expressed by cells within the retina, and may trigger the inflammatory response in the posterior segment of the eye (Kumar et al. 2004). Signaling through TLRs typically results in production of proinflammatory cytokines and chemokines, and upregulation of adhesion molecules on the cell surface (Kumar et al. 2004).

Bacterial cell wall components, including peptidoglycan and LPS, directly activate the complement system via the alternative pathway (Tosi 2005). Activation of the complement system triggers inflammation, attracts phagocytes to the site of infection via the release of powerful chemotoxins, promotes the opsinization and in some cases direct killing of bacteria, and promotes vasodilation and increased vascular permeability (Tosi 2005). Complement components are present at reduced levels within the eye (Engelbert and Gilmore 2005), but the activation of complement is limited by complement regulatory proteins CD55, CD46, CD59, and Crry (Sohn et al. 2000). Using C3$^{-/-}$ mice, it was found that complement played a nominal role in determining the outcome of *S. aureus* endophthalmitis (Engelbert and Gilmore 2005). These data suggested that although complement may contribute modestly to the early inflammatory response to *S. aureus* in the eye, it did not affect outcome.

In contrast to complement, Fas ligand, which is constitutively expressed within the normal eye and plays a critical role in maintaining the immune privilege (Griffith et al. 1995), was found to be important for mediating the clearance of *S. aureus* in C57B6 mice (Engelbert and Gilmore 2005). Although it was anticipated that C57B6 *gld* mice, defective in Fas ligand, might be more resistant to infection because of reduced immune privilege, the opposite was found. Mice defective in FasL expression were more vulnerable to infection and unable to clear an inoculum of 500 cfu from the vitreous, a dose routinely cleared by wild-type mice. It has been observed that the membrane form of FasL can also promote innate-mediated inflammation through the activation of neutrophils (Gregory et al. 2002), and this appears to be the activity that is most relevant in endophthalmitis.

A neutrophilic infiltrate is the main response in acute endophthalmitis (Callegan et al. 2002; Giese et al. 2003; Ramadan et al. 2006), although the cells that mediate the response in chronic endophthalmitis are yet to be quantified. Depletion of neutrophils in experimental *S. aureus* endophthalmitis transiently reduces the severity of host inflammation, but leads to the outgrowth of bacteria (Giese et al. 2003). From this it is clear that neutrophils play an important role in amplifying and perpetuating the inflammatory response, likely through cytokine and chemokine production. Within 6 h of intravitreal inoculation with *S. aureus*, TNF-α, IL-1β, and the rat homologue of IL-8 are detected within the vitreous (Giese et al. 1998). The adhesion molecules ICAM-1 and E-selectin are also upregulated early in iris, ciliary body, and retinal vessels, enhancing the infiltration of leukocytes to the site of infection (Giese et al. 2000). In the rat model of *S. aureus* endophthalmitis, IFN-γ

peaks at 24 h postinfection, correlating with an increased infiltration of macrophages and lymphocytes (Giese et al. 1998).

7 Treatment

The goal in the treatment of bacterial endophthalmitis, following cataract surgery, whether chronic or acute, is to eliminate the infection and minimize the destructive features of the host inflammatory response. However, the approach to treatment of acute and chronic endophthalmitis differs in important respects.

7.1 Acute Bacterial Endophthalmitis

In acute endophthalmitis, the infection is rapidly progressive and destructive; consequently, broad-spectrum treatment has to proceed before the results of an intraocular culture are available (Fig. 5). Thus, once a patient is suspected of having endophthalmitis, intraocular cultures are obtained and broad-spectrum antibiotics are then injected into the posterior chamber of the eye. Vancomycin and ceftazidime are commonly used to provide coverage of both Gram-positive and Gram-negative bacteria (Ciulla 1999). While intraocular cultures can be important in identifying and guiding the treatment of rare forms of postsurgical endophthalmitis, such as fungal infections, in most cases the clinical outcome is apparent by the time the results of the cultures and sensitivities are available. Patients are assessed daily and depending on the clinical progress and culture results consideration is given to reinjection of antibiotics beginning usually at 2 days.

The Endophthalmitis Vitrectomy Study (EVS) was conducted in the 1990s and addressed several contentious issues in the treatment of bacterial endophthalmitis (listed 1995). Prior to this study there were strong proponents for the use of intravenous antibiotics and pars plana vitrectomy for the treatment of endophthalmitis. The EVS addressed both these suggestions. A pars plana vitrectomy is a surgical procedure in which the vitreous gel is removed via instruments inserted through the pars plana of the eye. Advocates of this approach in endophthalmitis argued that removal of the vitreous during acute infection would result in more rapid clearance of bacteria and their toxins and antigens, as well as providing more uniform distribution of antibiotics throughout the eye.

In the EVS, 420 patients who presented with endopthalmitis within 6 weeks of cataract surgery were studied (listed 1995). Vitreous cultures were obtained from all patients and all patients received intravitreal injections of vancomycin and ceftazidime. Patients were then randomized to receive no systemic therapy or intravenous ceftazidime and amikacin, as well as no vitrectomy or pars plana vitrectomy.

The EVS found no benefit of using intravenous ceftazidime and amikacin in the treatment of endophthalmitis and found pars plana vitrectomy valuable only in patients presenting with visual acuity of light perception or worse (listed 1995). These results have significantly streamlined the treatment of endophthalmitis because most patients can receive all their treatment in the outpatient setting (74% of EVS patients had visual acuity better than light perception at presentation and thus would not benefit from vitrectomy). This less-complicated approach had the added benefit of reducing the time to antibiotic treatment, as patients did not have to wait for consultation and surgery with a posterior segment surgeon.

An issue not addressed by the EVS is the role of intraocular corticosteroids in the treatment of bacterial endophthalmitis. Corticosteroids have been established as adjunctive to antibiotics in a variety of infections ranging from bacterial meningitis in children (Williams and Nadel 2001) to tuberculous pericarditis (Dooley et al. 1997). The suggestion is that the broad anti-inflammatory and immunosuppressive effects of corticosteroids will prevent immune-mediated pathology associated with the infection. Of course, inhibition of the immune response with corticosteroids raises fears among clinicians that the steroids will also reduce the ability of the patient to clear the pathogen and may lead to prolonged and recurrent infections that are ultimately more destructive.

There is evidence from specific animal models that corticosteroids can have a benefit in the retention of retinal function when given in conjunction with antibiotics in the treatment of certain experimental infections, due to Gram-positive, as well as Gram-negative bacteria or fungi (Coats and Peyman 1992; Graham and Peyman 1974; Jett et al. 1995; Meredith et al. 1990; Park et al. 1995). However, the clinical literature has been less clear. A small prospective study by Gan and colleagues showed a tendency toward a benefit from treatment with intravitreal dexamethosone (Gan et al. 2005), whereas Shah's larger but retrospective study did not show any benefit (Shah et al. 2000). In the largest, prospective, randomized study to address this question that we are aware of, Das et al. (1999) found a reduction in early inflammation, but no overall effect on visual outcome associated with the use of intravitreal dexamethasone in patients with endophthalmitis. Because of these contradictory results, the decision of whether or not to use intravitreal corticosteroids in the treatment of bacterial endophthalmitis is usually based on the individual experiences and biases of the treating ophthalmologist.

7.2 Chronic Bacterial Endophthalmitis Following Cataract Surgery

The priorities in the treatment of chronic endophthalmitis following cataract surgery differ from those in the treatment of acute endophthalmitis. As stated earlier, because of the rapidly destructive nature of the condition, acute endophthalmitis must be treated immediately with broad-spectrum agents. This is usually an effective strategy because the vast majority of cases are caused by Gram-positive organ-

isms sensitive to vancomycin (Han et al. 1996). In chronic endophthalmitis the differential diagnosis is more broad, the potential for severe visual loss less likely, and the progression of the disease less rapid. All of these factors combine to make obtaining an accurate diagnosis a high priority in the treatment of chronic uveitis.

Chronic endophthalmitis following cataract surgery presents with mild intraocular inflammation months to years after the cataract is removed (Aldave et al. 1999; Ciulla 1999). It can be partially or completely responsive to topical corticosteroids. The differential diagnosis for this condition is extensive and can include a retained fragment of the crystalline lens, retained surgical material such as a suture or fiber, new presentation of uveitis not associated with the cataract surgery, inflammation stimulated by the IOL materials, and of course infection (Fig. 5). The noninfectious causes must be considered and excluded if possible.

P. acnes and coagulase-negative *Staphylococcus* are the most frequent infectious etiologies of chronic endophthalmitis (Recchia et al. 2005), but fungi are sometimes isolated. The nidus of infection is often located between the capsular bag and the IOL, sometimes producing a classic white plaque. Culturing these organisms is challenging because the number of bacteria circulating in the fluids of the eye is usually quite small. In addition, *P. acnes* is a slow-growing organism and special protocols specific for *P. acnes* culture should be used. In general, isolates of *P. acnes* can be grown on sheep blood agar anaerobically when incubated at 35°C for 48 h (Dali et al. 2001). Because the bacteria are often sequestered in between the IOL and the capsular bag, antibiotics injected into the eye may not reach these microbes in therapeutic concentrations. Additionally, growth in a biofilm may render these organisms more resistant to antibiotics than planktonic bacteria. This can lead to recurrent infections following seemingly effective treatment with intraocular antibiotics. In some cases, all or part of the capsular bag and the IOL must be surgically removed to cure the patient. Vitrectomy is also commonly combined with these procedures.

With these considerations in mind, a common pattern of treatment for a patient with suspected chronic bacterial endophthalmitis following cataract surgery would proceed as follows. Noninfectious causes of chronic endophthalmitis would be considered and excluded. A decision to obtain intraocular fluids would be made. A sample each of the aqueous and vitreous fluids is obtained for culture. Vancomycin is typically injected after the culture is obtained since it is active against both *P. acnes* and coagulase-negative *Staphylococcus*. If the patient is cured no further treatment is warranted. If inflammation recurs, consideration should be given to a repeat antibiotic therapy or surgical removal of the capsular bag and IOL, or both. Studies suggest that 50% or more of patients with *P. acnes* infection require only antibiotics to alleviate their infection (Aldave et al. 1999; Clark et al. 1999). Those not responding to antibiotic therapy generally respond to surgical removal of the lens capsule and IOL. In patients infected with coagulase-negative *Staphylococcus*, antibiotic treatment alone is usually adequate (Ciulla 1999).

8 Conclusion

IOLs play a role in the pathogenesis of bacterial endophthalmitis following cataract surgery. In the case of chronic endophthalmitis, the role seems clear as removal of the IOL is often necessary to cure the infection. This infection also displays many of the classic features of biofilm infections in that it is a chronic, recurrent infection that is poorly responsive to antibiotics and for which it is difficult to isolate the organism. In acute endophthalmitis, the picture is somewhat different. The IOL seems to provide a niche where bacteria can attach, divide, and secrete toxins and antigens relatively protected from the anti microbial defenses of the eye. However, after an adequate titer of organisms is reached, an acute infection does develop. A biofilm on an IOL in this case does not lead to chronic infection, but rather allows bacteria to survive long enough to grow to numbers sufficient to cause acute disease. Development of IOLs that broadly resist bacterial attachment will be challenging. As discussed earlier, materials that resist one microbe, may favor the attachment of another. Attention to *Staphylococcus* species is logical, since it represents the majority of postoperative infections. Such IOLs could be particularly significant in the developing countries that contain the majority of patients awaiting cataract surgery and where infection rates tend to be higher.

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Use of Immunodiagnostics for the Early Detection of Biofilm Infections

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Abstract Biofilm infections are a therapeutic and diagnostic challenge due to their location and persistence. Specimen culturing is the golden standard for diagnosing bacterial infections, but recent investigation of the ability of this technique to grow bacteria from a biofilm has indicated that it is not reliable. Other testing modalities, such as PCR and serology assays, are either nonspecific for biofilm infections or they include the risk of contamination during sampling. Therefore, accurate diagnosis of an infection usually takes days and requires extensive test procedures, leading to increased healthcare costs and discomfort to the patient. In recent years, many attempts have been performed to design and set up new serology diagnostic assays to obtain early, noninvasive diagnosis of infections sustained by biofilms colonizing native tissues and medical implants. This chapter describes such attempts, including some examples of tests that could eliminate many of the obstacles related to early diagnosis in biofilm infection. The new tools could also allow new medical and surgical approaches to monitor and treat biofilm infections associated with many medical implants.

1 Diagnosing Infections by Antibodies to Biofilm-Specific Epitopes

One of the main features of infections resulting from biofilm colonization of implanted medical devices is the persistent absence or paucity of local signs and general symptoms of infection/inflammation. Vascular graft infections usually remain asymptomatic for long periods; infections involving bone and joint prostheses can be associated with loosening of the device and local pain but, as we have seen, both of these are nonspecific signs that are of no use in differentiating septic inflammation from aseptic forms. Biofilm colonization of cardiac valves is often

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misdiagnosed, because signs of the infection appear long after dislocation of the ring sustaining the biologic valve has caused signs of regurgitation. Biofilms that form on indwelling vascular catheters can spread metastatic emboli to distant tissues without producing any signs of infection. On the whole, the absence of local and systemic symptoms is typical of chronic infections associated with biofilm colonization: it shows no correlation with the specific biomaterial used to manufacture the device or the anatomic district in which it is implanted.

The first signs of these infections are caused by dysfunction of the device itself (loosening of orthopedic prostheses, cardiac valve regurgitation) and device-related damage to the surrounding tissues (perforation of the gut wall with peritonitis and gastrointestinal hemorrhage in patients with aortic grafts, inflammation surrounding indwelling devices, etc.). *Calor, dolor, rubor, et tumor* – heat, pain, redness, and swelling, the four signs of inflammation described by the Roman encyclopedist Celsus in the first century A.D. – are undoubtedly classic, but they are also nonspecific. They may reflect the activation of an immune response to the microorganisms colonizing an implanted medical device and/or a host reaction to the simple presence of the device itself, as a foreign body.

In infections caused by the formation of biofilms on implanted devices, the immune/inflammatory response is incompletely activated or partially suppressed. Humans (and other complex eukaryotic organisms) have adapted to the constant presence of saprophytic biofilms on various mucosal surfaces within their bodies. The suppression of defensive mechanisms in these cases is part of a strategy that allows complex organisms to exploit the variegated functional capacities of microorganisms in order to carry out a myriad of specific tasks within the body (e.g., enzymatic digestion in the gut, biological barrier protection in mucous membranes and skin, etc.). A similar strategy was frequently adopted by Julius Caesar: by entrusting the government of newly conquered territories to local chiefs and preserving their status as leaders, he transformed potential enemies into faithful defenders of Rome's ever-expanding boundaries.

The human body can thus be envisioned as a complex ecosystem in which the maintenance of homeostasis depends on constant, reciprocal exchange and interaction between human and other prokaryotic (bacterial) and eukaryotic cells (fungus), and the acceptance of diversity and complexity is fundamental to the preservation of integrity. The system functions smoothly as long as each of the commensal populations remains in its own well-defined territory, but border violations are by no means uncommon. An implanted medical device serves as a "Trojan horse" for microorganisms, a vehicle that allows them to penetrate and colonize the *sancta sanctorum* of the human body, areas that are off-limits to their likes.

In this view, the body's defense systems, which are trained to tolerate the presence of biofilms formed by commensals in specific areas of the body, may also tolerate biofilms that form on medical devices, even when these devices are located within zones that are normally sterile. The mechanisms responsible for amplifying the immune/inflammatory response somehow fail to activate, and the clinical correlation of this misplaced tolerance is the absence of overt clinical signs of infection: fever and chills, leukocytosis, and anemia. Nevertheless, the presence of biofilm-forming

bacteria in areas of the body that are ordinarily sterile is indeed noted by the immune system, which responds with an incomplete local inflammatory reaction.

Prompt detection of artificial graft infections in the initial asymptomatic phases would allow earlier medical/surgical treatment, which should be associated with appreciable improvements in the prognosis and dramatic reductions in the health-care costs related to the use of implantable medical devices. In this chapter, we provide a critical review of some of the most important approaches that have been used for this purpose, including those based on imaging technology and others involving serological techniques. Particular attention will be focused on the efforts of our group and others to develop simple and reliable immunodiagnostic assays capable of revealing the faint but specific traces left by sessile bacteria on the immune response of the patient.

2 Diagnosing Biofilm Infections

Investigation of suspected infections is currently accomplished through a combination of diagnostic testing methods, including blood tests, microscopy, histology, optical studies, and culturing. Definitive diagnosis usually relies on several different tests with agreeable results as well as consistent repetition (i.e., two or three consecutive cultures identifying the same microorganism as the source). Some of these testing methods are described later. Biofilm infections on implanted medical devices can be divided into two groups: low-grade infections (indolent and subclinical) and those that are associated with clinical manifestations from the outset. The difference is related in part to the bacterial species involved.

Highly virulent organisms (e.g., *Staphylococcus aureus* and Gram-negative bacilli) are generally involved in infections characterized by the early appearance of symptoms, which is related to the production of toxins and virulence factors and to the tendency of these infections to spread to distant sites by means of septic metastasis (Yarwood and Schlievert 2003). Patients with high-grade infections involving orthopedic prostheses generally experience fever, persistent local pain, erythema, edema, massive hematomas, and disturbances of the wound-repair process. The pain is often particularly severe when the infection is caused by *Pseudomonas aeruginosa* (personal data), and this may be a reflection of the strikingly large number of proteases, leukocidins, and other virulence factors encoded in this pathogen's extensive genome. Device-related infections in this category are usually suspected early and rapidly treated.

Other bacteria that are less virulent (e.g., coagulase-negative staphylococci) are usually the cause of low-grade, indolent infections. These infections can remain clinically silent for years while bacteria slowly accumulate on the device. When the biofilm reaches maturity, planktonic cells detach from its surface and spread throughout the body, inducing a complete inflammatory reaction related to sepsis. However, exceptions to this rule are not infrequent. Late-onset infections (arbitrarily defined as those appearing six months or more after device implantation) can be

caused by highly virulent bacterial species, such as *S. aureus* (personal data). These infections are probably caused by hematogenous spreading from remote infection sites rather than by intraoperative contamination.

The difficulties encountered by clinicians in diagnosing infections due to bacterial colonization of implanted medical devices can be summed up in the following points.

Nonspecific Blood Tests. Several laboratory tests are used to detect inflammation and possible infection by a bacterial pathogen. These tests, however, are not specific for the presence of bacteria and can be misleading when interpreted incorrectly. One of the most common tests used to indicate inflammation is the level of C-reactive protein (CRP). CRP is elevated during systemic inflammation and can be seen in post-operative patients. Shortly after surgery, patient CRP levels rise but eventually return to normal. A second rise in CRP levels can indicate a post-operative infection, but again this is nonspecific for a pathogenic microorganism (Patel et al. 2005; Trampuz and Zimmerli 2006). Complete blood counts (CBCs) are commonly used to monitor hematological homeostasis and inflammation and are more specific for an invading pathogen than other tests. Differential elevation of certain leukocytes can suggest the presence of bacteria or viruses but cannot accurately specify what pathogen, if any, is causing the inflammation. Another indicator of inflammation is an elevation in the erythrocyte sedimentation rate (ESR). These tests, while nonspecific, are useful indicators for further work-up and are performed frequently because of their fast processing times and comparatively inexpensive costs (Table 1).

2.1 Diagnostic Imaging

The imaging modalities that have been used to detect and study device-related infections include plain-film radiology, computed tomography (CT), magnetic resonance imaging (MRI), endoscopy, angiography, ultrasound, and scintigraphy performed with labeled leukocytes or anti-neutrophil antibodies. In terms of safety, cost, and availability, plain-film radiology and ultrasonography are the only two imaging approaches that might be used for routine screening purposes. The real

Table 1 Summary of current diagnostic techniques used in healthcare facilities with their relative sensitivity, specificity, and processing time

	Sensitivity	Specificity	Processing Time
WBC, CRP	High	Low	< 1 h
Culturing	Low	High	24–30 h
Molecular (PCR)	High	High	13 h
Imaging (MRI, CT, Radiography)	Varies	Low	Hours
Microscopy (IFM, FISH)	High	Moderate	1–3 h
Serology (ELISA)	High	High	4–6 h

problem, however, is that their success in the detection of infections related to medical implants depends on the presence of substantial morphological changes in the infected area, such as the formation of an abscess or the erosion of bone tissue. And, with the exception of scintigraphy, the same limitation applies to all of the other imaging modalities listed above, which are also more expensive and invasive than conventional radiology and ultrasound. Consequently, these approaches are useful only in the advanced phases of the infection, when the tissues have already been damaged by bacterial enzymes and by the inflammatory reaction.

The scintigraphic approach is considered the gold standard for imaging diagnosis of biofilm infections involving implanted medical devices. It is based on the acquisition of functional data related to accumulation of neutrophils at the site of infection. The use of radioactive tracers enhances visualization even of small lesions, making it an extremely sensitive method. Unfortunately, however, it cannot distinguish between septic and aseptic inflammatory processes. Apart from the problem of low specificity, the scintigraphic approach is far too invasive, toxic, and expensive for routine use. Ultrasonography and plain-film radiology are cheep and safe, but they do not provide information in the early phases of biofilm infections.

In short, there is currently no imaging modality that meets the requisites for use as a routine tool for monitoring patients at risk for infections related to implanted medical devices. Additionally, none of the techniques described earlier actually result in identification of the genus or species of the microorganism causing the fouling of the device. Therefore, clinical treatment is at best a guess after such diagnostic approaches are employed.

3 Conventional Microbiology

A fundamental step in the laboratory diagnosis of any microbial infection is the collection of samples of biological fluids, tissues, or infected biomaterials for microbiologic analyses aimed at identifying and characterizing the causative organism at the species level. Intravenous catheters and endotracheal tubes can be removed fairly easily for culture. For more complex devices (artificial heart valves, vascular grafts, artificial joints, etc.), removal is much more complex, invasive, and expensive. In the absence of external fistulization, noninvasive collection of purulent material from deep infections is not possible, and in any case, with conventional microbiological techniques, it is difficult to culture bacteria from biofilm clusters contained in such material. New methods for detecting biomaterial colonization without withdrawal of the device are currently under investigation (Bouza et al. 2002).

An alternative to cultures of the device itself involves the use of multiple blood cultures. This approach has several drawbacks. For one thing, false-negative results can emerge when antibiotic treatment has been administered prior to blood sampling. However, the main problem is that the bacterial species most commonly responsible for biofilm infections on medical implants are not pathogens, as defined

by Koch's criteria: they are members of the human commensal flora. Consequently, the significance of their isolation from blood cultures may be difficult to determine. Coagulase-negative staphylococci are responsible for 70–80% of biofilm infections, but they also account for well over half of the contaminants (58–83%) found in positive blood cultures (Rupp and Archer 1994). For the same reason, positive titers of specific antibodies for the planktonic form of most species causing device-related infections are diagnostically irrelevant.

In summary, all the techniques used for decades to identify the etiologic agent of acute infections owe their good performance to the fact that they have been used for strict pathogens or bacteria displaying strict organ pathogenicity (e.g., *Brucella* or *Borrelia*). Our expanding knowledge of biofilm-related infections has broadened our view of possible infectious agents and created a strong demand for new diagnostic attitudes and techniques that overcome the main limitation for detection of biofilm infections; by definition, these communities of microorganisms are attached to a surface and not readily sampled by common diagnostic techniques.

A good alternative involves the use of immunodiagnostic assays for the detection of antibodies directed against antigens that are specific for the biofilm form of bacterial species, especially those that are traditionally considered saprophytes. Since staphylococci are one of the main causes of biofilm infections involving implanted medical devices, the first attempts have been devoted to the diagnosis of staphylococcal biofilm infections. Distinguishing pathogenic *S. epidermidis* and *S. aureus* strains from contaminant/commensal strains is one of the major challenges in clinical microbiology laboratories.

The ideal immunodiagnostic test for diagnosis of staphylococcal biofilm infections should have as many of the following characteristics as possible:

- Broad-spectrum specificity for all Staphylococcal species
- Capacity to detect infections while they are still in the very early phase (in particular, before the appearance of clinical signs and symptoms)
- Ability to differentiate between active and past infections
- Suitability for use during the follow-up after replacement of infected graft
- Low cost and simple execution
- Noninvasiveness
- Speed of obtaining results

Enzyme-linked immunosorbent assays (ELISA) can satisfy many of these prerequisites. They are simple, rapid, and repeatable, and they do not require removal of the implanted device. Furthermore, immunoenzymatic assays of antibodies to *S. aureus* antigens can identify cases of *S. aureus* bacteremia associated with false-negative blood cultures due to prior antibiotic treatment (Brakstad et al. 1989; Christensson et al. 1985; Ryding et al. 2002).

Another serological modality that fits the aforementioned criteria and can be explored for use in biofilm-specific detection is the lateral flow assay (LFA). An LFA has the potential to be as sensitive and specific as ELISAs and has a much shorter processing time. The disposable cassettes are easy to use, inexpensive, and require very little training of clinical staff to implement. Like the ELISA, a lateral

flow immunoassay works on the principle of antibody detection using antigens that are specific for an infectious agent.

4 Assays Based on the Use of Whole Bacterial Cells

A sensitive technique for the serodiagnosis of infections sustained by planktonic forms of bacteria involves the titration of serum antibodies against whole bacterial cells (Ryding et al. 2002). Since this approach does not require antigen extraction, it can be used to screen large number of samples.

Ryding et al. used ELISAs to measure IgG antibodies directed against seven different *S. aureus* antigens (peptidoglycan, teichoic acid, *S. aureus* ultrasonicate, whole *S. aureus* cells, alpha-toxin, lipase, and capsular polysaccharide) in the sera of 129 patients with *S. aureus* bacteremia, 78 patients with non-*S. aureus* bacteremia, and 100 febrile non-bacteremic patients. For the diagnosis of *S. aureus* bacteremia, the whole-cell ELISA proved to be the best performer in terms of sensitivity, but the specificity of all seven assays was low. Positive findings in two or more of the ELISAs (whole-cell, teichoic acid, alpha-toxin, lipase, and capsular polysaccharide) proved to be capable of distinguishing between *S. aureus* and non-*S. aureus* endocarditis, but this approach was not able to differentiate cases of transient *S. aureus* bacteremia from those involving endocarditis and/or septic embolization to other organs (Ryding et al. 2002).

Lamari et al. developed an ELISA for the titration of antibodies to antigens expressed on the surfaces of whole bacterial cells in intravenous immunoglobulin (IVIG) preparations and in human blood sera (Lamari et al. 2000a, b). Microplates coated with suspensions of various bacterial species (approximately 8,000 cells/mL, 100 mL per well) are incubated at 4°C for 16 h with serial dilutions of serum (or IVIG preparations). Antibody binding in each well was then assessed after application of peroxidase H-conjugated anti-human IgG. The titer of antibodies directed against the bacterial surface antigens represents the lowest serum concentration associated with absorbance of 0.2 units at 490 nm (Lamari et al. 2000b).

This method showed low intra-assay (C.V.: 5% for six wells) and inter-assay (C.V.: 7.5% for three repetitions) variation. The assay was used to evaluate the antibody content of two different intravenous immunoglobulin preparations. Twenty lots of each preparation were screened for the presence of bacterial species frequently isolated from clinical specimens, including *Escherichia coli*, *S. aureus*, *S. epidermidis*, *Klebsiella pneumonia,* and *Enterococci* spp. (Lamari et al. 2000b). The results revealed significant differences between the two IVIG preparations in terms of their specific antibody contents, differences that could prove to be clinically relevant in the treatment of immunodeficient patients (Lamari et al. 2000b).

Itoh et al. developed an easy and rapid ELISA system based on the use of a 96-well filtration plate fitted with a 0.22 mm membrane (MultiScreen ® -GV, Millipore) to detect antibodies against surface antigens present on the bacterial cell (Itoh et al. 2002). Suspensions of live bacterial cells (used as antigen) were applied

over the filter of the filtration plate wells, buffers were removed by vacuum filtration, and cells were resuspended in appropriate buffer. Assay reactions could be carried out in the wells without losing the solution. After blocking and inactivation of endogenous cellular peroxidases, the cells were incubated with biological samples (serum or feces). Peroxidase-labeled antihuman antibodies were then applied to detect bound antibodies. After 30 min of enzymatic reaction with chromogenic substrate, the solution was transferred to transparent 96-well microplates and optical density was measured at 490 nm in a microplate reader (Itoh et al. 2002). This assay was not developed to identify biofilm-related infections: it was conceived as a rapid means for identifying infections caused by planktonic forms of various bacterial species based on the antigens expressed on their cell surface. Therefore, it is not really a specific assay for biofilm-related infections.

The group of Laura Selan in the University of Rome "La Sapienza" began working in this field in 1990 using a whole-cell approach. The objective was the titration of specific IgG and IgM antibodies against antigenic structures present on the surface of staphylococcal biofilms. Sessile cultures of various staphylococcal strains were grown directly in microtiter wells. The biofilms were cultured 12, 24, 48, 72, and 120 h to simulate different stages of maturity, on the assumption that such stages would be characterized by more or less different surface antigenic expression profiles. Each plate was washed three times to remove planktonic cells, dried, and blocked with phosphate-buffered saline (PBS), pH 7.2, containing 10% bovine serum albumin (BSA).

This strategy was associated with two major problems. First of all, during ELISA, the wells were contaminated by planktonic cells that had been detached from biofilms during washing. In addition, neither the IgG nor the IgM titers obtained in these assays were capable of discriminating between the sera of patients harboring biofilm graft infection sustained by Staphylococci and sera of healthy controls.

5 Assays Based on Discrete Slime Antigens

5.1 Antibodies to Lipid S

Immuno-diagnostic systems can be based on the use of single, well-identified antigens, such as Lipid S, an exocellular, short-chain form of lipoteichoic acid, which is present in the cell wall and membrane of *S. epidermidis* (Lambert et al. 2000). It consists of six glycerophosphate units linked to a glycolipid, in contrast to the 40–42 units found in lipoteichoic acid.

In 2000, Lambert et al. described an in vitro assay based on the detection of Lipid S for serodiagnosis of infections caused by coagulase-negative staphylococci. According to their reports, detection of antibodies raised against this antigen can be useful in the diagnosis of intravascular catheter-related sepsis, biofilm colonization

of orthopedic prostheses, and endocarditis caused by these species (Lambert et al. 2000; Worthington et al. 2002; Rafiq et al. 2000; Connaughton et al. 2001). The assay was tested on sera from patients with proven central venous catheter-associated sepsis (based on strict clinical criteria that included blood-culture positivity) and controls with central venous catheters and no evidence of sepsis (Elliott et al. 2000). Sera were titrated against a standardized positive serum sample (IgG titer, 100,000; IgM titer, 25,000). Significant differences ($P < 0.001$) emerged between the mean IgG and IgM titers of the septic patients and control subjects (Elliott et al. 2000).].

The results were then analyzed in light of final diagnoses based on clinical and microbiologic criteria. An IgG titer cut-off of 20,000 diagnosed catheter-related sepsis with 75% sensitivity and 90% specificity. The diagnostic accuracy of the IgM assay was less encouraging (sensitivity and specificity 52% and 85%, respectively) when the cut-off was set at 5,000. Further studies on this test in different populations (patients with intravascular catheter-related sepsis caused by coagulase-negative staphylococci and those with Gram-positive bacterial endocarditis) led to increases in specificity that were not paralleled by enhancement of sensitivity. Based on their experience, the authors suggested that Lipid S might be a potential serodiagnostic marker of staphylococcal infections. This view is in contrast, however, with the results of previous studies. Wergeland et al. noted, for example, that assays based on lipoteichoic acid and cell-wall teichoic acid are of limited use for the diagnosis of *S. aureus* infections in clinical settings due to the substantial overlap between the range of titers observed in sera from infected patients and those obtained from blood donors (Wergeland et al. 1989).

5.2 Antibodies to a 20-kDa Acidic Slime Polysaccharide

The team headed by Karamanos identified several extracellular slime components that might be used in an ELISA for the diagnosis of *S. epidermidis* biofilm-related infections. In competitive ELISA experiments, a 20-kDa acidic polysaccharide (20 kDa PS) proved to be the best inhibitor of the reaction of slime with its homologous antibodies, suggesting that this fraction could be the major antigenic determinant of slime (Kolonitsiou et al. 2001).

In preliminary work to define the range of reactivity in ELISA of the 20 kDa PS, the investigators used sera from rabbits immunized with the same slime fraction obtained from different species of staphylococci. All the rabbit antibodies specifically recognized slime-producing *S. epidermidis*, the only species that displayed statistically significant ($P < 0.05$) reactivity across the entire dilution range. Cross-reactivity was observed with *S. haemolyticus*, *S. aureus*, and *S. saprophyticus* (in ascending order).

ELISA has also been used to detect the presence of anti-20 kDa PS antibodies in human sera (10 healthy controls and 25 patients with bacteremia caused by slime-producing *S. epidermidis* that had been blood-culture confirmed 10–15 days before collection of the serum specimen) (Karamanos et al. 1997). Anti-20 kDa PS

antibodies were present in the control sera but at titers that were significantly lower than those observed in the sera of the patients.

6 Assays Based on Mixtures of *S. Epidermidis* Slime Antigens

Three groups have used mixtures of slime antigens as probes for the detection of specific antibodies against *S. epidermidis* in biological samples.

Kjerulf et al. used combined ultrasonic extracts from different bacterial species (*S. aureus*, *S. epidermidis*, *Enterococcus faecalis*, and nonhemolytic streptococci) to detect antibody responses in sera from patients with various types of bacteremia ($n = 137$) or endocarditis ($n = 28$) and from healthy controls ($n = 275$) (Kjerulf et al. 1994). Bacterial endocarditis caused by *S. aureus* was successfully identified by the ELISA (diagnostic sensitivity 84%; diagnostic specificity 89%), but the test was less successful in diagnosing infections sustained by other bacterial species (including *S. epidermidis* and streptococci). Titers of IgG antibodies to *S. aureus* increased with age, whereas no age-related variations were observed for antibodies against other bacteria. This finding suggests that the assay is not absolutely specific for sessile forms.

Later work by Lambert et al. resulted in an assay based on the use of *S. epidermidis* exocellular carbohydrate antigens obtained by gel filtration chromatography of concentrated brain heart infusion culture supernatants (Lambert et al. 1996). They tested the assay in 16 patients with prosthetic joint infections sustained by *S. epidermidis*. Compared with the controls without joint infections, 13 of the 16 patients presented elevated serum IgG levels. However, the antigen was not specific for *S. epidermidis* bone infection: high levels of IgG were also detected in patients with other serious staphylococcal and streptococcal infections. Consequently, the assay was only capable of distinguishing between staphylococcal infection of joints and aseptic loosening of the joint prosthesis, that is, when immunoscintigraphy is already positive.

Selan and coworkers (Selan et al. 2002) developed an ELISA to detect serum antibodies against staphylococcal slime polysaccharide antigens (SSPA). Antigen-bound immunoglobulins were detected with peroxidase-conjugated antibodies against human IgG or IgM, and antibody titers were expressed as units of optical density. The assay was tested in patients with late-onset infections involving synthetic vascular grafts (LO-SVGI). These infections are characterized by a long, clinically silent period, and they are usually diagnosed at an advanced stage, after serious complications have occurred (e.g., suture erosion, abscess, or fistula formation) (Calligaro and Veith 1991).

Antibodies (IgG and IgM) against SSPA were titrated in sera collected from 38 patients with active LO-SVGIs caused by different staphylococcal species (group A), including *S. epidermidis* in six cases, *S. aureus* ($n = 2$), and coagulase-negative species other than *S. epidermidis* ($n = 24$). Six of these patients had mixed infections involving one or more staphylococcal species plus *P. aeruginosa* or *E. coli*. For

control purposes, assays were performed on sera from ten patients with active LO-SVGIs caused by bacteria other than staphylococcus, including *P. aeruginosa* ($n = 2$), mixed infections caused by Gram-negative bacilli (*Pseudomonas* spp. or Enterobacteriaceae; $n = 5$), mixed infection by *Enterococcus* spp. and *Enterobacter cloacae* ($n = 1$), mixed fungal–bacterial infections (*Candida albicans* plus *Enterococcus* spp. or plus *P. aeruginosa*) ($n = 2$) (group B); 16 healthy patients with histories of staphylococcal LO-SVGI that had been eradicated 6–72 months earlier by means of graft replacement (group C); 17 healthy patients with synthetic vascular grafts and no evidence of current or past graft infections (group D); and 58 healthy subjects with no implanted medical devices or prostheses of any type (group E). In this study, infections were classified as late-onset only when their first manifestations occurred 2 years or more after implantation of the vascular graft. All infections (ongoing and past) were microbiologically confirmed based on cultures of the explanted graft. The results of this study are summarized in Table 2 and Fig. 1.

The highest titers of IgG antibody to SSPA were noted in individuals with ongoing staphylococcal LO-SVGIs (group A). However, high titers were also seen in the control groups, which precluded the use of IgG titers for diagnostic purposes (Table 2).

In contrast, titers of IgM antibodies against SSPA were higher in the group A patients. There was virtually no overlap between the titers of these patients and those of controls. IgM antibody of 0.4 ELISA units (EU) or more indicated ongoing staphylococcal LO-SVGIs, with detection rates of 97% and no false-positives.

Table 2 Titers of IgM and IgG antibodies against staphylococcal slime polysaccharide antigens (SSPA) (ELISA units [EU]) in sera from patients with late-onset staphylococcal infections involving synthetic vascular grafts (LO-SVGIs) and from four control groups[a]

	Patient Groups					
	A ($n = 38$)	B ($n = 10$)	C ($n = 16$)	D ($n = 17$)	E ($n = 58$)	B + C + D + E ($n = 101$)
IgM titers (mean, SD) (EU)	0.69 (0.37)	0.28 (0.07)	0.26 (0.06)	0.18 (0.05)	0.17 (0.06)	0.19 (0.08)
IgG titers (mean, SD) (EU)	1.13 (0.36)	0.86 (0.46)	0.82 (0.57)	0.46 (0.27)	0.55 (0.26)	0.60 (0.37)
Positive tests based on an IgM titre threshold of (number, %) (EU)						
≥0.35	38 (100%)	2 (20%)	0	0	0	2 (2%)
≥0.40	37 (97%)	0	0	0	0	0

[a] A, patients with active, staphylococcal LO-SVGIs; B, patients with active, non-staphylococcal LO-SVGIs; C, patients with previous staphylococcal LO-SVGIs; D, healthy patients with uninfected synthetic vascular grafts; E, healthy patients with no implanted medical devices (see text for details)

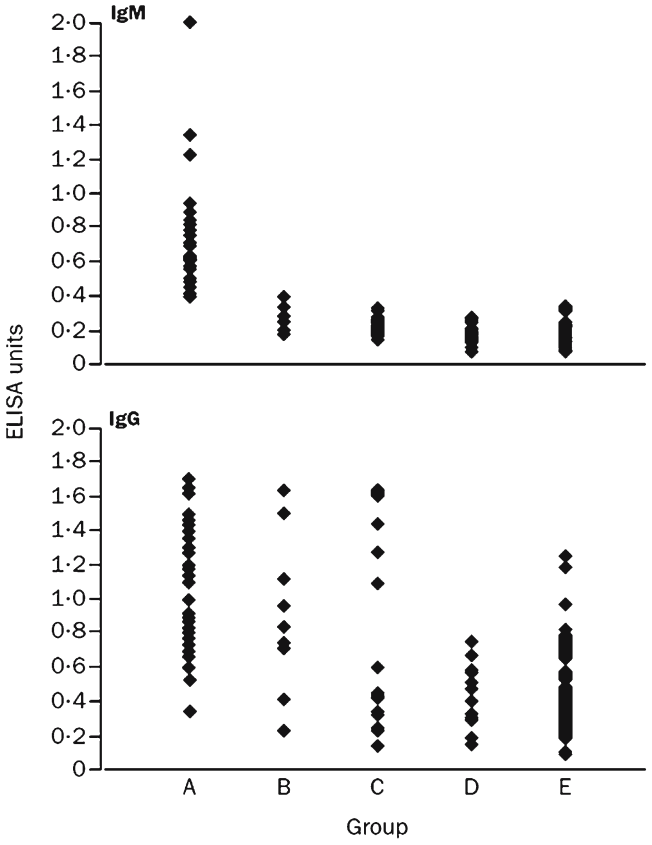

Fig. 1 Titers of IgM and IgG antibodies against staphylococcal slime polysaccharide antigens (SSPA) (ELISA units [EU]) in sera from patients with late-onset staphylococcal infections involving synthetic vascular grafts (LO-SVGIs) and from four control groups

When a cut-off of 0.35 ELISA units was used, the detection rate increased to 100%, but the false-positive rate also rose to 2%. Since these original studies, the group of Selan obtained comparable results in a larger population of patients with vascular grafts and a substantial group of patients with orthopedic prostheses (unpublished data; posters in the 1st International ASM Conference – Beijing, 6–10 April 2008).

The substantial difference observed between patients with ongoing vs. previous staphylococcal LO-SVGIs (group A vs. group C) suggested that levels of IgM antibody against SSPA decrease rapidly after successful graft substitution. Serological follow-up of three of the patients from group A after graft replacement confirmed this conclusion. In all three cases, IgM antibody titers fell below the level of 0.35 EU within 3 months of graft substitution. Recurrence of graft infection was associated with the return of elevated IgM antibody titers. These characteristics suggest that SSPA ELISA positivity can be used as a marker of active staphylococcal

graft infections. Periodic testing for IgM antibodies against SSPA could therefore prove useful in the follow-up of patients with implanted medical devices, including those whose devices have been replaced to eradicate previous infections.

The two false-positive diagnoses observed when the IgM titer threshold was set at 0.35 EU occurred in patients with active LO-SVGIs attributed to non-staphylococcal bacteria. These results might be somehow related to the polymicrobial nature of the two infections, which may have included a small staphylococcal component that was missed by microbiologic testing of the explanted graft. It is also possible that the positivity reflected a staphylococcal component that had recently been eliminated by the growth of other species. This latter observation renders the test particularly useful for diagnosing infections involving orthopedic grafts, which are frequently polymicrobial. In these cases, conventional microbiological tests are often insufficient to identify the causative bacteria.

The SSPA ELISA has a number of advantages over other available methods used to diagnose biofilm-related infections. First of all, it is versatile: the SSPA ELISA is capable of detecting antibodies in late-onset device-related infections caused by different staphylococcal species. Second, it has been validated in a group of patients suffering from late-onset graft infections, which represent the best clinical model of the majority of chronic infections related to biofilm colonization of implanted medical devices. The other ELISAs discussed earlier have been tested mainly in patient populations that reflect early-stage infections, mostly associated with acute bacteremia.

The SSPA antigen was chosen as the basis of this ELISA because it allows selective detection of infections sustained by sessile bacteria, which are the forms most frequently missed with conventional approaches. In a set of preliminary experiments in which sera from patients with LO-SVGIs were tested in experimental ELISAs based on different fractions of the biofilm matrix, SSPA appeared to be the most promising for development of an immunoenzymatic assay. During its validation (unpublished data) no evidence of interference was detected in cases of acute infection sustained by planktonic forms of staphylococci (bacteremia). This probably depends on the fact that the assay focuses on the immune response to antigens related predominantly (perhaps even exclusively) to the biofilm matrix. The information it provides is thus specifically related to device colonization by sessile bacteria.

The fact that the assay is based on a mixture of purified polysaccharide antigens extracted from the biofilm matrix explains the higher diagnostic value of the IgM titers. Polysaccharide antigens are known to elicit a thymus-independent humoral response based exclusively on IgM production. This response is maintained as long as the antigenic stimulus is present, and there is no shift to IgG production. Weller et al. showed that the IgMs elicited by antigens of this type are synthesized by a particular sub-population of B lymphocytes (B1): blood IgM + IgD + CD27 + cells that correspond to splenic marginal-zone B cells (Weller et al. 2004). Because of the absence of thymic involvement and IgG production, these responses are regarded as expressions of innate immunity. The peculiar behavior of the immune response to polysaccharide antigens represents a diagnostic advantage, since it can

be used at any time to evaluate the possibility of device infection, even during the post-replacement follow-up. If the infection is resolved, anti-SSPA IgM titers promptly return to normal levels, whereas persistence and relapse are reflected, respectively, by IgM titers that remain elevated or increase rapidly after a short period of normalization (Fig. 2).

On the other hand, IgM ELISAs have to be executed with great care and precision, and so adequate technician training is a must. It is also important to recall that the variations observed in IgM titers are generally very subtle, and clinicians who receive the results of an SSPA ELISA need to be informed that even small changes can reflect a potentially significant variation in the total bacterial load. This fact is one potential drawback to what is otherwise an encouraging approach for biofilm diagnostics. Since IgM levels show correlation with the presence (and possibly also the concentration) of the antigen, serial SSPA ELISAs should be able to identify infections in the very early phase, based on limited increases in titers with respect to the patient's own baseline.

The assay is simple to perform, noninvasive, and low-cost, features that render it ideal for use as a routine first-line screening method for the follow-up of patients harboring any kind of artificial medical device, even when there are absolutely no signs or symptoms of infection. It is important to recall, however, that, like all diagnostic tests based on conventional serum markers of infection, the results of the SSPA ELISA have to be interpreted in light of a larger and more comprehensive

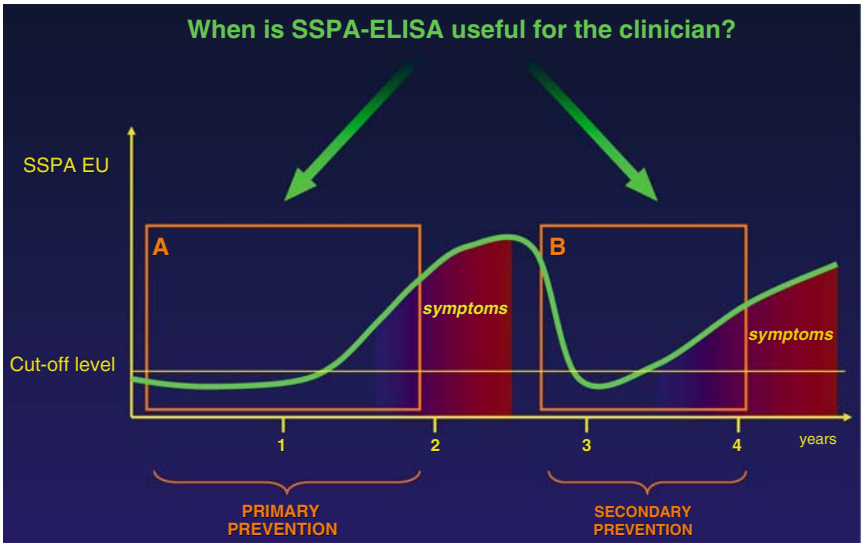

Fig. 2 Evolution of IgM antibodies against SSPA during the natural history of a hypothetical synthetic vascular graft infection caused by *Staphylococcus* spp., including treatment (with graft replacement) and relapse. Boxes (**a**) and (**b**) indicate stages during which the SSPA ELISA can be used for early diagnosis and post-therapy follow-up, respectively

clinical picture. If IgM titers exceed the preestablished threshold (or even when there is merely a steady rise with respect to the patient's baseline), an infectious disease specialist should be consulted and second-line diagnostic testing should be performed. These second-level approaches (CT, MRI, Leukoscan) are all expensive, specialist procedures, and some are also invasive. Again, none of these approaches identify the causative organism. Screening with the SSPA ELISA could result in more rational use of these studies, with substantial potential savings in terms of healthcare costs and patient risks. In addition, it should also result in earlier diagnosis of infections caused by biofilm colonization of medical implants. This effect would have a considerable impact on the prognosis of these infections – and ultimately on their epidemiology as well.

7 Upcoming Technologies

Leid and coworkers developed a lateral flow assay in collaboration with Dr. Mark Shirtliff's laboratory at the University of Maryland. Since 2000, Dr. Shirtliff has been elucidating those proteins in *S. aureus* biofilms that were immunogenic in animal models of infection. He investigated a number of proteins expressed in a mature MRSA biofilm for possible diagnostic application in a serology assay. These proteins were found to be upregulated during various stages of biofilm development when compared with planktonic expression and are highly immunogenic in rabbits with MRSA-infected tibias (Brady et al. 2006). Importantly, proteins ranged from early regulation (first 24 h of infection), median regulation (7–14 days), and late or chronic expression (up to 42 days post-inoculation in this study). These proteins could be utilized in a lateral flow assay system to detect early, developing, and chronic biofilm infections in humans. Indeed, this disposable assay could be easily and cheaply used to monitor the progression of device related infections in humans sustained by MRSA (and possibly by *S. aureus* in general). A general schematic of the MRSA biofilm-specific lateral flow assay is shown in Fig. 3.

The assay consists of a strip of nitrocellulose with a conjugation area of a colorimetric substrate, usually colloidal gold conjugated to an antibody-binding factor such as protein A. When serum is applied in a running buffer, the antibodies conjugate to the colloidal gold and accumulate at a test line (distal to the conjugation area), which is comprised of the antigen of choice. LFAs, such as the Rapid-Strep test™, serum pregnancy tests (SPT), and rapid drug screens, are successfully used in healthcare settings. Therefore, it is likely that the LFA platform can be modified to create a biofilm-specific diagnostic test by identifying and characterizing the antibody response to biofilm-specific proteins. Finally LFA is time-efficient and takes less than 10 min to perform.

Leid and coworkers used the MRSA biofilm specific proteins described earlier to develop diagnostic targets for the lateral flow assay. Each of these candidates was striped onto separate pieces of nitrocellulose and the sera samples drawn from rabbits with MRSA-infected tibias before infection sera and 42 days post inoculation were

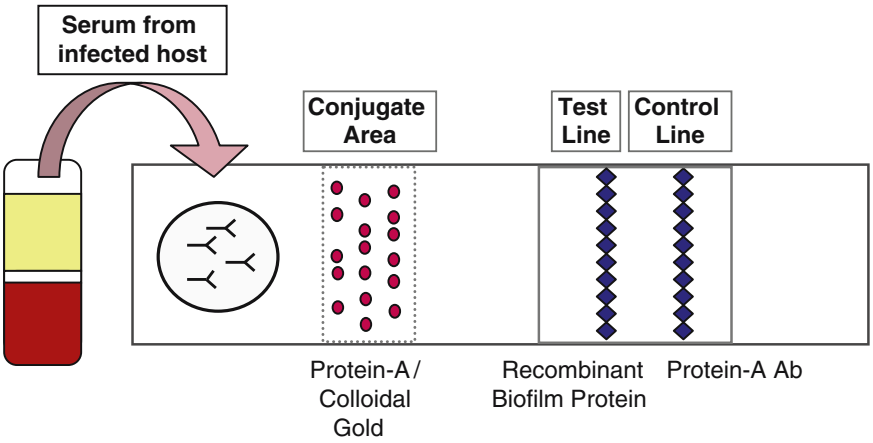

Fig. 3 Conceptual cartoon of the biofilm lateral flow assay (LFA). Serum from a patient is introduced to the assay, antibodies are bound to conjugates as they travel down the strip, and biofilm-specific antibodies bind to the test line while control antibodies bind the control line. For all assays, the control will be positive. For patients with biofilm infections, the test line will also be positive

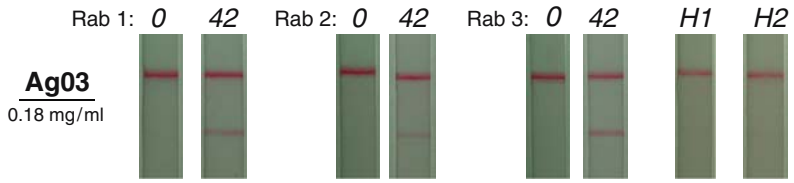

Fig. 4 Biofilm-specific lateral flow assay. Rabbits were infected with *Staphylococcus aureus* (MRSA) in a biofilm model of osteomyelitis. Sera was sampled at 0 and 42 days post infection. A biofilm-specific antigen (Ag03) was stripped down onto the LFA and sera from 0 and 42 days introduced to the assay. Note that at 0 days, none of the tests were positive whereas at 42 days post-infection, all of the LFAs were positive (Rab1, 2, and 3). Additionally, normal human serum did not react with the biofilm-specific antigen in this assay system (H1 and H2)

tested against each antigen in a lateral flow assay system (unpublished data). One antigen, labeled Ag03 here, demonstrated to work well in LFA. Figure 4 is a representative picture of this antigen working in the LFA. All three rabbits before infection were negative and during infection were positive in this LFA. Sera samples from healthy humans did not demonstrate cross-reactivity with Ag03, an important consideration for development of clinical tools to diagnose human biofilm infections. These data can be considered as preliminary information obtained in a preclinical setting worthy to be further validated on a well-selected population of humans with biofilm sustained infection.

All tests based on immunodiagnostic techniques are inexpensive, especially when compared with more elaborate tests such as PCR, CT scans, and MRI. The

need for cost-efficient diagnostic tests is apparent in light of the estimated cost to treat a nosocomial infection, ~$14,000 per patient (Cosgrove et al. 2005).

Decisions regarding the use of surgical or medical treatment in cases of suspected implant infections require confirmation of the suspicions raised by immunodiagnostic test results.

Unfortunately, conventional imaging techniques (ultrasound, plain-film radiology, CT-scan, and MRI) offer limited information. Techniques specifically designed to identify inflammation (Leukoscan) are more informative, but they are also invasive, costly, and nonspecific. In the initial phases of infection, detection of the subtle signs of inflammation in tissues surrounding the implanted device media and differentiation of septic and aseptic forms of inflammation are virtually impossible. Unequivocal CT or MRI confirmation is possible only in the presence of a substantial amount of purulent material. In this case, however, the diagnosis has probably already become obvious, based on the appearance of symptoms. In addition, the presence of severe sepsis and massive tissue damage dramatically reduces the options for effective treatment.

New possibilities will hopefully be provided by innovative imaging modalities. One of the more intriguing possibilities involves the development of novel contrast media based on advances in the field of molecular imaging. This rapidly emerging branch of biomedical research extends morphological observations in living subjects to a more meaningful dimension. It involves the visual representation, characterization, and quantification of biological processes at the cellular and subcellular levels, within intact living organisms (Weissleder and Ntziachristos 2003). Thus far, molecular imaging has been exploited mainly in the field of oncology, where specific molecular probes have been used to achieve contrast enhancement (Allen 2002; Bremer et al. 2003). A similar approach may be used to enhance the visualization of biofilms. Innovative contrast agents can be created, consisting of a conventional tracer (e.g., gadolinium for MRI) combined with a monoclonal antibody vector capable of specific binding to target molecules present within the biofilm. Detection of a signal produced by this tracer would provide highly specific evidence of the presence of living bacteria embedded in a biofilm within the site being examined. The authors of this chapter are actively working on this aspect of in vivo diagnostics.

The successful treatment of patients is directly correlated to time of diagnosis. Infections caught early and with accuracy are more likely to be resolved and to shorten the length of patient hospital-stay. The paradigm of biofilm formation in many bacterial pathogens has dramatically increased the difficulty of specific diagnosis and treatment. Standard culturing techniques are inefficient at growing bacteria in a biofilm form and diagnostic tests that are more sensitive to biofilms, such as PCR and MRI, are expensive and require extensive training and equipment to perform. These tests are also hampered by sample accessibility (PCR) or a readily available set of biofilm-specific diagnostic reagents (MRI). Serology assays are useful techniques to diagnose bacterial infections because they are fast and do not require sampling from the infected site. Immunodiagnosis by ELISA, LFA, and other platforms, combined with molecular imaging, are safe and noninvasive tools

that can be used repeatedly during the follow-up of patients with implanted medical devices. Their use is expected to dramatically modify the natural history of infections caused by biofilm colonization of implanted medical devices, and they might also pave the way to the development of new and more effective approaches to the treatment of these infections.

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Immune Responses to Indwelling Medical Devices

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Abstract Implanted medical devices have offered clinical hope to patients who either have critical illnesses or have more chronic problems such as joint destruction. No doubt, these devices have saved many lives and improved the quality of life of hundreds of thousands of people. Indeed, the use of indwelling devices has reached epic proportions in human medicine over the last three decades. One of the unintended consequences has been an accompanying rise in the infection rate in patients, which is directly related to the presence of these devices in humans. This is problematic because the devices are colonized by communities of microorganisms, termed biofilms, that are highly resistant to antimicrobial challenge and to destruction from the human host and its defenses. Over the past decade, there has been much progress on understanding how and why these communities are less susceptible to antimicrobial agents. However, many questions regarding the resistance of these communities to human host defenses are still unanswered . This chapter discusses the current knowledge of how the human immune system responds not only to the presence of indwelling medical devices, but also to the communities that colonize them.

1 Introduction

As the chapters in this book have repeatedly demonstrated, biofilm infections on implanted medical devices cause tremendous problems in medicine. Much of these chapters have documented on the antibiotic resistant nature of these communities and have touched upon the resistance of these communities to human host defenses. Indeed, a quick survey of all biofilm literature demonstrates that there is a preponderance of studies on antimicrobial resistance, yet there is a much smaller volume

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of knowledge about host resistance factors of biofilms. This knowledge gap on host defenses against biofilm infections is intriguing, since all implanted medical devices are quickly conditioned with host factors, including those factors that display antimicrobial activity. Additionally, it is clear that the human immune system responds to the presence of the implants: in some cases, as a result of the surgical procedure, and in other cases, as a result of the implant itself. Nonetheless, relatively few publications have focused on the host defenses and their responses to microorganisms specifically living the biofilm lifestyle. Since many previous chapters have included some information about either the immune response to implanted devices or the mechanisms associated with general host resistance, this chapter will review the main components of host factors involved in implant infections and document in detail innate immune responses to biofilms. Although the adaptive immune system plays an important role in defense, the role of the innate immune system will be the focus of the chapter, since it is the first line of defense against microbial colonization.

2 Implant Device Infections

The increased use of implanted medical devices such as intramedullary rods, screws, plates, and artificial joints has provided a physiological niche for pathogenic organisms to cause infections. Some bacterial species may initially colonize these implants during surgical implantation or subsequently by hematogenous seeding. An inherent problem associated with implants is their propensity to be coated by host proteins such as fibrinogen and fibronectin shortly after implantation (Francois et al. 1998). In the short term, fibrinogen/fibrin seems to be the dominant-coating host protein, while fibronectin becomes dominant in the long term, since fibrinogen/fibrin is degraded. Implants can then act as a colonization surface to which many bacterial species readily adhere. Implants are also often responsible for reduced blood flow and locally compromised immunity by impairing natural killer, lymphocytic and phagocytic cell activities. These implanted devices have also been linked to decreasing the amount of superoxide, a mediator of bacterial killing within professional phagocytic blood cells (Roisman et al. 1983). Another mechanism by which implanted medical devices produce locally compromised immunity is through frustrated phagocytosis (Roisman et al. 1983). In this case, professional phagocytes may undergo apoptosis when encountering a substrate of size that is beyond its phagocytic capacity. The resulting release of reactive products of oxygen and lysosomal enzymes may cause accidental host tissue damage and local vascular insufficiency, thereby increasing the predisposition of chronic infection development. A portion of the normal phagocytic processes is also devoted to the removal of the implant foreign material [particularly with metals, polymethylmethacrylate (PMMA), and polyglycolic acid], thereby utilizing the energy and resources of the immune system that would normally be used to fight infection (Santavirta et al. 1991; Santavirta et al. 1990; Wang et al. 1997). Therefore, prosthetic implants not only provide a substrate for bacterial adherence, but also limit the ability of the host

to adequately deal with the infection. Once colonized, bacteria (such as staphylococcal species) are able to synthesize a "slime" layer, termed the glycocalyx or biofilm. This layer prevents infection resolution by antimicrobial agents and host phagocytic cells (Brause 1986). Once an implant is colonized and chronic infection ensues, the only treatment option is implant removal. The best alternative for preventing these difficult-to-treat infections seems to be preventing the biofilm from forming in the first place. A strategy in which high risk patients (e.g., patients undergoing dialysis treatment, long-term intravenous catheterization, or joint arthroplasties) are administered an "anti-biofilm" vaccine may enable the immune system to quickly recognize early biofilm epitopes and remove the invading microbial community before the development of the fully mature community that is resistant to the host's immune system and antimicrobial agents.

Not only is treatment of implanted medical device infections difficult, but diagnosing these infections is also extremely problematic. However, a great step may have recently been made in the ability to diagnose these infections. An Italian group has developed an ELISA assay that was over 90% effective in detecting graft infections through the detection of serum antibodies against *Staphylococcus epidermidis* biofilm polysaccharide antigens (Selan et al. 2002). However, it is unclear whether this assay would be effective in diagnosing infections caused by other bacterial species.

The risk of implant infection may be increased by a number of factors. First, certain joint replacements are more susceptible to infection because they remain close to the surface and have poor soft tissue coverage (e.g., total elbow arthroplasties) (Sourmelis et al. 1986). Second, certain patient populations are at increased risk because of underlying conditions or systemic diseases, including those patients suffering from diabetes mellitus and rheumatoid arthritis (Dougherty and Simmons 1989). Patients who are elderly, obese, malnourished, or have undergone prior surgery at the implantation site are also at risk. Third, PMMA bone cement may be inhibitory to the activity of white cells and complement function. The heat released during PMMA polymerization may also kill the juxtaposed cortical bone, thereby creating a nonvascularized area. This provides the bacteria a lush growth environment while being sealed off from the circulating host defenses.

A number of bacterial species are particularly well suited to cause infections in artificial joints. Although coagulase-negative staphylococcal species are often isolated from perioperative infections, *S. aureus* was found to be the major mediator of prosthetic implant infection in a number of studies (Arciola et al. 2001; Sanderson 1991). *S. aureus* is a gram positive, ubiquitous bacterial species, with the predominant reservoir in nature being humans. The carriage rate of this organism in humans is reported to be between 11 and 32% in healthy adults (Millian et al. 1960; Tuazon and Sheagren 1974). In the pre-antibiotic era, *S. aureus* bacteremia resulted in a 90% death rate (Smith and Vickers 1960). Because of the increasing involvement of *S. aureus* in foreign body-related infections, the rapid development and exhibition of multiple antibiotic resistance by these organisms, and their great propensity to change from an acute infection to one that is persistent, chronic, and recurrent, this pathogen is once again receiving significant attention.

3 Host Factors Involved in Prosthetic Implant Infection

3.1 Host Factors and the Implant

Prosthetic implantation provides a physiological niche for pathogenic organisms to cause infection. As mentioned, some bacterial species may originally colonize these implants during surgical implantation or afterward by hematogenous seeding. An intrinsic problem connected with implants is their tendency to be coated in host proteins, such as fibrinogen and fibronectin, shortly after implantation (Francois et al. 1998). Earlier, fibrinogen/fibrin seems to be the dominant-coating host protein, while fibronectin prevails in the long–term, as fibrinogen/fibrin is degraded. Because of the ability of bacteria such as *S. aureus* to bind to fibrinogen and fibrin via receptors for these molecules, implants can become colonized, as bacteria easily adhere to these proteins. As well, implants are frequently responsible for reduced blood flow and compromise of local immunity by impairing the activity of natural killer, lymphocytic and phagocytic cells, as well as by reducing the amount of superoxide, a mediator of bacterial killing, within professional phagocytic blood cells (Roisman et al. 1983). Another means with which implanted medical devices create local immune compromise is through frustrated phagocytosis (Roisman et al. 1983), which occurs when professional phagocytes undergo apoptosis upon encountering a substrate of size that is beyond their phagocytic capability. The resulting release of reactive oxygen products and lysosomal enzymes may trigger accidental damage to host tissue as well as local vascular insufficiency, thereby increasing the predisposition for chronic infection development. Also, a portion of the normal phagocytic processes are devoted towards the removal of the implant itself (particularly with metals, methyl methacrylate, and polyglycolic acid), thus utilizing the energy and resources of the immune system that would usually be used to fight infection (Santavirta et al. 1991; Santavirta et al. 1990; Wang et al. 1997). Therefore, prosthetic implants not only present a substrate for bacterial adherence but also restrict the capability of the host to effectively deal with the infection. Once established, bacteria (such as staphylococcal species) are able to produce a “slime” layer, termed the glycocalyx or biofilm (discussed later). This layer prevents the inward diffusion of a number of antimicrobials and host phagocytic cells, allowing the bacterial population to escape the effects of antimicrobial therapy and host clearance (Brause 1986). Once an implant is colonized and chronic osteomyelitis develops, the only treatment option is implant removal.

The risk of implant infection may be increased by a number of factors. First, certain joint replacements are more vulnerable to infection because they stay near the surface of the body and have poor soft tissue coverage (e.g., total elbow arthroplasties) (Sourmelis et al. 1986). Second, PMMA bone cement may inhibit complement function and the activity of white blood cells. Also, the heat released during PMMA polymerization may kill the adjacent cortical bone, thereby creating a nonvascularized area. This offers the bacteria a lush growth environment while being sealed off from the circulating host defenses. Third, some patient populations are at elevated risk

due to underlying conditions or systemic diseases, including those patients suffering from diabetes mellitus and rheumatoid arthritis (Dougherty and Simmons 1989). Other risk factors include the development of infection at the site of the prosthesis that is not associated with the prosthesis itself, the presence of malignancy, and a history of joint arthroplasty (Berbari et al. 1998). A surgical patient index score of 1 or 2 also increases the risk of PII (Culver et al. 1991). As well, patients who are elderly, obese, malnourished, or who have undergone prior surgery at the implantation site are also at risk.

3.1.1 Elderly Patients

The elderly are more susceptible to many infections than younger adults, and thus may be considered immuno-compromised. These individuals have a lessening of innate and adaptive responses, which results in a generalized reduction in the response to foreign antigens. Specifically, there is a deficit in thymic and T lymphocyte function, mostly associated with the decreased production of, and reaction to, interleukin-2 (IL-2) and the associated decline in antibody production by B cells (Ben-Yehuda and Weksler 1992). Also, it has been postulated that the decrease in T cell activation could be due to a decrease in the expression of costimulatory molecules, such as CD28, on the T cell, which thus causes T cells to be tolerized in the absence of this "second signal" (Nociari et al. 1999). This decrease in immune function can be due to the effects of age on the immune system as well as suppression caused by age-related illness.

3.1.2 Phagocyte Defects

Defects of phagocyte function occur when the normal oxidative burst of the phagocytes, or their ability to adhere (for extravasation into infected tissues or for opsonization due to complement), is reduced. This decrease in proper function can lead to the inhibition of infection clearance as well as a series of deep infections such as PII. The three phagocyte defect disorders that have been linked with the onset of chronic osteomyelitis are chronic granulomatous disease (CGD), meloperoxidase (MPO) deficiency, and hyperimmunoglobulin-E-recurrent infection (Job's) syndrome (HIE). While there is no research illustrating a role for these defects in the inception of PII, because osteomyelitis and PII have such a similar disease course and because osteomyelitis often starts as an extension of PII, it is safe to assume that these illnesses can contribute to PII.

CGD patients have a defective cytochrome in the electron transport chain, which leads to a lack of production of reactive oxygen molecules (Gill et al. 1992; Tauber et al. 1983). These molecules are responsible for the oxidative burst that eradicates ingested microorganisms in phagocytes (Cunnion et al. 2001). As catalase-positive bacteria (such as *Staphylococcus spp., Pseudomonas aeruginosa, Escherichia coli, Aspergillus spp.*, and *Candida spp*.) are able to degrade the low levels of hydrogen

peroxide present in the phagocytes of CGD sufferers, these are the organisms often associated with deep infections in these patients (White and Gallin 1986). Patients deficient in MPO often go undetected as they do not generally get recurrent infections, unless they suffer from another disease, such as diabetes, simultaneously (Duff et al. 1996). HIE patients suffer from faulty interferon-γ (IFNγ) production by CD4 T cells. This leads to abnormal chemotaxis and heightened IgE levels (Donabedian and Gallin 1983). These individuals are susceptible to skin infections with *S. aureus* (White and Gallin 1986), which could spread to cause deeper infections such as PII, osteomyelitis, sepsis, and brain and bronchial abscesses (Khanna et al. 2005). These patients also have recurrent infections believed to be derived from a chemotactic disorder and a minor anomaly of neutrophilic killing of microbes. There are several other disorders that may affect phagocyte function, including diabetes, liver failure, antibody deficiency, complement deficiency, leukocyte adhesion deficiency types 1 and 2 (LAD 1 and 2), Chediak–Higashi syndrome (CHS), glycogen storage disease, and glucose-6-phosphate dehdrogenase deficiency (G6PD deficiency).

3.1.3 Immune System Disorders

Individuals who lack a fully functional immune system are at increased risk for osteomyelitis (Bahebeck et al. 2004; Restrepo et al. 2004; Rodriguez 1998; Talpada et al. 2002; Tehranzadeh et al. 2004). HIV is a worldwide epidemic, with 1.1 million Americans infected with the virus as of the end of 2002 (Tehranzadeh et al. 2004). Osteomyelitis is coupled with mortality rates over 20% in HIV-infected patients (Vassilopoulos et al. 1997), and is the second most frequent musculoskeletal infection in HIV-positive patients. Bone infections seem to be a common manifestation in young male homosexuals and intravenous drug users (Vassilopoulos et al. 1997; Belzunegui et al. 1997). Most of these infections are caused by *S. aureus* (up to 48%), though other species, such as *P. aeruginosa*, have also been found (Biviji et al. 2002; Major and Tehranzadeh 1997; Steinbach et al. 1993). In some cases, musculoskeletal infections such as osteomyelitis are the initial manifestations of HIV infection (Biviji et al. 2002), though these infections are not as common as infections affecting other organs (Major and Tehranzadeh 1997). The most commonly infected bones include the wrist, tibia, femoral heads, and thoracic cage, but other atypical sites, such as the patella and the mandible, have also been reported (Tehranzadeh et al. 1996). Though there is no reference to a correlation between HIV infection and PII, with its common linkage to osteomyelitis infections, it is safe to assume that HIV would predispose one to PII infection as well.

4 Role of the Innate Immune System

The innate immune system represents the first line of defense against invading microorganisms. It represents the most ancient defense mechanism and is found in plants, invertebrates, and vertebrates. Often ignored in the last two decades, its

importance has once again been brought to the forefront of immunology, some of which many argue was the direct result of the late Charles Janeway and his lifelong journey to understand the complexity and importance of these ancient antimicrobial defenses. While this may be debated, it is clear from the initial observations of Methnikoff, to the many contributions by Janeway and others, that the innate immune system is vital to many forms of life on this planet.

The innate immune system is composed of both soluble mediators, such as serum, enzymes, and other proteins, and cellular mediators such as macrophages and polymorphonuclear leukocytes (neutrophils). The main difference between these components, and the B and T lymphocytes of the adaptive immune system, is that while the innate immune system is highly capable of killing invading microorganisms and protecting the host from disease or death, it does not have a memory for these pathogenic events and therefore must continually fight these challenges from scratch. A good analogy is the posit that understanding the history of events, and then being able to plan accordingly for future challenges, results in more efficient and productive activities. This is really a description of the adaptive immune system. However, the innate immune system must respond to each challenge as if being invaded by a microorganism for the first time. In this context, the power of the innate immune system is truly amazing in that, in most cases, it protects humans and other animals from disease and death. The remaining chapter will focus on the soluble mediators and the cellular mediators of the innate immune system as they relate to published reports of biofilm-mediated infections. Although the authors have tried in earnest to be inclusive, we have chosen to focus on the most common mediators and on those mediators that have been studied in the context of biofilm infections.

5 Soluble Mediators

5.1 Lactoferrin

Lactoferrin is an iron-binding glycoprotein synthesized by neutrophils and in glands of the exocrine system. Its ability to bind free iron often protects the host simply by reducing the amount of iron available for invading microorganisms. By chelating iron, lactoferrin stimulates twitching motility in *Pseudomonas aeruginosa*. This twitching is a specialized form of motility causing bacteria to wander across a surface rather than aggregating to form a biofilm (Singh et al. 2002). By twitching motility, bacteria attach to surfaces using the type IV pilus (Mattick 2002). Retraction of the pilus frees bacteria from a surface, allowing them to roam, potentially searching the body for niches, such as an implanted medical device, where a community of organisms can be established (Skerker and Berg 2001). In the absence of lactoferrin, the daughter cells of attached bacteria remain near the origin of parent cell division, forming microcolonies and eventually biofilms. Conversely, the presence of lactoferrin induces motility and daughter cells move away from

parent cell origins, preventing the formation of microcolonies. That surface locomotion prevents *P. aeruginosa* biofilm formation suggests that microcolony development forms from the division of attached cells rather than the active aggregation of bacteria. In regards to host defense, lactoferrin prevents bacteria that survive initial killing from forming biofilms, buying time for adaptive responses to be recruited, and for antibiotics to be administered. Moreover, lactoferrin may take advantage of an evolutionary bacterial response. Since iron is an essential and difficult-to-acquire nutrient for microorganisms, lactoferrin-induced motility may actually benefit the bacteria. Through iron sequestration, twitching motility prevents bacteria from forming sessile biofilm structures in locations where iron is limited (Singh 2004). However, increased levels of superoxide dismutase activity have been observed in *P. aeruginosa* upon exposure to an iron chelator, reducing the efficacy of host respiratory burst response (Hassett et al. 1996, 1999).

Another unique ability of lactoferrin is its capacity to amplify apoptotic signals in infected cells, limiting necrotic tissue damage (Valenti et al. 1999). Necrotic tissue, a situation associated with implanted medical devices, serves as a wonderful ecological niche for microbial attachment and expansion at the site of necrosis. In addition, lactoferrin works synergistically with other compounds, including lysozyme, complement and prescription antibiotics. For example, combinations of lactoferrin and vancomycin demonstrated synergistic effects on biofilm reduction of *S. epidermidis* (Leitch and Willcox 1999a,b). *S. epidermidis* vancomycin resistance is attributed to the overproduction of cell wall materials that bind the drug at sites unrelated to its target location (Sanyal et al. 1993). When paired with lactoferrin, however, vancomycin penetrates the glycocalyx. This is attributed to the cationic lactoferrin binding the anionic cell wall materials of *S. epidermidis*, allowing vancomycin for greater access to its target (Leitch and Willcox 1999a,b). A similar mechanism of charge compensation is seen in conjunction with lactoferrin and Lysozyme (discussed later). Lactoferrin binds teichoic acid of *S. epidermidis*, allowing greater penetration of lysozyme and dissolution of the bacterial cell wall (Leitch and Willcox 1999a,b). In addition, the presence of lactoferrin has shown an increase in efficacy of antibiotics against Salmonella species (Naidu and Arnold 1994) and sensitization of E. coli to rifampin (Ellison et al. 1988). More recently, studies on the *Burkholderia cepacia complex* (Bcc), as a pathogen in cystic fibrosis (CF) lungs, demonstrated that lactoferrin, combined with rifampicin, decreased the viability of in vitro grown biofilms (Caraher et al. 2007).

The inactivation of lactoferrin may contribute to the chronic infection of *P. aeruginosa* biofilms common to CF patients. Cathepsin, a protease expressed by macrophages, fibroblasts, and in the epithelial cells of the lungs, becomes activated under acidic conditions (Chapman et al. 1997; Kirschke and Wiederanders 1994). The pH of the epithelial lining fluid of a CF patient is ~5.8, decreasing to 5.3 during infection (Tate et al. 2002). This acidic environment provides optimal conditions for cathepsin activity – shown to be several hundred-fold greater in CF patients than in non-CF patients (Rogan et al. 2004; Taggart et al. 2003). In addition, bacterial lipopolysaccharide (LPS) is also known to stimulate cathepsin release (Petanceska et al. 1996). Therefore, it seems that an initial bacterial infection in a CF patient

may increase concentrations of cathepsin, which cleaves and inactivates lactoferrin, predisposing the patient to *P. aeruginosa* biofilm formation and chronic infection (Rogan et al. 2004). However, lactoferrin binding of LPS may indirectly work in favor of the bacterium, decreasing the negative charge of the lipid A domain, enhancing the subsequent activity. This would in turn hinder the affects of other cationic antimicrobial molecules (CAMs, discussed later) (Na et al. 2004).

One of the most important works on lactoferrin production and anti-biofilm activity was published by Pradeep Singh's group in the journal Nature (Singh et al. 2002). For this study, Singh and colleagues demonstrated that lactoferrin, in concentrations below those commonly associated with antimicrobial activity, blocked biofilm formation by impacting twitching motility. In the presence of lactoferrin, the *P. aeruginosa* bacteria continuously wandered across the substrate surface and were never able to firmly attach and form mature biofilm communities. Follow up studies have suggested that lactoferrin production is decreased in CF patients (Rogan et al. 2004) and that the mucus environment in the CF lung prevents high concentrations of lactoferrin from reaching the biofilm bacteria (Matsui et al. 2006). However, all these studies were specifically associated with CF patients, not implanted with medical devices, and so it is unclear what role lactoferrin specifically plays in device-mediated infections. We have recently shown that cytokine crosstalk occurs between cellular mediators of the innate immune system in response to *P. aeruginosa* biofilms and that cytokine crosstalk eventually leads to lactoferrin secretion by human neutrophils (Leid et al. 2007). In these studies, *P. aeruginosa* biofilm bacteria that lacked the *flgK* gene (partly responsible for flagella production) were susceptible to lactoferrin-mediating killing. Overall, it is clear that lactoferrin is an important part of the innate immune defenses and it is likely that novel biofilm-specific mechanisms exist to defend against its broad antimicrobial activity.

Some treatment therapies received in health care facilities may actually encourage biofilm formation. Catecholamine inotropes, received by up to 50% of intensive care patients (Smythe et al. 1993), stimulate *S. epidermidis* growth as a biofilm on biomedical materials (Lyte et al. 2003). This mechanism of growth stimulation is due to the ability of catecholamines to supply iron to bacteria through interaction with transferrin and lactoferrin, allowing *S. epidermidis* to overcome iron restriction (Lyte et al. 2003; Freestone et al. 2000).

5.2 Lysozyme

Lysozyme is similar in its activity to the beta lactam antibiotics, in that it targets the microbial cell wall (peptidoglycan). However, unlike the beta lactams, which are most active against rapidly reproducing microorganisms because they block cell wall synthesis, lysozyme breaks down the cell wall and therefore may be active against microorganisms in all phases of growth. One of the first studies on implanted medical devices, bacterial communities, and lysozyme was conducted by Busscher et al. on voice prostheses (Busscher et al. 1997). Their study concluded

that voice prostheses preconditioned with saliva, and presumably lysozyme, retarded colonization by Streptococcal, Staphylococcal, and Candida yeast strains. A decade later, Hatti and colleagues reported that toothpaste containing salivary substitutes prevent biofilm colonization of healthy human teeth (Hatti et al. 2007). Interestingly, very few other reports have directly measured the activity of lysozyme against biofilm communities. This is especially important because the human body has many sources of lysozyme, including saliva (relevant to dental implants), serum (relevant to most other prosthetic implants), and neutrophils (relevant to most implanted devices). There is a clear need to better understand the role of lysozyme and biofilm bacterial interaction, as this enzyme may be employed as a defense against device colonization.

5.3 Cationic Antimicrobial Molecules

A recent review discussed antimicrobial peptides and biofilms and focused on the role of the exopolymers common in the bacterial matrix (Otto 2006). The main hypothesis was that the exopolymers either sequester or repulse these charged molecules. This is an intriguing approach and may lead to new therapeutics that either enhance activity of defensins and cathelicidins or block biofilm formation on implants containing these compounds. Defensins and cathelicidins are soluble mediators secreted by skin epithelial cells and sweat glands that act as first line representatives of innate host defense (Turner et al. 1998; Harder et al. 2001). Human cathelicidins and β-defensin have been shown to work synergistically against *S. aureus* (Ong et al. 2002). Defensins, in particular, are small peptides that form ion permeable channels in bacterial and mammalian cell membranes (Eckmann 2005). They are also part of the oxygen independent bactericidal mechanism used by human neutrophils (Schroder 1999). As mentioned earlier, these cationic peptides, however, are ineffective against *S. epidermidis* due to the production of polysaccharide intercellular adhesin (PIA). This gene-regulated response contributes directly to virulence and is often found in correlation with infections originating from indwelling medical devices. Furthermore, PIA seems specifically suited to protect against the antibacterial peptides of the skin. This is achieved by sequestering bacteria from proinflammatory products and inducing electrostatic repulsion of cationic antibacterial peptides. Vuong et al. (2004) pointed out that the specific characteristics of PIA may contribute to the reason why *S. epidermidis* is the predominant microbe on the human skin and major microorganism in nosocomial infections.

One of the hallmarks of biofilm infections is that they exhibit distinct developmental stages as they progress from initial attachment, which usually lasts from 6–48 h, to mature biofilm communities, sometimes observed as early as 4 days post-attachment. During this time, it is likely that these bacteria are exposed to components of the host defenses, but these defenses do not eradicate the expanding microorganisms. Long ago, we hypothesized that the human immune system likely

responds differently to these stages of biofilm maturation (Leid et al. 2002). More recently, Eberhard and colleagues quantified the different immune responses to early and late polymicrobial biofilms more clearly by using dental implants and the human oral cavity as a model (Eberhard et al. 2008). In their study, antimicrobial peptides such as human β-defensin, and two other newly characterized antimicrobial peptides RNase 7 and PSO, were dramatically upregulated in human epithelial cells at days 1 and 3 post-implant. As the implants were left longer and the biofilms were allowed to form for 9 days, the innate immune response of the epithelial cells switched to the recruitment of neutrophils through the secretion of IL-8. This work is the first of its kind to report a differential immune response to in vivo biofilms and, more importantly, to polymicrobial biofilms that develop in the oral cavity. Further studies along these lines will no doubt shed light on how the innate immune system either provides a protective response, or more importantly, how biofilm communities regulate the innate response such that biofilm growth and colonization occurs.

Although AMP activity is a broad response against microorganisms because of their ability to disrupt the cell membranes, as a therapeutic choice, one of the downsides of administration of AMPs may be their activity against the normal flora. To combat this, some groups have engineered Specifically (or selectively) Targeted AntiMicrobial Peptides (STAMPs). It two different studies, Eckert and colleagues demonstrated that manipulation of STAMPs resulted in selective killing of biofilms of *S. mutans* and *P. aeruginosa* (Eckert et al. 2006a, b). Interestingly, the STAMP version of the natural AMP G10 had increased activity against *P. aeruginosa* biofilms than the natural compound (Eckert et al. 2006b). Additionally, when coadministered with tobramycin, biofilm bacterial killing was enhanced, likely due to greater uptake of the antibiotic in the presence of increased membrane disruption (Eckert et al. 2006b). The combination of STAMPs and antibiotics may be a viable approach to treat biofilm infections in humans.

LL-37, the antimicrobial portion of cathelicidin, was shown to stimulate IL-8 and IL-1β secretion in human epithelial cells (Tjabringa et al. 2003; Elssner et al. 2004). This event indicates that LL-37 is able to mount an acute inflammatory response (Eckmann 2005). Furthermore, cathelicidins have shown to be effective at killing nosocomial pathogens such as *S. aureus*, *Enterococcus faecalis*, and *P. aeruginosa* that are resistant to most antimicrobial therapies (Zanetti et al. 2002). The mechanism of killing involves membrane disruption; however, some porcine cathelicidins have been shown to interrupt bacterial protein synthesis (Oren et al. 1999; Boman et al. 1993). LL-37 also acts to orient the immune system by promoting leukocyte recruitment to infection sites (Scott et al. 2002). Microbial killing is further amplified by increased cathelicidin production by activated neutrophils (Turner et al. 1998). Cathelin, a prodomain of cathelicidin, is a cysteine protease inhibitor that promotes both bacterial killing and host tissue injury prevention by inhibition of microbial and neutrophil derived proteases. Cathelicidin is also known to induce apoptosis in proliferating lymphocytes, thereby regulating the inflammatory response (Zaiou et al. 2003; Risso et al. 1998). It is important to note that cationic peptides are often inhibited by high salt concentrations, as that observed in the CF lung. LL-37 is no exception. This may be a contributing factor to the chronic

inflammation seen in CF patients, as the environmental conditions force a valuable protease inhibitor to become inhibited.

As a percentage of all implant-related infections, urinary catheters and central venous catheters (CVCs) make up a majority of cases in the hospital setting. The normal therapeutic treatment for these device-related biofilm infections is removal of the fouled catheter and replacement with a new, sterile catheter. However, this treatment regime adds enormous costs to health care and does not address the issue of catheter colonization and biofilm infections. A more appropriate approach would be catheters that are resistant to colonization, therefore, preventing infections. Cirioni and colleagues demonstrated that pretreatment of CVCs with the cathelicidin AMP BMAP-28, before implantation into a rat model of infection, markedly reduced colonization by challenge with *S. aureus* (Cirioni et al. 2006). In combination with antibiotics, BMAP-28 reduced bacterial colonization to less than 10 CFU. In another study, Burton and colleagues demonstrated that a GlmU enzyme inhibitor, in combination with the cationic antimicrobial peptide protamine sulfate, dramatically reduced colonization of urinary pathogens to urinary catheters when the compounds were impregnated into the catheter design (Burton et al. 2006). These two studies, directed related to biofilm growth on implanted medical devices, clearly demonstrate the potential role of antimicrobial peptides in the fight against device related infections.

One of the most interesting aspects of biofilm studies in the last decade has been the observation that not just bacteria form biofilms. Indeed, many other microorganisms, including protozoa, viruses, and yeasts have been shown to live in biofilm communities. Being that these other microorganisms are fairly new to biofilm study, there has been little work on innate immunity towards these types of infections, even though these microorganisms are a cause of implant-related infections. Of all non-bacterial microorganisms, the fungi are the best studied microorganisms in the biofilm mode of growth. Burrows and colleagues recently demonstrated that non-amphipathic cationic AMPs were highly active against biofilms of *Candida* species (Burrows et al. 2006). Impressively, in their study, even preformed fungal biofilms on plastic surfaces, directly relevant to implanted medical devices, were killed when these AMPs were administered. Martinez and Casadevall studied the efficacy innate immune AMPs to *Cryptococcus neoformans* biofilms in a model of medical device infections (Martinez and Casadevall 2006). Their study demonstrated that many purified AMPs, including recombinant human β-defensins, were highly inactive against the biofilm form of this fungus, especially when compared to their planktonic counterparts (Martinez and Casadevall 2006). These conflicting results are a clear demonstration of the need for further studies on biofilms of non-bacterial origin and the resultant innate immune responses that may or may not protect the host from the development of chronic biofilm infections.

5.4 Complement

The complement system in humans is composed of greater than 20 serum proteins that act in a cascade fashion to induce inflammation through C3a and C5a, increase

phagocytosis of microorganisms primarily through coating with C3b, and by the formation of the membrane attack complex as the complement cascade proceeds. Complement activation is stimulated by such components as bacterial LPS within *P. aeruginosa* biofilm matrices. Despite activation, *P. aeruginosa* biofilms persist in CF patients, resulting in diminished neutrophil responses. Therefore, biofilms may induce a constant low-grade complement response, contributing to chronic lung inflammation (Jensen et al. 1993). Furthermore, phagocytic killing of mucoid *P. aeruginosa* requires complement opsonization of the bacteria. Non-mucoid exopolysaccharide (MEP)-specific opsonins deposit complement component C3 on cell surfaces below the MEP layer. As a result, it is hypothesized that MEP serves as a barrier, preventing cell-surface bound complement opsonins from binding complement receptors on phagocytes (Meluleni et al. 1995).

The Meluleni et al. (Meluleni et al. 1995) study tested this hypothesis by tracking C3 deposition of MEP-specific and non-MEP-specific opsonins. They determined that location of C3 deposition more strongly influenced opsonization than did amount to C3 present in sera. MEP-specific opsonin action by C3b occurs broadly throughout the biofilm, whereas non-MEP specific oposonization by C3b occurs in clusters around small patches of bacterial cells. The inability of CF patients to produce MEP-specific opsonins likely contributes to the persistence of *P. aeruginosa* infection. It may be possible, through active and/or passive immunotherapy, to improve production of MEP-specific opsonins in CF patients. Doing so may limit lung destruction from chronic infection (Meluleni et al. 1995). Complement activation might further be hindered by the fact that some surfaces, such as those of manmade biomaterials, may not permit formation of C5 convertase. This would inhibit complement activation and thus cleavage of C3 to C3a and C3b (Janatova 2000).

More recently, Hoffmann and colleagues demonstrated that Azithromycin activity against mucoid *P. aeruginosa* biofilms was multifactorial and included an increased susceptibility of biofilm microorganisms to complement and hydrogen peroxide mediated killing (Hoffmann et al. 2007). Although complement itself did not result in increased biofilm killing, the combination with the antibiotic Azithromycin did. The synergy between antimicrobial, biofilm-specific killing and components of the human innate immune system are ripe for further exploration. As expected, complement by itself does little to enhance biofilm-specific killing. Simmons and Dybvig recently confirmed what has been known for some time in other microorganisms (Janatova 2000), when they demonstrated that biofilms of *Mycoplasma pulmonis* were protected against the lytic effects of complement (Simmons and Dybvig 2007). Finally, in a biochemical study of sialic acid bacterial intake in H. *influenzae*, Johnston et al. demonstrated that the ability of biofilm bacteria to take up sialic acid directly related to the ability of complement to kill biofilm bacteria (Johnston et al. 2008).

We have, in collaboration with Dr. Samuel Silverstein's laboratory at Columbia University, looked at both complement and antibody penetration (and eventual coating) and killing of biofilms of *S. epidermidis*. Although these studies are not complete, they do suggest that biofilm bacteria are easily opsonized by antibody and complement and that biofilm killing does occur if the appropriate conditions are present. Fig. 1 demonstrates the opsonization of *S. epidermidis* biofilm bacteria

by antibodies and complement. Although these biofilms were very mature (10 days old), it is clear that both antibodies and complement can opsonize the individual bacteria within the biofilms. However, for reasons yet to be elucidated, this interaction does not always lead to effective bacterial killing. We are currently investigating the mechanisms behind complement mediated opsonization and biofilm bacterial killing.

Bacteria have developed clever methods of evasion to escape host immune responses. CHIPS (chemotaxis inhibitory protein of *S. aureus*), for example, is a small protein secreted by *S. aureus* that blocks chemotaxis receptors, including complement C5a receptor (de Haas et al. 2004). In addition, *S. aureus* secretes staphylokinase to inhibit defensins (Jin et al. 2004). Bacteria can also incorporate molecules into their cell wall to alter its composition and avoid host defenses. The addition of D-alanine or L-lysine to the cell wall of *S. aureus* alters the net charge, making it more positive and more resistant to many antimicrobial peptides of the innate immune system. As a result, cationic AMPs (CAMPs) are hindered by electrostatic repulsion. *P. aeruginosa* employs a similar mechanism of CAMPs repulsion by incorporating the cationic aminoarabinose into its lipid A matrix (Fedtke et al. 2004). This kind of modification is often seen in CF patients and is suggestive of specific lipid A structures associated with the disease. In addition to CAM resistance, this alteration leads to an increased recognition by TLR-4, contributing to progressive lung degeneration (Ernst et al. 2003).

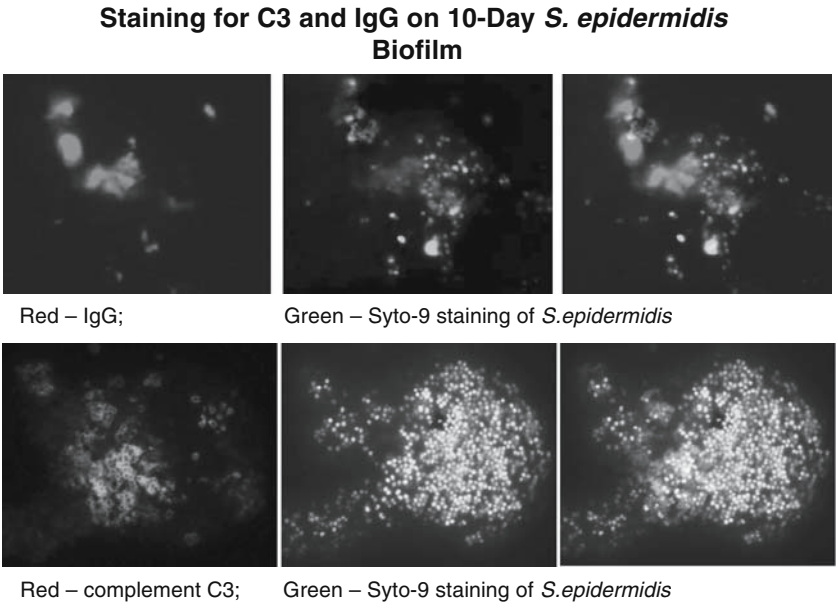

Fig. 1 Confocal micrographs of 10-Day-Old *Staphylococcus epidermidis* biofilms stained with Syto 9 (Green Fluorescence) and IgG (Red - top panel, specific for *S. epidermidis*) and Complement component C3 (Red - bottom panel). Notice that both antibody and complement bind to and opsonize the biofilm bacteria, although not at 100% efficiency

6 Cellular Mediators

Although many of the aforementioned molecules are released from cells of the human immune system, we have separated them above for clarity of their specific actions against biofilm-specific microorganisms. The final section of this chapter will detail cellular-specific mediators of the innate immune system and document their activity against biofilm microorganisms. Primarily, we focus on products secreted from activated neutrophils and macrophages.

S. mutans biofilms have been shown to absorb two-thirds the release of superoxide from neutrophils compared to planktonic bacteria. This may be accounted for by the presence of specific plasma proteins, such as albumin or immunoglobulins, which may hinder the attachment of cells to biofilms. These plasma proteins may block specific binding sites on neutrophils required for adhesion to bacteria and release of effective concentrations of microbicidal superoxide. The relatively high levels of plasma proteins in areas of gingival inflammation may affect neutrophil attachment, promoting cariogenic biofilm colonization (Shapira et al. 2000). What's more, extracellular polysaccharides produced by *S. mutans* inhibit neutrophilic detachment of biofilm bacteria (Steinberg et al. 1999). This might lead to prolonged PMN recruitment and accumulation due to frustrated phagocytosis. Evidence suggests that high neutrophil density is associated with reduced superoxide production as a protection mechanism for the host. Since superoxide is toxic to host cells, elevated concentrations could lead to neutrophil destruction and surrounding tissue damage (Shapira et al. 2000; Tanigawa et al. 1995).

Similar activity is seen with *P. aeruginosa* biofilms, as PMN oxidative burst responses are greatly reduced to only 25% of the response seen against planktonic bacteria (Jensen et al. 1990). Alginate production by *P. aeruginosa* biofilms protect against phagocytosis in the absence of specific host antibodies (Speert et al. 1986; van Oss 1978; Leid et al. 2005). This is not always the case, however, in the lungs of CF patients, as proteolytic enzymes from neutrophils may cleave immunoglobulins (Doring et al. 1985; Fick et al. 1985; Suter et al. 1984). Furthermore, alginate impedes chemotaxis, reducing PMN bactericidal potential and phagocytosis by macrophages (Simpson et al. 1988; Oliver and Weir 1990; Bayer et al. 1991; Stiver et al. 1988). Alginate may also act as a permeability barrier, trapping mediators and ions needed for an effective immune response (Jensen et al. 1992; Hoyle et al. 1990). Alginate further interferes with opsonization, induces proinflammatory cytokines, and suppresses lymphocyte transformation (Pedersen et al. 1992; Pedersen 1992). However, many of these studies were not done on alginate produced by biofilm microorganisms. Nonetheless, it is clear that *P. aeruginosa* biofilms result in defected neutrophil and monocyte killing (Leid et al. 2005; Jesaitis et al. 2003).

Initial colonization of CF lungs by *P. aeruginosa* can be eradicated by early and aggressive antibiotic therapy. However, once the bacteria convert to the mucoid form, characterized by alginate production, the organism can no longer be eliminated (Frederiksen et al. 1997). An experiment performed by Mathee et al. exposed non-mucoid *P. aeruginosa* to human PMNs and H_2O_2 and these scientists were

subsequently able to isolate mucoid variants. This suggested that mucoid conversion in the host is a defense mechanism of the bacteria in response to toxic oxygen byproducts. Therefore, H_2O_2 released by neutrophils, the predominant cells of inflammation in the CF lung, plays an import role in the conversion of *P. aeruginosa* to the incurable mucoid form (Mathee et al. 1999).

In general, *P. aeruginosa* biofilms show great resistance to toxic oxygen products such as H_2O_2, converting it to O_2 through catalase and superoxide dismutase activity (Lu et al. 1998; Brown et al. 1995; Hassett et al. 1995). Biofilm alginate may serve as a sink for O_2, H_2O_2, O_2 and myeloperoxidase products as a result of neutrophil immobilization. During an infection, neutrophils accumulate on the biofilm extracellular matrix, releasing toxic oxygen species, degradation enzymes, defensins, and lipid inflammatory mediators. Immobilized neutrophils become exposed to their own oxidants, resulting in self-injury (Bass et al. 1977; Pietarinen-Runtti et al. 2000). Lactoferrin, released by neutrophils and discussed earlier, falls prey to the high concentration of proteases in these areas, suffering degradation and losing its anti-biofilm properties (Singh et al. 2002). In addition, the biofilm extracellular matrix helps to replenish neutrophil-depleted oxygen needed for bacterial metabolism. Quorum sensing in *P. aeruginosa* also leads to biofilm bacterial tolerance to H_2O_2 and neutrophil killing (Bjarnsholt et al. 2005). One product of the quorum sensing system in *P. aeruginosa* is rhamnolipids. This same group has recently shown that rhamnolipid production rapidly killed accumulating neutrophils (Jensen et al. 2007), although results with other strains of *P. aeruginosa* in our hands has not resulted in killing of neutrophils, even though rhamnolipids were produced. It is yet unclear what this discrepancy of results is the result of. However, it is clear that neutrophils, and their products, are incapable of killing biofilms of *P. aeruginosa*. In other words, it appears that neutrophils help to provide the biofilm with the nutrients it needs to survive, while inflicting self-injury and hindering host immune response (Jesaitis et al. 2003). Neutrophils also lend to the pathology of lung disorders, providing a source of DNA and actin polymers that contribute to the congestive viscosity of CF lung mucous (Kirchner et al. 1996; Khan et al. 1995).

We demonstrated the ability of human leukocytes to penetrate *S. aureus* biofilms under shear force, mimicking those conditions found in vivo (Leid et al. 2002). Our data indicated that neutrophil (and other leukocytes) access to biofilm bacteria was not hindered by the biofilm structure, suggesting that other properties of biofilms are responsible for incapacitating host immune response (Leid et al. 2002). Consequently, once neutrophils settle and/or penetrate biofilms, they become unable to migrate from their point of contact and their secreted products are quickly overcome by the biofilm microorganisms. Microscopic evidence suggests that neutrophils maintain a rounded morphology, characteristic of unstimulated cells. Despite this inactivated appearance, neutrophils preserve their capacity for respiratory burst, degranulation, and phagocytosis. However, they are unable to effectively clear the infection through bactericidal activities (Jesaitis et al. 2003). The observed rounded cell morphology might be explained by production of *P. aeruginosa* exotoxins (ExoS and ExoT) injected into the neutrophil by Type III machinery (Singh et al. 2002). ExoT is a rho GTPase that works to inhibit actin polymerization, thereby

affecting cytoskeletal remodeling (Kazmierczak et al. 2001; Krall et al. 2000; Pederson et al. 1999). The induction of ExoS is capable of stimulating TLR-2 and TLR-4 simultaneously, inducing an extensive and damaging proinflammatory response that includes neutrophils (Epelman et al. 2004).

A study by Mittal et al. (Mittal et al. 2004) found that biofilm cells were able to survive neutrophil killing in *P. aeruginosa* infection of the bladder. These biofilm cells later reached renal parenchyma despite high numbers of neutrophils present at the site of inflammation (Mittal et al. 2004). This may have been facilitated by the ability of *P. aeruginosa* to induce cell death of PMNs through Type III machinery. In addition to the induction of neutrophil necrosis, bacterial induction of apoptosis may influence the severity of infection. *P. aeruginosa* virulence factors – pyocyanin, exoenzyme S, and cell surface porins – have been shown to induce apoptosis of neutrophils and macrophages (Usher et al. 2002; Kaufman et al. 2000; Buommino et al. 1999). Quorum sensing molecules (homoserine lactones) may also be associated with this apoptotic process, further influencing the resilience of *P. aeruginosa* (Charlton et al. 2000). Thus, reduced oxidative potential, the presence of quorum sensing molecules, and induced apoptosis through cytotoxins may explain *P. aeruginosa*-associated UTI resistance to host defenses (Mittal et al. 2004).

Chronic CF lung infection is largely attenuated to the mucoid *P. aeruginosa* phenotype (Govan and Deretic 1996). However, small colony variants (SCV) have been isolated from the CF lung (Haussler et al. 1999). Some SCVs express hyperpiliation, increased twitching motility, and a preference for stationary growth, possibly contributing to biofilm formation (Haussler et al. 2003). In fact, recovery of SCVs may be correlated to poor lung conditions and use of inhalative antibiotics (Haussler et al. 1999). Furthermore, genes involved in the Type III secretion system are strongly upregulated in some SCVs. This resulted in increased cytotoxicity and enhanced virulence in a murine model respiratory tract infection (von Gotz et al. 2004). These variants may be the result of mutation and selection induced by the environmental conditions of the CF lung (Haussler et al. 2003). *S. aureus* SCVs in CF patients show similar resilience to phagocytosis and cationic peptides. In fact, positively charged antimicrobial peptides may actually select for SCVs. Consequently, AMPs are further hindered by the high salt concentration common in the CF lung (Sadowska et al. 2002).

As mentioned throughout this book, there is a tendency for implanted medical devices to become coated with fibrinogen and fibronectin host proteins, resulting in bacterial colonization of the device (Francois et al. 1998). Reduced blood flow to the area surrounding the implanted medical device is another consequence of implantation, compromising host defenses by impairing natural killer cells, lymphocytes, phagocytosis, superoxide activity, and in general, leukocyte inflammation (Roisman et al. 1983). The process of frustrated phagocytosis is also associated with indwelling medical devices (Roisman et al. 1983). This occurs when professional phagocytes undergo apoptosis in response to encountering a substrate too large to engulf and destroy. Reactive oxygen products and lysosomal enzymes are released, causing host tissue injury and poor local vascularity. The host is then susceptible to the development of biofilm and/or chronic infection (Santavirta et al. 1991, 1990;

Wang et al. 1997). Furthermore, heat released during PMMA bone cement polymerization may kill surrounding bone tissue creating a nonvascularized area. This would provide bacteria with an ideal growth environment, protected from circulating host defenses.

One preponderance of the collective amount of data relating to the innate immune system and biofilms is that neutrophils recovered from implant infection sites are highly activated yet unable to control the infection (Wagner et al. 2004). A loss of migratory ability is observed while cytotoxic potential is maintained, contributing to tissue destruction (Wagner et al. 2003). Because neutrophils release nitric oxide, cytotoxins, and other bactericidal agents that are toxic to host tissues, PMN response is limited in terms of time and space. This is usually achieved by macrophages, which infiltrate the infected area and clean up spent and apoptotic neutrophils (Ward and Lentsch 1999; Dallegri and Ottonello 1997; Kaplanski et al. 2003). Uptake by macrophages is necessary to prevent tissue damage from neutrophil release of cytotoxic and proteolytic mediators (Melnikoff et al. 1989; Savill 1997). This, however, is not the case in posttraumatic osteomyelitis, a device-associated infection resulting in progressive inflammatory disease. Colonization of the implant by a Staphylococci biofilm may require implant removal and even reconstructive surgery due to tissue destruction and osteolysis (Wagner et al. 2004). Here, macrophage infiltration is not observed and neutrophil mediated tissue destruction remains unrestrained (Wagner et al. 2003). CD16, the low-affinity receptor for IgG, may be a possible explanation in the observed neutrophil longevity as loss of CD16 is associated with cell apoptosis (Dransfield et al. 1994; Homburg et al. 1995). Neutrophils observed in posttraumatic osteomyelitis patients expressed elevated levels of CD16, which may explain why neutrophils remained viable at the infection site, escaping apoptosis. As a result, chronic inflammation and eventual osteolysis associated with posttraumatic osteomyelitis occurs. The exact mechanism of CD16 regulation in this setting is not yet understood (Wagner et al. 2003).

γδ T-lymphocytes represent an additional cellular mediator in the innate immune response. Their role in biofilm defense certainly warrants further investigation. Elevated levels of these lymphocytes are commonly seen in the blood of CF patients (Perez-Payarols et al. 1994). In addition to possessing cytotoxic properties, γδ T-cells also release cytokines that activate macrophages needed to clear infection cites of bacteria as well as apoptotic host defense cells (Julia et al. 1998). However, there have been no studies to date that specifically correlate γδT cells and biofilm persistence.

Toll-like receptors (TLR) are surface molecules on innate immune cells and are important structures in activating the host's immune system cascade. For example, monocytes and macrophages rely on TLR-4 to recognize bacterial LPS, releasing Interleukin-1 (IL-1) that promotes formation of β-defensin (Hoffmann 2003). Transcription of proinflammatory cytokines by TLR binding requires myeloid differentiation factor 88 (MyD88) (Takeda et al. 2003). Even non-TLR proinflammatory mediators, such as complement fragments, are unable to facilitate efficient neutrophil recruitment in MyD88 deficient mice (Skerrett et al. 2004). TLR-4 also induces NF-$_{\kappa}$B activation, which can lead to downstream gene activation. This

pathway, however, is also reliant on functional NADPH oxidase (Sadikot et al. 2004). Production of superoxide in the respiratory burst is dependent on NADPH oxidase, as is H_2O_2 and OH ions (Babior 2004). Presence of reactive oxygen species liberates neutrophil elastase and cathepsin G from extracellular matrix, promoting host tissue injury (Reeves et al. 2002). Neutrophil elastase may cause a downregulation of TLR-4 in human bronchial epithelial cells (Devaney et al. 2003), resulting in a severely hindered innate immune system, predisposing the host to chronic infection (Hauber et al. 2005).

Upregulation of TLR-2 may occur through *P. aeruginosa* activation of TLR-4. TLR-4 is known to build tolerance against bacterial LPS, leading to hyporesponsiveness in instances of prolonged exposure (Medvedev et al. 2000). This indicates that TLR-4 is most active during the early phase of *P. aeruginosa* infection and that TLR-2 becomes more involved as the infection progresses (Power et al. 2004). TLR-2 expression works to reduce host signaling in response to adhesins during infection. This effect is troublesome in the CF patient. Mucins are constantly produced in the CF airway, resulting in excessive mucous secretion. Adhesins are needed to bind mucins; however, should the adhesins interact with the host, unblocked by TLR-2, an overexpression of calgranulins would result. In fact, excessive calgranulins are often observed in CF patients. Therefore, reduced TLR-2 expression may promote lung environment hostility and inflammation while hindering host defenses (Lorenz et al. 2004).

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