Molecular Microbiology: New Dimensions for Cutaneous Biology and Wound Healing

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The role of bacteria in the pathogenesis of chronic, nonhealing wounds is unclear. All wounds are colonized with bacteria, but differentiating colonizers from invading organisms is difficult, if not impossible, at the present time. Furthermore, robust new molecular genomic techniques have shown that only 1% of bacteria can be grown in culture; anaerobes are especially difficult to identify using standard culture methods. Recent studies utilizing microbial genomic methods have demonstrated that chronic wounds are host to a wide range of microorganisms. New techniques also show that microorganisms are capable of forming highly organized biofilms within the wound that differ dramatically in gene expression and phenotype from bacteria that are typically seen in planktonic conditions. The aim of this review is to present a concise description of infectious agents as defined by new molecular techniques and to summarize what is known about the microbiology of chronic wounds in order to relate them to the pathophysiology and therapy of chronic wounds.

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INTRODUCTION

Chronic wounds are a major health-care problem; nonhealing wounds lead to disability, decrease quality of life, and are very expensive. In the United States, the cost of chronic wounds exceeds $10 billion annually and constitutes over half of the total cost for all skin diseases (Bickers *et al.*, 2006; Kuehn, 2007). There are also substantial indirect costs through loss of income, depression, deconditioning, and impact on friends and family. Although a wide variety of antimicrobial treatments are used, including complex dressings and systemic antimicrobials, minimal evidence exists with regard to their efficacy.

Almost all chronic wounds occur in a host with a predisposing condition, such as venous disease, arterial and vascular compromise, and neuropathy (Fonder *et al.*, 2008). Systemic conditions affecting wound healing include diabetes mellitus, central nervous system compromise, trauma, inflammatory illnesses, metabolic abnormalities, coagulopathies, immunosuppression, smoking, malnutrition, and obesity. Many of these predisposing factors impair blood flow, resulting in local hypoxia that may decrease leukocyte bactericidal action by affecting oxidant-producing enzymes such as myeloperoxidase (Bowler *et al.*, 2001; Mustoe, 2004).

There are also a variety of factors that promote inflammation within the chronic wound. The combination of necrotic tissue and low oxygen tension promotes the proliferation of facultative or obligate anaerobes within the wound. High microbial burden leads to the presence of polymorphonuclear leukocytes within and around the wound. This inflammatory response encourages a sustained release of cytotoxic enzymes, matrix metalloproteinases, and free oxygen radicals (Sibbald *et al.*, 2007; Bjarnsholt *et al.*, 2008). These proinflammatory agents degrade the extracellular matrix, inhibit cell migration, and prevent wound closure (Mustoe, 2004). As a result, bacterial proliferation and colonization are believed to retard wound healing.

Chronic wounds are colonized by polymicrobial flora, originating from the external environment, local skin flora, the enteric tract, the vagina, and oral mucosa. Further complicating the local ecology of chronic wounds are their metabolic and morphological characteristics, which can affect the genotypic expression of the wound bacteria. Chronic wounds lead to persistent bacterial populations that are typically arranged into highly organized biofilms. Biofilms are cooperative communities of sessile bacteria that are embedded in an extrapolysaccharide matrix with altered phenotype and growth characteristics. As biofilms often contain bacterial communities as opposed to a single species, it is important to understand the metabolic relationships and morphological arrangements of bacteria within a wound, which, however, remain unexplored.

Biofilms are increasingly appreciated as being important in the pathogenesis of persistent infections, particularly in chronic mucosal or skin surface infections. However, biofilm-associated bacteria are also difficult to culture and even harder to treat with antimicrobials (Stewart and

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Abbreviations: c-DNA, complementary DNA; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; RNA, ribonucleic acid; rRNA, ribosomal ribonucleic acid; RT-PCR, real time polymerase chain reaction

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Costerton, 2001; Costerton, 2007; Brady *et al.*, 2008). Until recently, the complexity of wound microflora was underappreciated because standard culture techniques could not identify most bacteria—particularly anaerobic organisms, which are notoriously difficult to isolate (Bowler *et al.*, 2001; Davies *et al.*, 2001; Kaeberlein *et al.*, 2002). Therefore, it has been difficult to establish the roles of microorganisms within the wound, and to ascertain which microorganisms are deleterious and impair healing, which are ''benign'' colonizers, and which may even facilitate healing processes. One interpretation suggests that some commensal biofilms may prevent colonization by pathogens (Costerton *et al.*, 2003).

Despite the paucity of data on the bacterial composition of chronic wounds or on how to effectively treat them, systemic antibiotics are used liberally in these situations. Although antibiotics can be useful when there is local invasion or systemic infection, there is little evidence to support their use in chronic wound care, especially when there is clear observational evidence that wound characteristics remain unchanged over time. To determine the most effective method of treatment of chronic wounds, it is essential to understand the relationship between the presence of bacteria and delayed wound healing. Furthermore, it is critical to determine the metabolic relationships that exist between bacteria and the morphological arrangement of bacteria within a wound. There is increasing evidence that specific bacterial species or a high bacterial load may inhibit wound healing.

To establish the role of bacteria in wound healing, it is necessary to define the full panoply of organisms within a wound. Genomic tools such as nucleic acid amplification, rapid DNA sequencing such as pyrosequencing, and development of 16S ribosomal clone libraries have revolutionized our ability to understand the microbiology of chronic wounds and may revolutionize our approach to the use of antimicrobial agents in the therapy of chronic wounds. These new technologies facilitate a full investigation of the flora within a wound, as they identify the large number of organisms that are fastidious or noncultivable. This fundamental information can be correlated with clinical course to develop testable hypotheses and evidence-based algorithms for the treatment of bacteria in chronic wounds. This paper reviews the most current data to provide an analysis of the opportunities now available to investigate the role of bacteria in chronic wounds.

MICROBIAL GENOMICS

Bacterial genomics has revolutionized the field of infectious diseases. For example, the causative bacterial agents of cat-scratch disease and Whipple's disease were only conclusively identified with the use of genomic methods. Without highly specialized equipment and procedures, traditional cultures cannot detect fastidious or slowly growing organisms such as anaerobes and mycobacteria (Costerton, 2007; Dowd *et al.*, 2008).

Defining the bacterial microbiome was made possible by the discovery of 16S ribosomal DNA sequences, known as the universal primer. These sequences are universally found in bacterial species and are not found in eukaryotes. Furthermore, species-unique sequences, which can be used to identify individual bacterial species, are typically found adjacent to the 16S ribosomal DNA. To identify bacterial DNA within a specimen, the 16S DNA is first amplified, which identifies the bacterial DNA, and then the flanking sequences are identified and compared with a known library of bacterial sequences, which then identifies the species. In situations in which multiple bacteria may be present, the initial approach was to develop clone libraries of bacterial DNA obtained from the specimen (typically 100–1000 clones), and to sequence each individual library and identify the species source. This technique involved a substantial amount of repetition.

Newly developed techniques, such as pyrosequencing and microarrays (Whitley, 2008), automate this process and have demonstrated that the human microbiome is incredibly complex, with only a minority of bacterial organisms in the environment and human host identifiable by culture. Genomic techniques have the capacity to overcome the bacterial sampling problems that are often encountered in chronic wounds or in other settings in which multiple species coexist. In complex bacterial mixtures, it is technically difficult to separate and identify more than three to six species because of different concentrations of bacteria and interspecies competition. Full-genome sequencing alleviates this problem. Molecular techniques can also define patterns of genetic and protein expression under different clinical and morphological conditions. This is particularly important in understanding the role of biofilms in chronic wounds.

These new techniques are already dramatically changing our understanding of the ecology and evolution of microorganisms. Goldenfeld and Woese (2007) point out that our current understanding of horizontal gene transfer (which is responsible for much of the spread of antibiotic resistance) indicates that microorganisms ''absorb and discard genes as needed'' in response to changes in their environment. As genes are rapidly passed between species, the boundaries between ''species'' in the microbial world are blurred (Jain *et al.*, 1999). Extensive communication between microbes in biofilms indicates that the microbial ecosystem might be best viewed as a cooperative community rather than as individual organisms or species.

One caveat in interpreting genomics data is that sequencing identifies DNA from both viable and nonviable organisms. DNA fragments from organisms that have lyzed in response to host defense activity or antimicrobials may be incorrectly identified as viable species within the specimen. Future generations of genomics will likely focus on identifying the transcriptome, which is a reflection of RNA messenger activity within the specimen and thus reflective of the bacteria currently living in the specimen.

Molecular techniques and metagenomics

Many different techniques have been developed to identify microorganisms in a specific environment (Table 1). Whole-genome DNA–DNA hybridization, which compares the

Table 1. Common methods used to analyze bacterial populations in chronic wounds

Method	Technique description	Processing time and costs	Advantages	Disadvantages	Practice implications
Qualitative cultures	Swab culture of wound; Standard bacteriology culture	1 h for Gram stain; 1–3 days for culture results. Inexpensive	Inexpensive, widely available	Low sensitivity, not quantitative	Rapid and widely available, used to screen for methicillin-resistant *S. aureus* or *Pseudomonas*
Quantitative culture	Tissue specimen homogenized and organisms identified and quantified	3–4 days for speciation and quantification; expensive	Accurate and reproducible	Labor intensive, requires specialized facilities and not widely available. Long lag time	Widely used in Burn units, but clinical correlation not well defined
Nucleic acid amplification-PCR	DNA is extracted from specimens and amplified, using organism-specific primers	4–6 h; moderate cost	Rapid turnaround, highly sensitive. Equipment increasingly available	Requires testing for known organisms. Not useful for identifying ''unknowns'', not clinically standardized	Research settings largely. Used for methicillin-resistant *S. aureus* surveillance
Metagenomic methods	DNA is extracted from wound specimens, amplified and sequenced. All bacterial clones in specimen are sequenced	Several days; requires dedicated facility and personnel; expensive	Identifies all bacterial sequences within a specimen, including fastidious organisms and organisms in low numbers	Expensive, long turnaround time, not standardized. Sequences identified may include surface contaminants and nonviable organisms	Research settings at present, needs clinical correlation

genomes of two different species under standardized conditions, was one of the first techniques used, but is limited because it can only be used in cultured microorganisms and remains a time-intensive process. PCR, genetic fingerprinting techniques, and metagenomic techniques have largely replaced it as a tool for species identification (Pontes *et al.*, 2007). PCR is used to amplify DNA or c-DNA, which is derived from RNA. DNA samples identify all of the organisms that are present in an ecosystem, whereas c-DNA samples can identify those species that are metabolically active at the time of sampling. Once amplified, the DNA or c-DNA products can be analyzed with a variety of techniques, such as denaturant-gradient gel electrophoresis, temperature-gradient gel electrophoresis, terminal restriction fragment length polymorphism, single-stranded conformation polymorphism, probe hybridization, microarrays, high-throughput pyrosequencing, or shotgun Sanger sequencing. Specific bacterial populations can also be identified in tissue samples using fluorescent *in situ* hybridization.

Many of these techniques have been adopted for use in metagenomic analysis, which is the most recent development in molecular studies. Metagenomics is used to determine the entire genetic composition of a microbial community in an ecosystem by directly isolating DNA from the environment, tagging and classifying each component, and amplifying it with PCR. Metagenomic methods can detect almost all the DNA signatures of microorganisms within a wound, even those present in low numbers or in a dormant metabolic state (Costerton, 2007). For example, one study of *Staphyloccus aureus* colonization in the female reproductive tract compared 16S rRNA-directed fluorescent *in situ* hybridization probes with culture; *S. aureus* was identified in 100% of samples using the probes (confirmed using additional molecular studies), whereas swab and culture only yielded *S. aureus* in 10.8% of samples (Veeh *et al.*, 2003). Recently developed systems also bypass the need for cloning, which allows the identification of microflora that cannot be cultured (Pontes *et al.*, 2007; Blow, 2008). The hope is that metagenomics will give additional information with regard to the genetics, physiology, and relationships in microbial communities (López-García and Moreira, 2008).

Molecular technology has also revolutionized the study of bacterial morphology and localization in biological systems. Fluorescent *in situ* hybridization with probes for 16S RNA permits the localization of specific bacterial ecosystems within the wound to determine the spatial relationships that are formed between these microorganisms in microbial communities or biofilms.

Molecular techniques and chronic wounds

Molecular techniques have not been used widely to characterize chronic wounds, although preliminary small studies have characterized the microflora of some chronic wounds and the human skin microbiome. There have been no published molecular studies that have characterized and compared skin microbiome in disease (e.g., diabetes) and nondisease states.

The few molecular studies of chronic wounds and intact skin have powerfully demonstrated the importance and utility of these methods (Melendez JH, Frankel YM, An AT, Williams L, Price LB, Wang NY, Lazarus GS, Zenilman J (under final review) Evaluation of multiplex real-time PCR assays in comparison to culture-based approaches for species identification of bacteria in chronic wounds. *J Clin Microbiol*).

We recently used reverse transcription–PCR to identify common pathogens in chronic wounds and recovered microorganisms in eight of 11 specimens that were negative by quantitative culture; reverse transcription–PCR was also 91.7% sensitive for common Gram-negative and Gram-positive organisms recovered by quantitative culture (Frankel YM, Melendez JH, Wang NY, Price LB, Zenilman J, Lazarus GS (in press) Defining wound microbial flora; molecular microbiology unlocks new horizons. *Arch Derm*). Other studies used molecular techniques to examine human skin microflora and the manner in which it changes over time, and the associations between skin pathology and microorganisms (Dekio *et al.*, 2005, 2007; Gao *et al.*, 2007; Grice *et al.*, 2008; Paulino *et al.*, 2008). Although these methods have not yet been widely applied in clinical laboratories, the advantage of reverse transcription–PCR and similar techniques is that they have rapid turnaround times, typically within 4–6 hours.

The availability of large-scale genomic sequencing has further increased our understanding of chronic wound pathology. We recently evaluated the complete bacterial genomics of 25 chronic wounds seen at our referral center. Using pyrosequencing, we found an average of 10 different bacterial families in the wounds, which is approximately four times more than that estimated by culture. The most prevalent bacteria belonged to the *Clostridiales* family XI, which suggests that fastidious anaerobes have a critical function in the pathogenesis of chronic wounds (Price LB, Liu CM, Melendez JH, Frankel YM, Engelthaler D, Aziz M *et al.* (in press) Community analysis of chronic wound bacteria: impact of diabetes and antibiotics. *PLoS*). Although this finding has been observed in a few small studies (Bowler *et al.*, 2001), genomic studies suggest that anaerobic predominance is much higher than previously suspected.

Furthermore, chronic wounds include a variety of anaerobic species found in the gut, such as *Bacteroides* and *Peptostreptococci*. However, we also identified the *Mobiluncus*, *Gardenella*, and *Atopibium* species. The association between mixtures of anaerobic species and mucosal inflammatory infection has been observed in other body sites, such as oral or periodontal infections, and in the female genital tract. In particular, the *Mobiluncus*, *Gardenella*, and *Atopibium* species have been found in the vaginal flora of patients with bacterial vaginosis and have been identified as potential pathophysiological agents (Fredricks *et al.*, 2005; Oakley *et al.*, 2008). One theory for approaching both bacterial vaginosis and chronic wounds might be to consider them as anaerobic synergistic communities (or infections) wherein there is an environment that promotes the growth of species that interact both within themselves and with the host to produce an inflammatory response. Further research is needed to assess whether a unifying anaerobic hypothesis is valid.

BIOFILMS IN CHRONIC WOUNDS

Biofilms are communities of sessile microbial cells that attach to a surface and secrete a hydrated extracellular polymeric substance matrix. The organisms become embedded in this matrix, which is composed of polysaccharides, proteins, glycoproteins, glycolipids, and extracellular DNA (Whitchurch *et al.*, 2002; Percival and Bowler, 2004). The extracellular polymeric substance matrix supports microcolonies of cells, allows cell–cell communication, forms water channels, retains and concentrates nutrients, and can support gene transfer through conjugation, transformation, and transduction (Costerton *et al.*, 1999; Flemming *et al.*, 2007; Davis *et al.*, 2008). Biofilms are the predominant phenotype in natural environments and chronic infections, whereas the planktonic state is typically transient (Costerton, 2007; Wolcott and Ehrlich, 2008). It seems that the same is true of chronic wounds. Kirketerp-Møller *et al.* (2008) recently published a study examining the structural organization of bacteria in chronic wounds using PNA-fluorescent *in situ* hybridization. The study established that *Pseudomonas aeruginosa* aggregated in microcolonies embedded in extracellular polymeric substance in these chronic wounds. There were few planktonic or single organisms observed in the wounds.

The formation of a biofilm is a dynamic process, and the earliest stages of formation can occur within hours of inoculation (Schaber *et al.*, 2007; Davis *et al.*, 2008). Bacterial cell–cell communication is a critical function for ensuring survival in harsh environments, and mechanisms for this communication include cell–cell signaling and quorum sensing. Quorum sensing is a bacterial signaling mechanism that regulates organism multiplication in relation to the local bacterial population. For example, as the population reaches a threshold, bacterial cell turnover slows by a regulation of transcriptional factors. Quorum sensing relies on small molecules to act as ligands for transcriptional regulators, and has been elegantly described in *P. aeruginosa* biofilms (Davies *et al.*, 1998; Singh *et al.*, 2000). Owing to their importance in maintaining biofilms, quorum-sensing molecules have been identified as, to our knowledge, hitherto undisclosed targets for antimicrobial agents (Balaban *et al.*, 2005).

Bacterial biofilms are important in the pathogenesis of a variety of mucosal site infections, such as urinary tract infections (Nickel *et al.*, 1994), periodontal infections, chronic wounds, and chronic bronchitis, in patients with cystic fibrosis (Singh *et al.*, 2000); in persistent infections such as osteomyelitis (Brady *et al.*, 2008), endocarditis (Presterl *et al.*, 2005), and prostatitis (Costerton *et al.*, 2003); and in a variety of infections of prosthetic devices and medical instruments, including orthopedic implants, heart valves, and indwelling catheters (Costerton *et al.*, 1999; Hall-Stoodley *et al.*, 2004).

There is evolving evidence to show that biofilms are also involved in the chronicity of wounds and in abnormal wound healing. James *et al.* (2008) showed that 60% of chronic wounds contained a biofilm on light and scanning electron microscopy, compared with only 6% of acute wounds. Their group also found that these biofilms were composed of diverse polymicrobial communities, and many bacterial species were identifiable only by PCR.

Biofilm resistance to antimicrobial agents and host defenses

Owing to the properties of extracellular polymeric substance and quorum sensing, cells can change their proteome to exist in a sessile state with low metabolic levels and downregulated cell activity (Brady *et al.*, 2008). In addition, bacteria in a biofilm express very different sets of genes than does the planktonic form of the same species; up to 50% of the proteome may differ from the same microorganism in a planktonic state (Costerton *et al.*, 2003; Fux *et al.*, 2005; Clutterbuck *et al.*, 2007). These changes have important implications for clinical therapeutics. Most antimicrobials work by altering processes in reproducing or metabolically active organisms. As a result of their low metabolic activity, microorganisms in biofilms are far more difficult to eradicate with conventional antimicrobials than are planktonic microorganisms (Davis *et al.*, 2008; Stewart and Franklin, 2008). Treatment will often suppress the symptoms of an infection without eradicating the causative organisms (Clutterbuck *et al.*, 2007), which explains why infections often recur once an antimicrobial treatment is stopped (Stewart and Costerton, 2001). A recent study by Davis *et al.* (2008) demonstrated that *S. aureus* existing in a biofilm on a wound surface displayed greater antimicrobial resistance to topical mupirocin and triple antibiotic ointment cream than did planktonic communities of *S. aureus*. Indeed, it has been reported that some biofilms can persist with concentrations of antibiotics and antimicrobial agents 100–1000 times higher than the concentration that can inhibit planktonic cells (Nickel *et al.*, 1985; Wolcott and Ehrlich, 2008).

Biofilm-associated organisms also tend to be resistant to host defenses (Costerton *et al.*, 2003). The polysaccharide matrix of the biofilm can block complement activation, inhibit chemotaxis and degranulation of polymorphonucleocytes, and can render antibodies ineffective (Percival *et al.*, 2008). Leid *et al.* (2002) demonstrated that human leukocytes could penetrate an *S. aureus* biofilm, but could not engulf any bacteria despite the proximity of *S. aureus*. Conversely, human leukocytes had no difficulty phagocytosing planktonic *S. aureus* (Leid *et al.*, 2002). *S. aureus* in biofilms also exhibit different immunogenic proteins than the proteins expressed in acute, septic infections. Although the immunogenic proteins of *S. aureus* biofilms lead to a significant antibody response, this response is still generally ineffective in clearing the infection (Brady *et al.*, 2006). This ineffective phagocytosis results in tissue damage, which in turn induces a migration of increased numbers of polymorphonuclear leukocytes and macrophages into the area. Although they are unable to kill the microorganisms in the biofilm, they release large amounts of proinflammatory cytokines and enzymes that lead to a destruction of surrounding tissue (Percival *et al.*, 2008).

Treatment of biofilms

Treating suspected biofilm infections in chronic wounds is challenging. The most important intervention is to effectively treat the underlying systemic disease that led to the development of the chronic wound. For the direct treatment of a wound, there are three widely used approaches, although the evidence supporting these interventions is limited. The first approach is debridement, a cornerstone of wound management and certainly a sensible strategy. The second approach is the use of local dressings, particularly those that contain silver. Several studies have found that silver-containing wound dressings are active against *in vitro* biofilm models, particularly in concentrations of 5–10 micrograms per milliliter (Bjarnsholt *et al.*, 2007; Percival *et al.*, 2007, 2008). The third treatment approach is the use of systemic antimicrobials when the infected area spreads beyond the wound border, involves underlying bone or tissues, or becomes systemic. Systemic antimicrobials should be used rationally, using both known organism susceptibility and the probability of specific organisms being present in various wound types.

There are currently very few published alternative options to these treatment strategies (Brady *et al.*, 2008). Clearly, this is an attractive area for intervention. At the present time, the best strategy remains aggressive debridement, silver-containing dressings and ointments, and antibiotic therapy, particularly if there is a method for obtaining an accurate characterization of microorganisms in the wound.

CLINICAL APPROACHES TO SAMPLING

Wound cultures are obtained using a variety of sampling techniques, including superficial swabs, deep swabs, deep tissue biopsies, curettes, and aspiration. Superficial swabs are commonly used in clinical setting, but are believed to be inaccurate. Superficial swabs do not properly identify anaerobes and instead may isolate noninvasive ''innocent bystanders'' in the wound that have colonized the surface (Urbancic-Rovan and Gubina, 2000; Lipsky *et al.*, 2004). Several investigators have demonstrated that anaerobic yield is higher for curette or deep tissue biopsy than it is for superficial swab (Urbancic-Rovan and Gubina, 2000; Davies *et al.*, 2007), although facultative and aerobic populations demonstrate more similarity (Davies *et al.*, 2007).

Deep tissue biopsy provides an enhanced sensitivity and specificity for invasive organisms, but requires technical skill and can aggravate the wound if performed improperly. Some researchers have also suggested that deep tissue biopsy may underrepresent the full diversity of wound microflora (McGuckin *et al.*, 2002). Curettage correlates better with deep tissue biopsy, is relatively noninvasive, can sample surface and invading microflora, and can be used to reliably isolate anaerobic bacteria (McGuckin *et al.*, 2002). Our group recently demonstrated that using a single brisk harvest with a 3 mm curette at the advancing border of a wound reliably and reproducibly produces 20 mg of tissue, an amount that is sufficient to perform quantitative cultures and multiple genomic analyses (Frankel YM, Melendez JH, Wang NY, Price LB, Zenilman J, Lazarus GS (under review) Defining wound microbial flora; molecular microbiology unlocks new horizons. *Arch Derm*).

The sample should be cultured quantitatively rather than qualitatively, if molecular techniques are not available. Studies, including work performed in our laboratory and in a clinical setting, have consistently shown that careful

quantitative cultures are reproducible, more reliable, and more accurate than the traditional qualitative approaches (Danilla *et al.*, 2005). The superiority of quantitative cultures is related to specimen acquisition and standardization. Quantitative cultures are performed on carefully collected, weighed, and processed specimens, and are usually performed on tissue, in a research setting. Qualitative cultures are often based on an unstandardized swab sweep of a wound, and cannot be standardized. Furthermore, in practice, culture accuracy may be affected by structural variables such as transit time and conditions.

Implementing quantitative culture in practice is limited because of increased expense, decreased availability, and the longer turnaround time required. Clinicians, however, should be cognizant of the limits of qualitative culture. We believe that the utility of qualitative culture is largely limited to ruling out the presence of certain key pathogens, such as methicillin-resistant *S. aureus* and *Pseudomonas*, which in turn drive the therapeutic decisions with regard to treatment for these organisms.

MICROBIOLOGY OF CHRONIC WOUNDS

Chronic wounds are colonized by a diverse array of endogenous microorganisms derived from the skin, oral mucosa, enteric tract, and environment. The microbial population in wounds is significantly different from that found on normal skin; chronic wounds typically have a diverse polymicrobial community with a mix of aerobic and anaerobic bacteria. The microflora also seems to be influenced by the underlying etiology of the chronic wound, as the ratio of aerobic to anaerobic bacteria may differ depending on whether the wound is a venous, diabetic, or pressure ulcer (Dowd *et al.*, 2008).

Pressure ulcers

Pressure ulcer develops when force on a bony prominence obstructs capillary flow to the skin, leading to tissue necrosis (Eaglstein and Falanga, 1997). It is estimated that 1.3–3 million adults have a pressure ulcer in the United States, which costs about $5 billion dollars per year (Lyder, 2003; Medina *et al.*, 2005). Multiple studies have examined the

Table 2. Average number of bacterial species isolated from pressure, diabetic, and venous ulcers in each study using traditional culture methods

Ulcer type	Article	Average number of organisms per culture	Common aerobic organisms isolated in culture	Common anaerobic organisms isolated in culture
Pressure	Sapico *et al.* (1986)	5.8 (necrotic tissue)	*E. coli*	*Bacteroides* spp.
		1.7 (necrotic tissue)	*Proteus* spp.	*Peptostreptococcus* spp.
	Sopata *et al.* (2002)	1.2	*S. aureus*	*Propionibacterium* spp.
			S. epidermidis	*Clostridium* spp.
			P. aeruginosa	
			Klebsiella pneumoniae	
			Enterococcus faecalis	
Diabetic	Citron *et al.* (2007)	5	*S. aureus*	*Peptostreptococcus* spp.
	Gerding, 1995)	4.1–5.8	Coagulase-negative *Staphylococcus* spp.	*Peptococcus* spp.
	Johnson *et al.* (1995)	3 anaerobes	*Streptococcus* spp.	*Prevotella* spp.
	Louie *et al.* (1976)	5.8	*Corynebacterium* spp.	*Bacteroides* spp.
	Raja (2007)	1.47	*Enterobacteriaceae (Proteus, Klebsiella, Enterococcus, E. coli)*	*Clostridium* spp.
	Sapico *et al.* (1980)	4.7		
Venous	Bowler and Davies (1999)	5.1 (infected ulcers)	*S. aureus*	*Peptostreptococcus* spp.
		3.6 (noninfected ulcers)	*S. epidermidis*	*Peptococcus* spp.
	Brook and Frazier (1998)	2.3	*Streptococcus* spp.	*Finegoldia* spp.
	Davies *et al.* (2007)	2.7 (swab)	*Enterococcus* spp.	*Bacteroides* spp.
		2.8 (deep tissue biopsy)	*P. aeruginosa*	*Propionibacterium acnes*
	Gilchrist and Reed (1989)	2.7	*Proteus mirabilis*	
	Hansson *et al.* (1995)	2.4		
	Lookingbill *et al.* (1978)	1.8		
	Davies *et al.* (2004)	3.25 (swab)		
		2.6 (deep tissue biopsy)		

Common aerobic and anaerobic microorganisms isolated from pressure, diabetic, and pressure ulcers using traditional culture methods.

Table 3. Common aerobic and anaerobic microorganisms isolated from pressure, diabetic, and pressure ulcers in recent investigations using molecular techniques

Ulcer type	Article	Common aerobic organisms isolated using molecular techniques	Common anaerobic organisms isolated using molecular techniques
Pressure	Dowd *et al.* (2008)	*S. dysgalactiae*	*Anerococcus* spp.
		Serratia spp.	*Peptococcus* spp.
		Streptococcus spp.	*Dialister* spp.
			Peptoniphilus spp.
			Finegoldia magna
Diabetic	Dowd *et al.* (2008)	*S. aureus*	*Peptoniphilus asaccharolyticus*
		Rhodopseudomonas spp.	*Clostridium* spp.
		Bacteroides fragilis	*Veillonella atypia*
		Morganella morganii	*Finegoldia* spp.
		Pseudomonas spp.	*Anaerococcus vaginalis*
		Enterococcus spp.	
		Hemophilus spp.	
		Stenotrophomonas spp.	
		Citrobacter spp.	
Venous	Dowd *et al.* (2008)	*S. aureus*	*Clostridium* spp.
	Davies *et al.* (2004)	*Pseudomonas* spp.	*Peptoniphilus* spp.
	Hill *et al.* (2003)	*Proteus* spp.	
		Stenotrophomonas maltophilia	
		Enterobacter cloacae	
		Serratia spp.	
		Sphingomonas sp.	
		Corynebacterium spp.	
		Acinetobacter spp.	

microflora that affect these wounds; both superficial and deep tissue cultures have been used, and the results are fairly consistent. The predominant organisms isolated by culture are *S. aureus, S. epidermidis,* and *Streptococcus* spp. (Table 2) (Daltrey *et al.*, 1981; Sopata *et al.*, 2002; Heym *et al.*, 2004). Other bacteria that consistently appear include *Proteus mirabilis, P. aeruginosa,* and *Propionibacterium* spp. Unfortunately, although some of the wounds were cultured for anaerobes, the anaerobic incubation period and manner of collection in these studies were not described. By contrast, Sapico *et al.* (1986) incubated anaerobic cultures for 14 days, and isolated significant numbers of anaerobes, including *Bacteroides* spp., *Peptostreptococcus* spp., and *Clostridium* spp., in addition to typical aerobic microorganisms.

Dowd *et al.* (2008) used molecular techniques to confirm that anaerobes are significant pathogens in pressure ulcers, and found that these bacterial communities are extremely diverse (Table 3). A total of 62% of the sequences isolated from pressure ulcers were from anaerobic microorganisms. The sequencing and culture results demonstrate that the predominant organisms in pressure ulcers seem to be strict anaerobic Gram-positive cocci.

Gram-positive anaerobic cocci have several mechanisms by which they may stall or halt wound healing. These bacteria release a variety of hydrolytic enzymes, including hyaluronidase, gelatinase, and collagenase, which might lead to increased extracellular matrix turnover and inflammation (Steffen and Hentges, 1981). They also release short-chain fatty acids that have been shown to inhibit proliferation of fibroblasts, keratinocytes, and endothelial cells (Heerdt *et al.*, 1997). Short-chain fatty acids may also be the causative agent of the malodor associated with anaerobic organisms, and may also contribute to hyperlipidemia associated with very large wounds, which slows wound healing in diabetic and nondiabetic patients (Wall *et al.*, 2002).

Diabetic ulcers

Diabetic ulcers are a common cause of disability: up to 25% of individuals with diabetes suffer from a foot ulcer during their lifetime (Singh *et al.*, 2005). Although diabetic ulcers are primarily caused by peripheral neuropathy and arterial insufficiency, diabetic patients also have impaired humoral immunity to microorganisms. Diabetic polymorphonuclear leukocytes have impaired bactericidal activity against

P. aeruginosa as compared with normal leukocytes, and diabetic serum has a persistent inhibitory effect on both normal and diabetic leukocytes, even in the presence of intensive diabetic management (Naghibi *et al.*, 1987). In addition, increased plasma glucose stimulates the growth of Gram-positive organisms while inhibiting the growth of Gram-negative organisms (Robson and Heggers, 1969a). High glucose levels have been associated with an increased risk of wound infection in both humans and animal models (Krizek and Davis, 1964; Guvener *et al.*, 2002; Hirsch *et al.*, 2008), and hyperglycemia has been demonstrated to be associated with Gram-positive septicemia (Robson and Heggers, 1969b; Robson, 1970).

Diabetic ulcer healing is frequently exacerbated by infection. The Eurodiale study reported that 58% of diabetic foot ulcers were infected at presentation, on the basis of the presence of at least two of the following clinical signs: frank purulence, warmth, erythema, lymphangitis, edema, pain, fever, and foul smell (Prompers *et al.*, 2007). However, many diabetics have an impaired inflammatory response and may not show the classical signs of infection in a wound with a high microbial burden (Gardner and Frantz, 2008). Indeed, Gardner *et al.* (2001) demonstrated little correlation between microbial burden and signs of infection (including classical signs such as pain, erythema, edema, purulent exudate, and secondary signs such as serous exudate, friable granulation tissue, foul odor, and so on), thereby suggesting that the true numbers of infected diabetic ulcers are being underestimated. Diabetic wounds may also heal more slowly *a priori*; researchers have demonstrated that a diabetic pig model shows decreased levels of growth factors such as IGF-1 and has delayed reepithelialization of wounded tissue as compared with controls (Velander *et al.*, 2008).

Microbiological studies of diabetic foot infections generally show polymicrobial flora, and most studies have isolated *S. aureus*, coagulase-negative staphylococci, *Streptococcus* spp., *Corynebacterium* spp, and *Enterobacteriaceae* (including *Proteus* spp., *E. coli, Proteus* spp., *Klebsiella* spp., and *Enterococcus* spp.) from diabetic ulcers (Table 2) (Louie *et al.*, 1976; Sapico *et al.*, 1980; Wheat *et al.*, 1986; Gerding, 1995; Johnson *et al.*, 1995; Howell-Jones *et al.*, 2005; Raja, 2007). The percentage of anaerobes isolated varies depending on the study, but despite the differences in the quantity of anaerobic colonization, the predominant anaerobes in all studies were the same: *Peptostreptococcus* spp., *Peptococcus* spp., *Prevotella* spp., and *Bacteroides* spp. (Louie *et al.*, 1976; Wheat *et al.*, 1986; Gerding, 1995; Johnson *et al.*, 1995; Citron *et al.*, 2007). It is important to note that a sizable number of diabetic ulcers seem to be colonized by anaerobic bacteria, and the role of anaerobes in retarding wound healing has generally been overlooked (Bowler, 2002).

The only study that specifically examined diabetic ulcers using molecular techniques isolated 30% anaerobic bacteria from the samples (Dowd *et al.*, 2008). The strict and facultative anaerobes included *Peptoniphilus, Finegoldia, Rhodopseudomonas, Enterococcus, Veillonella, Clostridium, Morganella, Anaerococcus*, and *Citrobacter* spp. The most common aerobic organisms recovered were *Staphylococcus, Pseudomonas, Haemophilus,* and *Stenotrophomonas* spp (Table 3) (Dowd *et al.*, 2008). As expected from a study using molecular techniques, the number and types of organisms showed much more variability than those isolated by culture techniques alone.

Venous ulcers

The majority of lower leg ulcers are caused by venous disease and account for over $3 billion annually and a loss of 2 million working days (McGuckin *et al.*, 2002). An estimated 0.2–1% of the population in developed countries suffers from venous ulcers (Nelzén *et al.*, 1991; Medina *et al.*, 2005), and treatment costs an average of $9685 per patient (Olin *et al.*, 1999). These ulcers can be challenging to treat and are prone to recurrence. Venous ulcers occur in patients with venous hypertension, stasis, or thrombosis, and the tissue surrounding the wound is often edematous with hyperkeratosis, hemosiderin deposition, dilated venules, and dermatitis (Hansson, 1988; Bergan *et al.*, 2006). Histologically, venous ulcers are characterized by fibrotic cuffs around dermal capillaries that may interfere with the exchange of oxygen and nutrients between the dermis and blood (Medina *et al.* 2005).

Venous ulcers are colonized by a wide variety of aerobic and anaerobic organisms. The most common aerobic bacteria cultured from venous ulcers include *S. aureus, S. epidermidis, Streptococcus* spp., *Enterococcus* spp., *P. aeruginosa*, and *Proteus mirabilis* (Table 2) (Eriksson *et al.*, 1984; Gilchrist and Reed, 1989; Hansson *et al.*, 1995; Brook and Frazier, 1998; Davies *et al.*, 2007). The most common anaerobes isolated from these wounds are *Peptococcus* spp, *Peptostreptococcus* spp., *Finegoldia* spp., and *Bacteroides* spp. (Gilchrist and Reed, 1989; Hansson *et al.*, 1995; Brook and Frazier, 1998).

Several investigators have studied the microflora of chronic leg ulcers without restricting the type of chronic leg ulcer examined. However, because the majority of chronic leg ulcers studied were probably venous ulcers, the results from these studies are likely reflective of the microflora of venous ulcers. Indeed, these studies isolated a similar microbial profile as compared with those that only investigated venous ulcers (Lookingbill *et al.*, 1978; Bowler and Davies, 1999). Bowler and Davies (1999) also examined the difference in microflora between ''infected'' and ''noninfected'' chronic leg ulcers (as defined by clinical criteria), and found a higher percentage of anaerobic isolates in infected leg ulcers as compared with those in noninfected leg ulcers (49 *versus* 36%, respectively).

Unfortunately, not all of these studies used standardized techniques to isolate and culture bacteria, and several did not use adequate methods to recover significant pathogens. For example, Schraibman (1990) was able to isolate β-hemolytic streptococci from 18% of chronic leg ulcers using selective streptococci media to culture the samples, which is significantly higher than that reported in other studies in which selective media were not used (Eriksson *et al.*, 1984). In addition, several of the studies did not appropriately assess the presence of anaerobes by using a

long incubation time. The highest yield of anaerobic bacteria (58% of samples) was obtained in a study that incubated samples in a thioglycolate broth for 14 days (Brook and Frazier, 1998).

Recently, venous ulcers were investigated using molecular techniques (Table 3). Hill *et al*. (2003) was the first group to compare 16S rRNA gene sequencing with culture data in one chronic venous leg ulcer. *Acinetobacter* spp, *S. epidermidis, Proteus* spp., and *C. tropicalis* were isolated from tissue swab and biopsy. In contrast, 26 different clones were isolated using 16S rDNA sampling, including clones that were related to uncultured organisms such as *Morganella morganii* or *Bacteroides ureolyticus*. Several phylotypes, which to our knowledge are previously undisclosed, were also isolated (Paster *et al*., 2002).

Davies *et al*. (2004) published a larger-scale study comparing 16S rRNA gene sequencing with culture and found similar results. Eight healing and 10 nonhealing venous ulcers were examined; *Staphylococcus* spp., *Pseudomonas* spp., *Micrococcus* spp., and *Streptococcus* spp. were isolated from the majority of cultures. However, 40% of the bacterial strains recovered by PCR and denaturant-gradient gel electrophoresis were not cultured from swabs of the same wounds. In addition, most of the strains isolated by molecular techniques had >95% sequence identity with known wound microflora. This finding indicates that many of the species recovered by PCR and denaturant-gradient gel electrophoresis can be cultured, but were likely not isolated by culture because of factors such as competition, overgrowth, or an ''uncultivable'' or sessile growth state. The findings also reveal that there is substantially more variability in these venous ulcers than has been appreciated.

When Dowd *et al*. (2008) examined venous ulcers, the predominant organisms isolated were *Enterobacter, Pseudomonas, Proteus* spp., *S. aureus*, *Stenotrophomonas* spp., and *Serratia*. As in the studies by Hill *et al*. (2003) and Davies *et al*. (2004), significantly more microorganisms were isolated using molecular techniques than by culturing methods. Overall, far fewer anaerobic species were isolated from venous ulcers than from diabetic or pressure ulcers.

CONCLUSION

The microflora of chronic wounds is far more complicated than that previously suspected. Not only are there more species of microorganisms inhabiting wounds than previously realized, it is also clear that these microorganisms live in complex structures that are remarkably different from populations of planktonic bacteria. Most microorganisms in chronic wounds exist in biofilms, and relatively few bacteria in natural environments and chronic infections exist in the planktonic form. This information has important clinical and therapeutic implications. Antimicrobials are excellent at killing planktonic bacteria, but are unable to eradicate the nidus of infection in biofilms. As a result, many chronic wounds become infected repeatedly with the same microorganisms.

To appropriately treat chronic, poorly healing ulcers, it is necessary to understand the flora of the lesion compared with that in normal skin, and determine the composition of biofilms in the wound. To understand the role of microorganisms in the pathophysiology of delayed wound healing, we must define the spectrum of the species of bacteria present and determine how differing ecological systems correlate with wound healing. We also need to understand the synergistic relationships between bacteria and the communication that occurs within biofilms. The molecular techniques described in this review will enable these advances. Molecular techniques, which are rapid and precise (if performed correctly), will become the new gold standard for determining the presence of organisms within a wound. These new data may enable us to design evidence-based antimicrobial interventions that will decrease the scourge of chronic wounds in our rapidly aging population.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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