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Influence of Microorganisms on the Healing of Skin Grafts from Chronic Venous Leg Wounds

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1. Introduction

Chronic wounds are associated with a significant morbidity and continue to affect an increasing number of patients. In developed countries an estimated 1-2% of the population will suffer from a chronic wound at some point in their lifetime. Chronic wound treatment is associated with considerable expenses and in the United States alone more than 25 billion dollars are spent on treatment annually (Percival et al., 2011).

Although it is well known that infection is involved in making the wound chronic and unable to heal, the process for identifying bacterial flora within wounds is not standardized (Frankel et al., 2009). The microorganisms associated with chronic wounds are usually investigated by traditional culture–dependent methods by taking a swab or biopsy from the wound and applying it as inoculate for various microbial cultures. The introduction of molecular biological methods and improved sampling techniques has illustrated that the culture-dependent methods presumably underestimate the microorganisms present in the wound. This is often due to a combination of inadequate growth conditions and colonization with slow, unculturable, fastidious or anaerobic bacteria growing in biofilms (Price et al., 2010).

It is well known that naked bacterial DNA can persist in a sample for a while after the infection has been eliminated, and the risk of detecting DNA from dead (non-viable) bacteria is a recognized bias when using molecular methods. The commercially available kit Molysis (Molzym, Bremen, Germany) degrades the naked bacterial DNA residing in a sample, thereby, improving the detection of viable and hence more clinically relevant bacteria in the subsequent molecular analysis. RNA has a much shorter half-life than DNA making it suitable as a measure of microbiological activity in a sample. RNA extracted from

a sample can be converted into cDNA and used as a target in polymerase chain reaction (PCR) or the RNA can be targeted directly within the cell for instance by fluorescence in situ hybridization (FISH).

The exact role and function of microorganisms in chronic wounds is not fully understood, but bacteria seem to have an important influence on the prognosis of the wound. In a study by Høgsberg et al. (in press) the bacterial flora was examined in chronic wound beds determined for split-skin grafting using conventional culture methods. In the study the presence of *Pseudomonas aeruginosa* was found to be particularly important. If *P. aeruginosa* was present, only 33% of the grafted chronic wounds healed successfully in contrast to 77% when *P. aeruginosa* was not detected. This emphasizes the importance of a careful sampling technique, characterization of the wound and clinical correlation.

In this chapter we describe techniques to characterize the microorganisms from a swab or skin grafting biopsy from chronic wounds. New and previously obtained results are presented and discussed.

2. Molecular methods for studying the microbial flora in chronic wounds

Various methods have been applied for the study of microorganisms in chronic wounds and each method has its advantages and disadvantages. In most clinical settings the gold standard is traditional culture-based methods. The weakness of cultivation is that it can take several days to obtain a final identification, and that fastidious and slow-growing microorganisms in the sample might be overlooked. However, cultivation usually provides additional information such as phenotypic resistance patterns that are highly relevant from a clinical perspective. Molecular diagnostic approaches on the other hand offer a more rapid and deep identification but have other limitations. In the following section some of the well-established molecular techniques are described as well as a few of the latest methods in the field.

2.1 16S rDNA cycle

Culture-independent identification of bacteria can be carried out by performing a ribosomal RNA (rRNA) gene cycle e.g. (Nielsen et al., 2008; Thomsen et al., 2010). This method is based on the fact that the composition of the gene encoding the bacterial 16S rRNA (16S rDNA) is unique for each bacterial species. Total DNA is extracted from a sample and the 16S rDNA is amplified nearly in its full length by PCR using broad-range primers. If the sample is polymicrobial the resulting PCR product will consist of amplicons with varying sequences making it unsuitable for direct sequencing. The individual amplicons need to be separated before sequencing, and construction of clone libraries and denaturant gradient gel electrophoresis (DGGE) are two different ways of achieving that (Andersen et al., 2007; Muyzer et al., 1993; Thomsen et al., 2010).

To construct clone libraries the amplicons are ligated into plasmid vectors and cloned into *E. coli* cells. The cells are spread on agar plates and grown over night, and each colony appearing on the plate represents one amplicon. The vectors are then extracted from each individual colony, and the 16S rDNA amplicons are sequenced. By performing BLAST (Basic Local Alignment Search Tool) searches in public databases (Altschul, 1997), the obtained sequences can be assigned to specific bacteria, and an improved phylogenetic resolution can be obtained by performing a phylogenetic analysis on basis of the sequences.

In DGGE the amplicons are separated by providing them with a GC-rich primer tail and loading them on a polyacrylamide gel containing a gradually increasing concentration of chemical denaturant. The unique base composition of each amplicon results in individual melting points meaning that the amplicon can only move through the gel until a certain point, where the double stranded structure is fully melted and only held together by the GC tail. Upon staining, each band on the gel theoretically represents a unique 16S rDNA amplicon which can be excised and extracted from the gel and sequenced. Construction of clone libraries and DGGE are relatively labour-intensive methods with many individual steps and it takes several days to obtain the sequences that eventually reveal the bacteria present in a sample. This makes the methods unsuitable for diagnostic purposes where a rapid answer is required, whereas, they are more relevant as a research tool. The sequence information provided by the clone libraries and the DGGE can be used to make more specific analyses on the original sample, such as FISH or quantitative PCR (qPCR).

2.2 qPCR / RT-qPCR

qPCR is a culture-independent method for determining the number of a specific gene in a sample. qPCR basically proceeds like a regular PCR but has the advantage that the formation of PCR product can be followed as it progresses, making laborious post-PCR steps such as gel electrophoresis unnecessary. In qPCR the formation of PCR product is usually detected by one of two techniques: addition of a dye that fluoresces upon binding to double stranded DNA, or by application of a sequence-specific probe that fluoresces only when the target DNA is present. In both cases, the fluorescence is measured by a detector in the PCR machine after each PCR cycle. The intensity of the fluorescence progresses exponentially as does the PCR product formation because the increase in fluorescence is proportional to the increasing amount of target sequence. By analyzing standards with known concentrations of the target sequence, it is possible to quantify the initial amount of target DNA in the sample. In order to focus only on the active bacteria the RNA can be extracted from the sample, converted into cDNA and used as target in qPCR in a method called reverse transcriptase qPCR RT-qPCR).

2.3 FISH

DNA-FISH and peptide nucleic acid (PNA)-FISH rapidly identify microorganisms in complex samples and provide information about number, morphology and spatial distribution. The FISH technique is based on fluorescent probes of varying specificity that are complementary to a ribosomal RNA sequence, and the target cells can be visualized using fluorescence microscopy. A detailed procedure has been published by Amann (1995) and Stender (2003).

2.4 Pyrosequencing

Pyrosequencing is a method of DNA sequencing based on the "sequencing by synthesis" principle and it is likely to replace the relatively labour-intensive procedure of constructing clone libraries. It differs from the classic Sanger sequencing, in that it relies on the detection of pyrophosphate release upon nucleotide incorporation, rather than chain termination with dideoxynucleotides (Ronaghi et al., 1998). This deep sequencing technique has been used to evaluate the diversity of microbial populations where thousands of reads/sequences can be

obtained from each sample (Andersson et al., 2007; Bogaert et al.; Dowd et al., 2008; Price et al., 2009; Price et al., 2010; Smith et al., 2010; Stewart et al., 2010; Wolcott et al., 2009).

2.5 Ibis T-5000

The Ibis T5000 Biosensor system combines automated sample preparation, PCR amplification, electrospray ionization mass spectrometry, and information management to rapidly characterize known and unknown organisms. The system is based on base ratios (not base sequences) and can provide test results in six to seven hours (Ecker et al., 2008). The method was recently identified as the molecular system most likely to fulfill the requirements of routine diagnosis in orthopedic surgery (Costerton et al., 2011).

2.6 Method evaluation

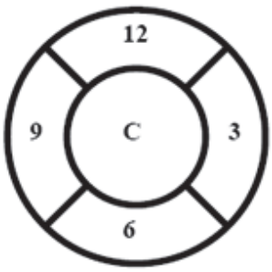
All the mentioned methods are somehow biased and application of multiple methods is generally recommended for research studies. For example, the nucleic acid extraction efficiency is crucial for all methods except FISH, where sample fixation is important. Primer specificity, differential amplification efficiency and incomplete databases for bioinformatic search are other possible biases. In addition, it is important to be critical to molecular findings based on DNA. Previous studies have shown that naked bacterial DNA can reside in a sample for a long time after an infection has been eradicated fx Pichlmaier et al. (2008). The PCR techniques cannot distinguish between DNA from active and dead bacteria and this raises the question of the clinical relevance of the findings. Targeting RNA instead of DNA (for instance using FISH or RT-qPCR) could help exclude irrelevant findings and focus only on the active bacteria.

3. Results

In a study by Thomsen and coworkers (2010) the bacterial flora in different types of wound material from 14 skin graft operations was examined. Routine cultivation as well as a panel of DNA-based methods were performed and the results compared. Cultivation showed polymicrobial growth in all but one wound, and *S. aureus*, *P. aeruginosa*, *K. oxytoca* and enterococci were identified most often, whereas no anaerobic species were found. In most of the samples the finding of *S. aureus* and *P. aeruginosa* by cultivation was confirmed by the molecular methods. However, for all samples DNA from additional species was identified, including anaerobic bacteria. Among others DNA from *Alcaligenes* sp., *Anaerococcus* sp., *E. faecalis* and *Stenotrophomonas* sp. was identified.

Furthermore, the study comprised an examination of the spatial location of *S. aureus* and *P. aeruginosa* within two wounds using species specific qPCR. This showed a great variation in the distribution of bacteria at different locations in the same wound. As an example, the number of *P. aeruginosa* varied with three orders of magnitude for two biopsies taken from the same wound indicating that the bacteria grow locally in micro colonies (biofilms) (Figure 1). This tendency to proliferate in biofilms is illustrated in Figure 2, where a biopsy from a skin graft operation is examined for the presence of *P. aeruginosa* using PNA-FISH. In a recent analysis we examined the bacterial diversity in four samples (referred to as A-D) taken from the same wound by standard cultivation techniques as well as construction of clone libraries, PNA FISH and qPCR on basis of DNA and RNA (Table 1). Cultivation identified three bacterial species: *Staphylococcus pettenkoferi*, *Enterobacter cloacae* and

Stentrophomonas maltophilia. These findings were confirmed in one or more of the clone libraries (*S. pettenkoferi* DNA was found in clone library A and C, *S. maltophilia* DNA in C and D and *E. cloacae* DNA in D). DNA from additional bacteria was found in all four clone libraries of which *Propionibacterium* sp., *Massilia timonae*, *Brevibacterium pityocampae*, *Streptococcus mitis*, *Achromobacter* sp. and *Acinetobacter* sp. were the most abundant. The presence of *Propionibacterium acnes*, a common inhabitant of the human skin, was confirmed and quantitated with species specific qPCR.



		S. aureus		*P. aeruginosa*	
	method	Wound E	Wound F	Wound E	Wound F
C	q-PCR	**89**±11%	**200**±13%	**510**±18%	**920**±9%
	cultivation			+	
	DGGE	+	+		
3	q-PCR	No sample	**86**±8%	No sample	**300**±13%
	cultivation				
	DGGE		+		
6	q-PCR	**240**±10%	**290**±8%	**760**±7%	**8200**±8%
	cultivation			+	
	DGGE	+	+		
9	q-PCR	**310**±13%	**80**±5%	**47**±9%	**800**±10%
	cultivation			+	
	DGGE	+	+		
12	q-PCR	**180**±8%	**93**±12%	**280**±3%	**15**±5%
	cultivation			+	
	DGGE	+	+		

Fig. 1. Quantitative PCR data obtained from two wounds (wound E and F, respectively) for *S. aureus* and *P. aeruginosa* (copies/ng DNA ±standard error of the mean, n=3). Cultivation and DGGE results are mentioned as well + : Samples were taken under skin graft operations at the center (C), and at approximately 3, 6, 9, and 12 o'clock around the wounds' periphery. From Thomsen (2010).

Some of the 16S rDNA sequences from clone library A, C and D matched *Staphylococcus* sp. upon BLAST search.

To further annotate these bacteria, RT-qPCR assays targeting *S. aureus femA* mRNA and *Staphylococcus* sp. 16S rRNA were applied. The *S. aureus* specific qPCR was negative for all four samples whereas the *Staphylococcus* sp. assay was positive, indicating that the clones matching *Staphylococcus* sp. originated from coagulase-negative staphylococci (CoNS).

mRNA from *P. acnes* and *Staphylococcus* sp. was detected in the four wound samples indicating that these bacteria were active at the time of sampling. This partially corresponds with the cultivation results where *Staphylococcus pettenkoferi* was observed, whereas *P. acnes* was not. *P. acnes* is considered to be a fastidious bacterium that requires anaerobic growth conditions and has a slow growth rate. This might explain why it was not detected by cultivation.

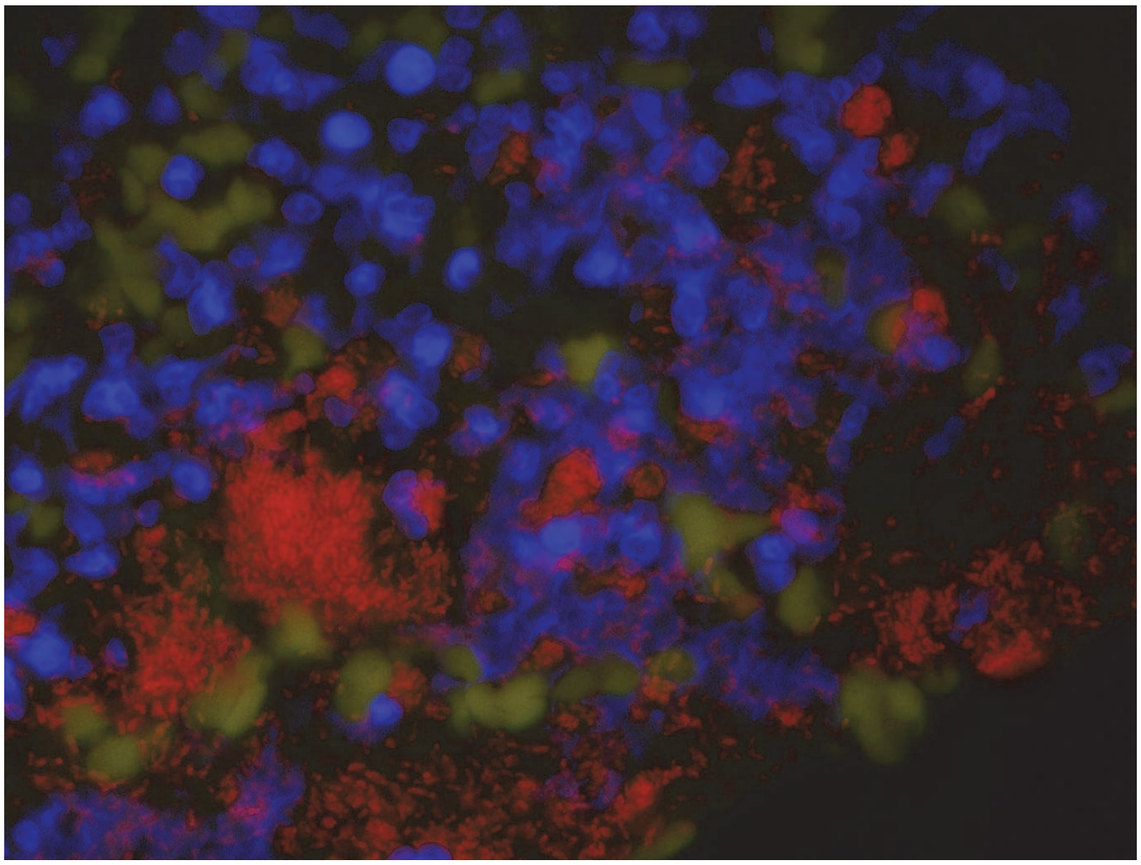


Fig. 2. *P. aeruginosa* identified in a biopsy from a skin graft operation by a specific PNA FISH probe (red stain). Host cells were stained with DAPI (blue).

P. aeruginosa and *S. aureus* are common in chronic wound infections but in this specifically examined case not found by cultivation, or in any of the clone libraries or by species specific qPCR. When comparing the results of our recent analysis to those obtained by Thomsen and coworkers (2010) it is seen that in both cases DNA from *Brevibacterium* sp., *Anaerococcus* sp., *Achromobacter* sp. and *Alcaligenes faecalis* was identified using the clone library approach but not by cultivation. Furthermore, both studies show that molecular methods identify additional bacteria compared to cultivation and that the bacterial composition differs depending on the location within the individual wound.

Sample	Cultivation	Clone libraries (genomic DNA) Parentheses numbers of clones	qPCR (genomic DNA)	RT-qPCR (cDNA)
A	*Staphylococcus pettenkoferi*, *Enterobacter cloacae*, *Stentrophomonas maltophilia*	*Brevibacterium pityocampae* (7), *Staphylococcus* sp. (8), *Propionibacterium* sp. (19), *Massilia timonae* (1), *Staphylococcus epidermidis* (1), *Xanthomonas* sp. (1), *Xanthomonadaceae* sp. (2), *Stenotrophomonas* sp. (3), *Herbaspirillum* sp. (1) and 12 uncultured bacteria best matching: *Ralstonia solanacearum* (1), *Massilia timonae* (5), *Propionibacterium* sp. (1), *Propionibacterium acnes* (3) and *Acidobacterium* sp. (2).	1200 *P. acnes* CFU/g tissue	Positive for *P. acnes* Positive for *Staphylococcus* sp.
B		*Propionibacterium* sp. (23), *Streptococcus mitis* (4), *Achromobacter* sp. (2), *Kocuria* sp. (3), *Micrococcus* sp. (2), 12 uncultured bacteria best matching: *Ralstonia solanacearum* (3), *Anaerococcus* sp. (2), *Kocuria* sp. (4), *Propionibacterium* sp. (1), *Achromobacter xylosoxidans* (1) and *Neisseriales* sp. (1).	1200 *P. acnes* CFU/g tissue	Positive for *P. acnes* Positive for *Staphylococcus* sp.
C		*Acinetobacter* sp. (4), *Staphylococcus* sp. (1), *Stenotrophomonas maltophilia* (4), *Stenotrophomonas* sp. (5), *Propionibacterium* sp. (4), *Alcaligenes faecalis* (1), *Micrococcus* sp. (2), *Mesorhizobium* sp. (1), *Enterobacter hormachei* (1) and 22 uncultured bacteria best matching: *Propionibacterium acnes* (3), *Enterbacter hormachei* (4), *Anaeromyxobacter* sp. (2), *Lactobacillus iners* (7), *Ralstonia solanacearum* (2), *Corynebacterium accolens* (3), *Bradhyrizobium* sp. (1).	1700 *P. acnes* CFU/g tissue	Positive for *P. acnes* Positive for *Staphylococcus* sp.
D		*Achromobacter denitrificans* (5), *Achromobacter* sp. (4), *Enterobacter hormachei* (7), *Stenotrophomonas maltophilia* (6), *Enterobacter* sp. (1), *Enterobacter cloacae* (1), *Propionibacterium* sp. (1), *Staphylococcus* sp. RNA (1) and 25 uncultured bacteria best matching: *Enterobacter hormachei* (12), *Enterobacter cloacae* (6), *Streptococcus alactolyticus* (4), *Enterococcus ludwigii* (2) and *Massilia* sp. (1).	1000 *P. acnes* RNA CFU/g tissue	No *P. acnes* detected Positive for *Staphylococcus* sp.

Table 1. Comparison of 4 separate biopsies from one wound investigated by cultivation, clone libraries, qPCR and RT-qPCR. Genomic DNA and RNA extracted from the four samples were examined using qPCR and RT-qPCR assays specific for *Propionibacterium acnes, Staphylococcus sp., P. aeruginosa* and *S. aureus.* RT-qPCR was only used to prove the presence of active bacteria and not to quantitate the bacteria.

4. Discussion

4.1 Microbial diversity

Over the years the microbial flora in chronic wounds has been target for thorough investigation and the advancing culture-independent techniques become more and more common as research tools. In such studies the molecular methods generally tend to reveal a much greater microbial diversity than the traditional cultivation methods. The study by Thomsen and coworkers (2010) is an example of this, where culture-independent methods revealed multiple species in each investigated wound. All of the wounds contained *S. aureus*, and *P. aeruginosa* was also frequently found, but aside from this, rather different floras were identified for the individual wounds. Furthermore, the molecular biological methods detected a varied anaerobic flora in some of the wounds and pathogenic species not found previously in chronic venous leg ulcers were identified. No anaerobes or new species were detected with culture methods. This corresponds well with the results of our recent examination of four biopsies taken from the same wound. Only aerobic bacteria were identified by cultivation whereas DNA and RNA from the anaerobic *P. acnes* were found by molecular methods. Furthermore, the molecular methods indicated varying polymicrobial communities in all four samples. Surprisingly, *S. aureus* and *P. aeruginosa* were not identified neither by cultivation or the cultivation independent methods in this specific wound.

The examination of wound flora has mainly focused on bacterial diversity, but a study from Dowd and collagues (2010) indicated that fungi might also be important in chronic wound infections. In their retrospective study, 915 chronic wounds were examined using molecular methods and fungal DNA was identified in 208 samples (corresponding to 23% of the investigated samples). DNA from a total of 48 different fungal species was identified of which yeasts of the *Candida* genus were the most abundant. In a retrospective analysis of the DNA resulting from the wounds included in the Thomsen (2010) study, qPCR with universal fungal primers and probe revealed fungal DNA in one of 14 samples.

In a recent study by Smith (2010) pyrosequencing was used to identify bacterial populations in 49 pressure ulcers. Diversity estimators were utilized and wound community compositions analyzed in relation to metadata such as age, race, gender, and comorbidities. Pressure ulcers are shown to be polymicrobial of nature with no single bacterium exclusively colonizing the wounds. The microbial community among such ulcers is highly variable and typically contains 3-10 primary populations. Up to hundreds of different species can be present in each wound; however, many in trace amounts only. There are no clearly significant differences in the microbial ecology of pressure ulcers in relation to metadata except when considering diabetes. The microbial populations and composition in the pressure ulcers of diabetics may be significantly different from the communities in non-diabetics. However in this particular work, the location of the pressure ulcer was not recorded. As diabetes tends to facilitate pressure ulcers on lower extremities due to neuropathy, the variability could reflect variations in location of the ulcer rather than diabetes itself.

The biofilm mode of growth seems important in chronic wounds with many different species present and it is generally accepted that the microbial composition of the entire wound is not represented in only one wound biopsy. Using FISH, we detected bacteria in biofilms in a biopsy from a skin graft operation (see Fig. 2) and this might explain how the bacteria survive inside the wound bed. In chronic wounds individual micro colonies might exist that only consist of single species, and it is very likely that mono- and polymicrobial

biofilms can be found in the same ulcer, but the importance and relevance of this has yet to be established (Kirketerp-Moller et al., 2008; Burmølle et al., 2010). There are also biofilms in healing wounds, and other factors like host response, virulence and antibiotic resistance seem important for the fate of the wound.

4.2 Heterogeneous distribution

A chronic wound is a heterogeneous environment that varies at different locations with regard to the accessibility of oxygen and the flow of fluid. Furthermore, external influences such as the application of a wound dressing can cause local changes in the wound. This means that different microorganisms have more or less favorable growth conditions at different locations which often results in a heterogeneous microbiological distribution throughout the wound (Price et al., 2010). Wolcott (2009) concluded that individual wounds have distinct ecological footprints, and within the individual wounds there can be both significant site specific differences and relative uniformity in the bacterial ecology.

The heterogeneous distribution of microorganisms in chronic wounds has been described in several studies and the clinical relevance of this is discussed (e.g. by Fazli et al., 2009; Kirketerp-Moller et al., 2008¸Wolcott et al., 2009). Using qPCR and FISH it has been illustrated that the number of the pathogens *S. aureus* and *P. aeruginosa* varied depending on the location and depth of the wound examined (Fig. 1) (Thomsen et al., 2010). *S. aureus* was primarily located close to the wound surface while *P. aeruginosa* was primarily located deeper in the wound (Fazli et al., 2009). Some bacterial species were present all over the chronic venous leg ulcer while some were only present in parts of the wounds (Thomsen et al., 2010).

The macroscale spatial variation in wound microbiota was also investigated by Price et al. (2010). A total of 31 curette samples were collected at the leading edge, opposing leading edge, and/or center of 13 chronic wounds. Bacterial community composition was characterized using a combination of 16S rDNA-based pyrosequencing and various other methods. A total of 58 bacterial families and 91 bacterial genera were characterized among the 13 wounds. While substantial macroscale spatial variation was observed among the wounds, bacterial communities at different sites within individual wounds were significantly more similar than those in different wounds ($p=0.001$). Our recent study of four biopsies from one wound showed a similar tendency regarding the spatial heterogeneity. The clone libraries constructed for each sample had sequences in common, for instance *P. acnes* that appeared in all libraries, but also contained bacterial sequences that were unique to the particular location. Even though the results are based on only one wound they support the prevalent opinion that a careful adjustment of sample sites may improve the quality of wound microbiota studies. However, the significant similarity in bacterial communities from different sites within individual wounds indicates that studies failing to control for sampling site should not be disregarded based solely on this criterion. Ideally a composite sample from multiple sites across the surface and in the depth of individual wounds may provide the most robust characterization of wound microbiota (Price et al., 2010); however, this is often not possible clinically.

The introduction of next-generation sequencing methods such as pyrosequencing has made it possible to achieve huge amounts of information about the microbial composition in a sample in a relatively short time. However the large amount of data generated by the new methods gives rise to new challenges considering data handling and especially

interpretation. As stated in the review by Rogers and coworkers (2010), different species in a polymicrobial infection can interact and thereby result in a different pathogenesis than the individual species would cause. This could mean that microorganisms that are usually considered non-pathogenic might play an important role in a polymicrobial community. For the time being it cannot be concluded if the new molecular methods are helpful to the clinicians or if all the extra information only complicates the overall picture. More studies have to be carried out to elucidate this.

The uneven distribution of bacteria in the wounds is highly relevant for the clinician and how and when the sample is taken highly influences the outcome of the diagnostic analysis. The ideal solution would be to examine the entire wound and identify every single pathogen, but again this is not possible nor does it provide the full answer. New questions would arise: Which bacterial strain or even subgroup is important and what influence do they have on the healing of the wound? Evaluating the result of a culture-independent analysis is after all still a paramount challenge for the clinician.

4.3 Consequences

4.3.1 Optimal sampling and diagnosis

No diagnostic methods can compensate for the three-dimensional uneven distribution of microorganisms in chronic ulcers. Several studies have indicated that the microbial flora varies at different locations in a wound (fx Kirketerp-Moller et al, 2008; Fazli et al, 2009, Thomsen et al., 2010), emphasizing the importance of adequate sampling techniques and ideally the use of multiple swabs or biopsies when examining wounds.

In situations where infected tissue is removed with the purpose of attaching a skin graft, instead of focusing on the microbial flora in the removed tissue it might be more relevant to focus on the remaining tissue on which the graft is to be attached (Bitsch et al., 2005). Microbial growth reduces the chance of the skin graft healing, and therefore examining the debrided wound before placing the graft, for instance by using a swab, might be of value. The surface of the chronic wound is likely to host commensal flora, and it is more likely that an in-depth residing bacterium is more pathogenic than a superficial one.

When designing a protocol for sampling we propose the following to be considered: 1. Revise the ulcer before sampling. 2: Swap a large area or take a big or multiple biopsies.

Melendez et al. (2009) developed a panel of qPCR assays targeting 14 common, clinically relevant pathogens for rapid identification of bacteria directly from tissue samples. Thirty-nine tissue samples from 29 chronic wounds were evaluated and the results compared with those obtained by culture. As revealed by culture and PCR, the most common organisms were methicillin-resistant *Staphylococcus aureus* (MRSA) followed by *Streptococcus agalactiae* (Group B *Streptococcus*) and *P. aeruginosa*. Under optimal conditions, the turnaround time for PCR results is only 4-6 h. Furthermore, qPCR is an inexpensive approach that easily can be introduced into clinical practice for detection of organisms directly from tissue samples. However, the downside to qPCR is that the primers and probe dictate the specificity and therefore there is a risk of overlooking species. For instance the qPCR assays reported by Melendez et al. (2009) only monitored aerobic pathogens but other studies have indicated that it is highly relevant to include assays for anaerobic pathogens and probably also fungi. The anaerobic and also slow-growing pathogen *P. acnes* was fx detected in the investigated wound presented in this study. In addition to the DNA identified in the clone libraries RNA from *P. acnes* was also identified indicating that the bacterium had been active very recently.

Which method is the optimal to examine the microbial flora of a chronic wound is a subject of discussion but in order to get as much information as possible the sample could be examined using a combination of traditional cultivation methods and molecular methods especially for research purposes.

4.3.3 The role of microorganisms in chronic wounds

Non-healing ulcers of mixed origin could heal if the underlying, but in many cases, still unknown cause was resolved. One of these factors is bacterial biofilm. But what is the difference between the biofilm in the healing group and in the non-healing group? Right now it is uncertain which microorganisms are the real important and truly pathogenic ones in chronic wounds, so should we treat for all of them? The most abundant microorganisms are not necessarily the most important in the wound from a clinical perspective. Furthermore, the pathogenicity between different strains and phenotypes differ, and they probably differ over time within the same phenotype. The most abundant bacteria found by traditional methods might just be the one with the most optimal growth conditions. Other factors like virulence genes, communication between the bacteria, the physical environment for the microorganisms and the host-biofilm interaction are also important. Hemolytic streptococci for instance are more virulent than certain other bacteria and a relatively small load of these are needed to initiate the infection of a wound. The beta-haemolytic *Streptococcus* and *Staphylococcus aureus* are examples of species that are highly virulent and co-exist very well with other species. So far it is unknown whether the virulence of a certain species is dependent upon another. Cell–cell communication in bacteria is accomplished through the exchange of chemical signal molecules called autoinducers. This process, called quorum sensing (QS), allows bacteria to monitor their environment for the presence of other bacteria and to respond to fluctuations in the number and/or species present by altering particular behaviors (Miller & Bassler, 2001). The N-acyl homoserine lactone QS signal molecule in *P. aeruginosa* will trigger the production of virulence factors such as rhamnolipids that have been shown to eliminate neutrophils in vitro (Van Gennip et al., 2009). Adding detection of known virulence genes to the molecular methods would be helpful in the process of interpretation.

Ideally, each wound should be carefully evaluated and in addition to traditional biofilm-based wound care strategies, an antimicrobial/antibiofilm treatment program with individualized therapeutic approaches should be identified for each patient's respective wound microflora (Smith et al., 2010; Wolcott et al., 2009). However, we do not have the tools for interpretation yet. First some questions should be asked when studying chronic wounds: What are the microorganisms doing to prevent healing of the wound? Can markers of the microbial community be identified that predict a change in infection dynamics and clinical outcomes? Would it be sufficient to search only for the presence of *Pseudomonas*? Can these new strategies directly characterize the impact of antimicrobial therapies, allowing treatment efficacy to be both assessed and optimized (Rogers et al., 2010)?

5. Conclusion

Longitudinal studies correlating multiple analyses of the microorganisms present in a chronic wound to patient metadata will increase our understanding of the problems that are caused by the microorganisms. The questions researchers should ask are: What role does

every single bacterial and fungal species have in the ulcer? Which role does the biofilm formation play and is it the same for all species? Which virulence factors are the most important, and does QS play a role? The understanding of the exact function of the microorganisms in chronic wounds will make the use of molecular methods as a diagnostic tool highly relevant in the future. Only by obtaining thorough knowledge of this we will be able to develop sufficient treatment strategies for each individual ulcer. We find it premature to implement the new culture independent methods into daily clinical practice as it will drive the clinician to act on the results. The result of that could very well be the application of additional antibiotics because "if the bug is there, it must do harm". For now we do not know that, but we need to find out. Until then, the clinicians will have to rely on "Best-Practice Principals".

6. Acknowledgement

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7. References

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research,* 25, 3389-3402.

Amann, R. I. (1995). In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In: *Molecular Microbial Ecology Manual* A. D. L. Akkermans, J. D. van Elsas & F. J. de Bruijn (Eds.), pp. MMEM-3.3.6/1-MMEM-3.3.6/15. London: Kluwer Academic Publications.

Andersen, A., Hill, K. E., Stephens, P., Thomas, D. W., Jorgensen, B., & Krogfelt, K. A. (2007). Bacterial profiling using skin grafting, standard culture and molecular bacteriological methods. *J Wound Care,* 16(4), 171-175.

Andersson, A., & mfl. (2007). Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS ONE, 3*(7), e2836.

Bitsch, M., Saunte, D. M., Lohmann, M., Holstein, P. E., Jorgensen, B., & Gottrup, F. (2005). Standardised method of surgical treatment of chronic leg ulcers. *Scand J Plast Reconstr Surg Hand Surg,* 39(3), 162-169.

Bogaert, D., Keijser, B., Huse, S., Rossen, J., Veenhoven, R., van Gils, E., et al. Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. *PLoS One,* 6(2), e17035.

Burmølle, M., Thomsen, T.R., Fazli, M., Dige, I., Christensen,L., Homøe, P. et al. (2010). Biofilms in chronic infections - a matter of opportunity - Monospecies biofilms in multispecies infections. *FEMS Immonol Med Micro,* 59(3), 324-336.

Costerton, J. W., Post, J. C., Ehrlich, G. D., Hu, F. Z., Kreft, R., Nistico, L., et al. (2011). New methods for the detection of orthopedic and other biofilm infections. *FEMS Immunol Med Microbiol,* 61(2), 133-140.

Dowd, S., Sun, Y., Secor, P., Rhoads, D., Wolcott, B., James, G., et al. (2008). Survey of bacterial diversity in chronic wounds using Pyrosequencing, DGGE, and full ribosome shotgun sequencing. *BMC Microbiology,* 8(1), 43.

Dowd, S. E., Delton Hanson, J., Rees, E., Wolcott, R. D., Zischau, A. M., Sun, Y., et al. (2010). Survey of fungi and yeast in polymicrobial infections in chronic wounds. *J Wound Care,* 20(1), 40-47.

Ecker, D. J., Sampath, R., Massire, C., Blyn, L. B., Hall, T. A., Eshoo, M. W., et al. (2008). Ibis T5000: a universal biosensor approach for microbiology. *Nat Rev Micro,* 6(7), 553-558.

Fazli, M., Bjarnsholt, T., Kirketerp-Moller, K., Jorgensen, B., Andersen, A., Krogfelt, K., et al. (2009). Nonrandom distribution of Pseudomonas aeruginosa and Staphylococcus aureus in chronic wounds. *J Clin Microbiol,* 47(12), 4084-4089.

Frankel, Y. M., Melendez, J. H., Wang, N.-Y., Price, L. B., Zenilman, J. M., & Lazarus, G. S. (2009). Defining Wound Microbial Flora: Molecular Microbiology Opening New Horizons. *Arch Dermatol,* 145(10), 1193-1195.

Kirketerp-Moller, K., Jensen, P., M, F., Madsen, K., Pedersen, J., Moser, C., et al. (2008). The distribution, organization and ecology of bacteria in chronic wounds. *J Clin Microbiol,* 46(8), 2717-2722.

Melendez, J. H., Frankel, Y. M., An, A. T., Williams, L., Price, L. B., Wang, N. Y., et al. (2009). Real-time PCR assays compared to culture-based approaches for identification of aerobic bacteria in chronic wounds. *Clin Microbiol Infect,* 16(12), 1762-1769.

Miller, M. B., & Bassler, B. L. (2001). Quorum sensing in bacteria. *Annu Rev Microbiol,* 55, 165-199.

Muyzer, G., De Waal, E. C., & Uitterlinden, A. G. (1993). Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S rRNA. App Env Micro, 59(3), 695-700.

Nielsen, M., Thomsen, T. R., Moser, C., Hoiby, N., & Nielsen, P. H. (2008). Use of cultivation-dependent and -independent techniques to assess contamination of central venous catheters: a pilot study. *BMC Clin Pathol,* 8(10).

Percival, S. L., Hill, K. E., Malic, S., Thomas, D. W., & Williams, D. W. (2011). Antimicrobial tolerance and the significance of persister cells in recalcitrant chronic wound biofilms. *Wound Repair Regen,* 19(1), 1-9.

Pichlmaier, M., Marwitz, V., Kühn, C., Niehaus, M., Klein, G., Bara, C., et al. (2008). High prevalence of asymptomatic bacterial colonization of rhythm management devices. *Europace,* 10(9), 1067-1072.

Price, L., Liu, C., Melendez, J., Frankel, Y., Engelthaler, D., Aziz, M., et al. (2009). Community analysis of chronic wound bacteria using 16S rRNA gene-based pyrosequencing: impact of diabetes and antibiotics on chronic wound microbiota. *PLoS ONE,* 4(7).

Price, L. B., Liu, C. M., Frankel, Y. M., Melendez, J. H., Aziz, M., Buchhagen, J., et al. (2010). Macroscale spatial variation in chronic wound microbiota: a cross-sectional study. *Wound Repair Regen,* 19(1), 80-88.

Rogers, G. B., Hoffman, L. R., Whiteley, M., Daniels, T. W., Carroll, M. P., & Bruce, K. D. (2010). Revealing the dynamics of polymicrobial infections: implications for antibiotic therapy. *Trends Microbiol,* 18(8), 357-364.

Ronaghi, M., Uhlen, M., & Nyren, P. (1998). A sequencing method based on real-time pyrophosphate. *Science,* 281(5375), 363, 365.

Smith, D. M., Snow, D. E., Rees, E., Zischkau, A. M., Hanson, J. D., Wolcott, R. D., et al. (2010). Evaluation of the bacterial diversity of pressure ulcers using bTEFAP pyrosequencing. *BMC Med Genomics,* 3, 41.

Stender, H. (2003). PNA FISH: an intelligent stain for rapid diagnosis of infectious diseases. *Expert Rev Mol Diagn,* 3(5), 649-655.

Stewart, F. J., Ottesen, E. A., & DeLong, E. F. (2010). Development and quantitative analyses of a universal rRNA-subtraction protocol for microbial metatranscriptomics. *ISME J,* 4(7), 896-907.

Thomsen, T. R., Aasholm, M. S., Rudkjøbing, V., Saunders, A., Bjarnsholt, T., Givskov, M., et al. (2010). The bacteriology of chronic venous leg ulcer examined by culture-independent molecular methods. Wound Repair and Regen 18(1), 38-49.

Van Gennip, M., Christensen, L. D., Alhede, M., Phipps, R., Jensen, P. O., Christophersen, L., et al. (2009). Inactivation of the rhlA gene in Pseudomonas aeruginosa prevents rhamnolipid production, disabling the protection against polymorphonuclear leukocytes. *Apmis,* 117(7), 537-546.

Wolcott, R., Gontcharova, V., Sun, Y., & Dowd, S. (2009). Evaluation of the bacterial diversity among and within individual venous leg ulcers using bacterial tag-encoded FLX and titanium amplicon pyrosequencing and metagenomic approaches. *BMC Microbiology,* 9(226).

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The procedure of skin grafting has been performed since 3000BC and with the aid of modern technology has evolved through the years. While the development of new techniques and devices has significantly improved the functional as well as the aesthetic results from skin grafting, the fundamentals of skin grafting have remained the same, a healthy vascular granulating wound bed free of infection. Adherence to the recipient bed is the most important factor in skin graft survival and research continues introducing new techniques that promote this process. Biological and synthetic skin substitutes have also provided better treatment options as well as HLA tissue typing and the use of growth factors. Even today, skin grafts remain the most common and least invasive procedure for the closure of soft tissue defects but the quest for perfection continues.

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