Non-Random Distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in Chronic Wounds.

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Abstract

In this study the spatial organization of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in chronic wounds was investigated. Wound biopsies were obtained from patients diagnosed as having chronic venous leg ulcers, and bacterial aggregates in these wounds were detected and located by the use of peptide nucleic acid-based fluorescence in situ hybridization (PNA-FISH) and confocal laser scanning microscopy (CLSM). We acquired CLSM images of multiple regions in multiple sections cut from five wounds containing *P. aeruginosa* and five wounds containing *S. aureus*, and measured the distance of the bacterial aggregates to the wound surface. The distance of *P. aeruginosa* aggregates to the wound surface was significantly higher than that of *S. aureus* aggregates, suggesting that the distribution of the bacteria in the chronic wounds was non-random. The results are discussed in relation to our recent finding that swab culturing techniques may underestimate the presence of *P. aeruginosa* in chronic wounds, and in relation to the hypothesis that *P. aeruginosa* located in the deeper regions of chronic wounds may play an important role in keeping the wounds arrested in a stage dominated by inflammatory processes.

Introduction

Chronic wounds, such as diabetic foot ulcers, pressure ulcers and venous leg ulcers are an increasing problem worldwide. 1-2% of the population in developed countries develops chronic wounds; a condition associated with severe patient suffering, employment loss, reduced life quality, and high costs to the health care systems (13). Detailed knowledge about chronic wounds is required in order to develop better wound treatment and management strategies.
A normal wound healing process involves four main phases: (i) Coagulation, (ii) inflammation, (iii) cell proliferation and repair of the matrix, and (iv) epithelialisation and remodelling of the scar tissue (23). However, chronic wounds are believed to be captured in the inflammatory phase, where a persistent influx and elevated cell activity of polymorphonuclear neutrophils (PMNs) occur (1). Although PMNs play a critical role in the host defence and wound healing, they release cytolytic enzymes, free oxygen radicals, inflammatory mediators, and matrix metalloproteases (MMPs) which cause local tissue damage in the host (22, 23, 26).

It is known that the microflora of chronic wounds is multispecies. In a bacterial profiling study Gjødsbøl et al. found that chronic venous leg ulcers harboured *S. aureus* (in 93.5% of the ulcers), *Enterococcus faecalis* (71.7%), *P. aeruginosa* (52.2%), coagulase-negative staphylococci (45.7%), *Proteus* species (41.3%), and anaerobic bacteria (39.1%) (12). *S. aureus* and *P. aeruginosa* are opportunistic pathogenic bacteria, which are widely known as causing chronic biofilm based infections in their hosts. *S. aureus* is most commonly isolated from chronic wounds (8, 12, 15, 17) and, in certain situations, can express a number of potential virulence factors and surface proteins which promote its adherence to the damaged tissue, and decrease neutrophil functions and immune responses of the host (10, 11). *P. aeruginosa* often causes biofilm-based chronic infections and expresses virulence factors, in particular rhamnolipid, that can eliminate the activity of PMNs (4, 16). A number of studies have demonstrated that *P. aeruginosa* is present frequently in chronic wounds (12, 17), and have provided evidence that the bacteria are located in aggregates enclosed in extracellular polymeric matrix material as found in biofilms (17).
Furthermore, the chronic wounds harbouring \textit{P. aeruginosa} were larger than those that did not, and they also seemed to be more severely hindered in the healing process (12, 14, 20).

Biofilms are bacterial aggregates enclosed in a self produced extracellular polymeric matrix (6, 21, 25). In clinical environments biofilms can form on dead or living tissues, mucosal surfaces, or surfaces of medical devices in the host. Bacteria in biofilms often display different characteristics from their planktonic counterparts, such as increased resistance against the activities of the host immune system and tolerance to antimicrobial treatments (7). Such characteristics are important since biofilms are involved in many chronic bacterial infections. Recent studies have shown the presence of bacterial biofilms in chronic wounds (9, 15, 17). Although the role of biofilms in chronic wounds is not yet fully understood, it is believed that their existence can be one of the reasons of impaired wound healing (4, 16).

We previously demonstrated that there is a lack of correlation between the bacteria detected by standard culturing and those detected directly by PNA-FISH in chronic wound samples (17). While \textit{S. aureus} was detected more frequently by swab sample cultivation than by PNA-FISH, the opposite was true for \textit{P. aeruginosa}. This lack of correlation between detection by swab sample cultivation and PNA-FISH may be due to an ability of different bacterial species to colonize different regions of chronic wounds. Swab sample cultivation identifies the microorganisms present in the surface region of the wound, but may not detect microorganisms located inside the wound bed. Accordingly, in the present report we present evidence that \textit{S. aureus} primarily colonises the region of chronic wounds which is close to the surface, whereas \textit{P. aeruginosa} primarily colonizes the deeper regions of chronic wounds. The ability of \textit{P. aeruginosa} to colonize the deeper
regions of chronic wounds may be due to the capability of this organism to produce virulence factors which destroys PMNs (4, 16), and it may play an important role in keeping the wounds arrested in a stage dominated by inflammatory processes.

Materials and methods

Tissue sample collection and preparation

Nine patients diagnosed as having chronic venous leg ulcers were included in the study. As described below, four patients had *S. aureus*-containing wounds, four patients had *P. aeruginosa*-containing wounds, and one patient had a wound that contained both *S. aureus* and *P. aeruginosa*. Material (4 mm punch biopsies) from chronic venous leg ulcers (see Fig. 1) was obtained with acceptance of patients and in accordance with the biomedical project protocols (H-B-2008-023 and KA-20051011), which were approved by the Danish Scientific Ethical Board. Wound biopsy material was collected by a surgical team before cleansing, and surgical preparation of the wound (2), and was immediately transferred to phosphate buffered saline with 4% paraformaldehyde, and stored at 5 °C before further preparation for microscopic investigation. The biopsy material for microscopic investigation was imbedded in paraffin, cut in 4 μm sagittal sections and mounted on glass slides. Prior to microscopic investigation paraffin was removed from the tissue sections by immersing the glass slides twice in xylene (total 10 min), twice in 99.9% ethanol (total 6 min), twice in 96% ethanol (total 6 min) and three times in distilled sterile water (total 9 min).

PNA-FISH and conventional tissue staining
The deparaffinised tissue sections were analysed by means of conventional hematoxylin and eosin (H&E) staining and fluorescence in situ hybridization (FISH) using peptide nucleic acid (PNA) probes. PNA probe in hybridization solution (AdvanDx, Inc., Woburn, MA) was added dropwise to each tissue section, which then was covered with a coverslip and hybridized in a PNA-FISH work station (AdvanDx, Inc., Woburn, MA) covered with a lid at 55 °C for 90 min. Three separate PNA probe solutions were used: 1) a Texas-Red (TxR) labelled _P. aeruginosa_-specific probe, 2) a FITC labelled _S. aureus_-specific probe, and 3) a mixture of the Texas-Red (TxR) labelled _P. aeruginosa_-specific probe and the FITC labelled _S. aureus_-specific probe. The slides with tissue sections were washed in wash solution (AdvanDx, Inc., Woburn, MA) at 55 °C for 30 min, air dried, mounted with Vectashield mounting medium with 4’,6’-diamidino-2-phenylindole (DAPI) (Vector Labs, Ca) and covered with a coverslip. The tissue sections were examined as described below.

### Image acquisition and analysis

Microscopic observations of the tissue sections were performed using an Olympus epi-fluorescence microscope (Olympus, Hamburg, Germany), or a Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) equipped with an argon laser and helium-neon laser for excitation of the fluorophores. Multichannel simulated fluorescence projection images were generated by using the IMARIS software package (Bitplane AG, Zurich, Switzerland), and further processed for display by using PhotoShop software (Adobe). Background subtraction was performed on the images to remove the host-tissue autofluorescence by using the IMARIS software package. The images were converted to 8-bit gray scale images by using ImageJ v1.41o software (http://rsb.info.nih.gov/ij/index.html), and the moment calculator tool
(http://rsb.info.nih.gov/ij/plugins/moments.html) of the same software was used to locate
the center of mass of the bacterial population displayed on the images.

157 **Statistical evaluation**

To evaluate whether the data obtained from the distance measurement of *P. aeruginosa* and *S. aureus* aggregates to the wound surface were statistically significant, unpaired t-test was performed. *P* values of ≤0.05 were considered significant. The statistical program Stat-View (SAS Institute Inc., Cary, NC) was used to calculate *P* values.

164 **Results**

Initially we identified *P. aeruginosa* and *S. aureus* in biopsy material from chronic wounds by the use of PNA–FISH with species-specific probes. Based on this identification we selected five wounds containing *P. aeruginosa* and five wounds containing *S. aureus*. (One of the selected wounds contained both *P. aeruginosa* and *S. aureus.*) In order to study the spatial distribution of *P. aeruginosa* and *S. aureus* in these wounds, we cut five sections sagittally at 50 µm intervals from each wound biopsy and performed PNA–FISH with *P. aeruginosa* and *S. aureus* specific probes on these sections. We subsequently acquired CLSM images at three different regions on each section. The bacteria were predominantly present as large aggregates. To get a measure of the distance of the bacteria to the wound surface we located the center of mass of the bacterial aggregates identified on each image by using the moment calculator tool of ImageJ software, and measured its distance to the wound surface. This analysis showed that the *S. aureus* aggregates were located close to the wound surface whereas the *P. aeruginosa* aggregates were located deeper in the wound bed (*P* < 0.0001) (Table 1). The center of
mass of the *S. aureus* aggregates were primarily located at a distance of 20-30 µm to the wound surface, whereas the center of mass of the *P. aeruginosa* aggregates primarily were located at a distance of 50-60 µm to the wound surface (see Fig. 2). Fig. 3 shows representative CLSM images of the location of *P. aeruginosa* and *S. aureus* in the chronic wounds. The range of the distribution of *P. aeruginosa* and *S. aureus* was limited so that co-localisation of the two bacterial species was rare. In order to visualize host cells and bacteria in the wound biopsies we performed combined PNA-FISH and DAPI staining as well as H&E staining in biopsies from wounds containing *P. aeruginosa* or *S. aureus*. As shown in Fig. 4, the analysed sections from wounds with *P. aeruginosa* had a higher number of PMNs than the analysed sections from wounds with *S. aureus*, suggesting that wounds with *P. aeruginosa* may have a higher degree of inflammation than wounds with *S. aureus*.

**Discussion**

Although the microflora of chronic wounds is polymicrobial and heterogeneous, *S. aureus* and *P. aeruginosa* are among the most frequently isolated bacteria from these wounds (8, 12, 15). In the present study, we characterised the distribution of *P. aeruginosa* and *S. aureus* in nine chronic wounds. Four wounds with *S. aureus*, four wounds with *P. aeruginosa*, and one wound with both *S. aureus* and *P. aeruginosa*. Analysis of the images obtained using PNA-FISH and CLSM indicated that *P. aeruginosa* was located significantly deeper in the wound bed than *S. aureus*.

We previously investigated the presence of bacteria in samples from 22 chronic venous leg ulcers by standard culturing and PNA-FISH (17). By swab sample cultivation we
found that 12 of the wounds were colonized with *S. aureus*, whereas 5 of them were colonized with *P. aeruginosa*. Conversely, by using PNA-FISH, *P. aeruginosa* was detected in 9 of the wounds, while *S. aureus* was detected in only 2 of them. Our present finding that *P. aeruginosa* is located in the deeper regions of the wound bed offers an explanation for the different results obtained by swab sample cultivation and PNA-FISH. Because the swab sample technique detects bacteria in the upper region of the wounds bacteria that primarily colonises the deeper regions may not be detected.

For a good healing response, the bacterial load of chronic wounds needs to be optimally managed. Topical antimicrobials can in some cases effectively control superficial bacterial burdens if the infection is localized, but may not be appropriate for highly infected wounds. Systemic antibiotics may be effective in some cases of severe infection with tissue invasion (23). Usage of a nanocrystalline silver dressing was shown to decrease the superficial bacterial burden as assessed by surface swab investigation, but had no effect on the bacterial burden of the deep wound compartment as measured by tissue biopsy (24). Thus, it is of great importance to define the spatial organisation of bacterial species within a chronic wound for the most effective infection management. A relevant picture of the spatial organisation of the bacteria in a chronic wound might be obtained by using molecular methods such as denaturing gradient gel electrophoresis (DGGE) (2, 8) and FISH (16) in combination with traditional culturing of swab as well as biopsy samples.

The biofilm mode of growth provides bacteria different characteristics compared to their planktonic counterparts such as protection against the activities of the host immune system and increased tolerance to antimicrobial treatments (7). *P. aeruginosa* in biofilms expresses quorum-sensing controlled virulence factors that can kill or eliminate the...
activity of host immune cells. It has been shown that rhamnolipid, a leukocidal toxin 
produced by *P. aeruginosa*, causes rapid necrosis of PMNs *in vitro* (16). Bjarnsholt et al. 
proposed that rhamnolipid offers a protective shield against the activities of host immune 
cells, and demonstrated that aggregates of *P. aeruginosa* in chronic wounds were 
surrounded by host cells, possibly PMNs, but not penetrated (5, 17), similar to what was 
observed in *in vitro* biofilms of *P. aeruginosa* overlaid with freshly isolated PMNs (4). 
The bacteria in chronic wounds are expected to compete with each other for the available 
nutrients. The ability of *P. aeruginosa* to migrate via type IV pili and flagella-mediated 
motility in biofilms (3, 18, 19), and to produce virulence factors that can eliminate the 
activity of host defence systems (4, 16) may explain the presence of these bacteria in the 
deeper regions of chronic wounds. Destruction of PMNs via virulence factors produced 
by *P. aeruginosa* located in the deeper regions of chronic wounds may be one of the 
factors causing a persistent influx of PMNs and keeping the wound in an inflammatory 
stage. However, more research is required before specific bacterial species in specific 
modes of growth can be identified as causative agents in chronic wounds.

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Table 1. Average distance of bacterial aggregates to the surface of wound samples.

<table>
<thead>
<tr>
<th>Wound biopsies</th>
<th>Bacterial species detected by PNA-FISH</th>
<th>Average distance to wound surface (µm)(^b)</th>
</tr>
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<tbody>
<tr>
<td>LGA02</td>
<td>S. aureus</td>
<td>28.3 (6.6)</td>
</tr>
<tr>
<td>BIJ04</td>
<td>S. aureus</td>
<td>8.8 (1.7)</td>
</tr>
<tr>
<td>HAH08</td>
<td>S. aureus</td>
<td>28.1 (5.0)</td>
</tr>
<tr>
<td>Pt17</td>
<td>S. aureus</td>
<td>26.1 (5.1)</td>
</tr>
<tr>
<td>M2(^a)</td>
<td>S. aureus</td>
<td>23.7 (3.7)</td>
</tr>
<tr>
<td>M3(^a)</td>
<td>P. aeruginosa</td>
<td>57.5 (9.4)</td>
</tr>
<tr>
<td>Pt11</td>
<td>P. aeruginosa</td>
<td>50.0 (13.4)</td>
</tr>
<tr>
<td>Pt20</td>
<td>P. aeruginosa</td>
<td>53.5 (9.9)</td>
</tr>
<tr>
<td>Pt23B</td>
<td>P. aeruginosa</td>
<td>68.7 (11.2)</td>
</tr>
<tr>
<td>Pt31</td>
<td>P. aeruginosa</td>
<td>46.1 (6.0)</td>
</tr>
</tbody>
</table>

\(^a\) M2 and M3 are biopsies obtained from the same wound. Both S. aureus and P. aeruginosa were detected in these biopsies. In the case of M2 the distance of S. aureus to the wound surface was analysed, whereas in the case of M3 the distance of P. aeruginosa to the wound surface was analysed.

\(^b\) The centre of mass of the bacterial aggregates on each image was located and its distance to the wound surface was measured. Average distances of the centre of mass to the wound surface were obtained from 15 images acquired for each wound sample. The values in parentheses are the standard deviations.
Figure legends

Figure 1. The sampling region on a chronic venous leg ulcer. Biopsies were taken from a central region within the wounds. The arrows point to a representative sampling region.

Figure 2. The distribution of the distances from the wound surface to the center of mass of *S. aureus* aggregates (□) or *P. aeruginosa* aggregates (■). The distances are average values obtained from analysis of 15 images for each wound sample.

Figure 3. Representative CLSM images of *S. aureus* (A, B), *P. aeruginosa* (C, D), and both organisms (E) in chronic wounds. The bacteria were detected via PNA-FISH with a FITC-labelled *S. aureus*-specific probe (green), or a TxR-labelled *P. aeruginosa*-specific probe (red), or a mixture of the probes. Arrows point to the wound surfaces. The scale bars correspond to 30 µm.

Figure 4. Epi-fluorescence micrographs (A and B) and bright field micrographs (C and D) showing red PNA-FISH stained *P. aeruginosa* (A), green PNA-FISH stained *S. aureus* (B), blue DAPI stained host cells (A and B), H&E stained host cells and *P. aeruginosa* (C), and H&E stained host cells and *S. aureus* (D). Some of the bacteria or host cells are encircled and labelled b and h respectively. Arrows point to the wound surfaces. The scale bars correspond to 35 µm.