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Use of 16S rRNA sequencing and quantitative PCR to correlate venous leg ulcer bacterial bioburden dynamics with wound expansion, antibiotic therapy, and healing

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Abstract

Clinical diagnosis of infection in chronic wounds is currently limited to subjective clinical signs and culture-based methods that underestimate the complexity of wound microbial bioburden as revealed by DNA-based microbial identification methods. Here, we use 16S rRNA next generation sequencing and quantitative polymerase chain reaction to characterize weekly changes in bacterial load, community structure, and diversity associated with a chronic venous leg ulcer over the 15-week course of treatment and healing. Our DNA-based methods and detailed sampling scheme reveal that the bacterial bioburden of the wound is unexpectedly dynamic, including changes in the bacterial load and community structure that correlate with wound expansion, antibiotic therapy, and healing. We demonstrate that these multidimensional changes in bacterial bioburden can be summarized using swabs taken prior to debridement, and therefore, can be more easily collected serially than debridement or biopsy samples. Overall, this case illustrates the importance of detailed clinical indicators and longitudinal sampling to determine the pathogenic significance of chronic wound microbial dynamics and guide best use of antimicrobials for improvement of healing outcomes.

Venous leg ulcers (VLU) account for 70% of all chronic leg wounds and have an annual prevalence of 1.7% among people aged 65 years and older.^{1,2} VLU have high bacterial bioburden that is believed to contribute to delayed healing, though the role of benign colonization vs. pathogenic infection has not been clearly defined.^{3–5} Without a clear definition of colonization vs. infection, clinicians are currently limited to subjective clinical signs and patient-reported symptoms of infection when prescribing antibiotic therapy for chronic wounds.⁶ The inability of clinicians to objectively define a wound infection likely

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Conflicts of Interest

The authors have no conflicts of interest to disclose.

Disclosure

The authors have no further disclosures.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site

Figure S1. Stacked graphs of other sampling depths and regions.

contributes to the lack of improved healing outcomes with the use of topical and systemic antibiotics.^{7,8} Bacterial culture requires several days and cannot be relied upon for decision making when signs of infection are first noted; thus, only 12% of wound care professionals routinely perform bacterial culture prior to antibiotic therapy.⁹ Additionally, culture-based methods are known to be biased toward microbes that thrive under standard growth conditions, thereby underestimating the complexity of wound microbial bioburden as revealed by high-resolution DNA-based microbial identification methods.⁴

Though currently primarily available for research use, DNA-based methods do not require the time and resources required for growth of microbes, thus, offering many promising advantages over clinically available culture-based methods, including improvement of both the speed and accuracy of infection diagnosis. Initial clinical integration of these technologies, including quantitative polymerase chain reaction (qPCR) and DNA sequencing, has spurred a paradigm shift in microbial pathogenesis of chronic wounds, incorporating ecological concepts such as the multidimensional nature of microbial bioburden.³ In this sense, bioburden integrates not only the identification of microbes, but also the total abundance and diversity of the host-associated microbial community. Collectively, these three aspects—microbial identification, load, and diversity—give a more complete accounting of the host–microbe interactions present in a chronic wound, and more completely identify microbial-associated barriers to healing. However, previous studies linking microbial bioburden to wound healing outcomes have weaknesses, including: (1) cross-sectional study designs with limited patient information on the healing outcome or antibiotic use and focus on clinically noninfected wounds; (2) use of only debridement samples without comparison of different sampling methods or indication of the depth at which the wound is being sampled; and (3) analyses limited to one aspect of microbial bioburden—usually the taxonomic identity—without considering the abundance or overall diversity of the microbial community.^{4,10,11} Here, we utilize 16S rRNA qPCR and DNA sequencing for an unprecedented view of the change in bacterial bioburden in a VLU over a 15-week healing course, with the following aims: (1) prospectively examine bacterial bioburden longitudinally through different phases of healing, including wound expansion, stalled healing, and resolution; (2) compare different wound regions (wound center and distal edge) at three depths (swab before debridement, debridement curettings, and swab after debridement); and (3) determine the overall bacterial abundance (qPCR) as well as the relative abundance of the specific bacteria involved (16S sequencing) to determine the role of benign colonization vs. infection and lay the groundwork for more evidence-based guidance for the diagnosis and treatment of infections in chronic wounds.

MATERIALS AND METHODS

Patient history

The patient was a 40-year-old African American man with known venous insufficiency. Previous venous insufficiency scans showed venous reflux in the right greater saphenous vein at the level of the mid-calf as well as in the left small saphenous vein. Varicosities were noted from the right knee to distal calf and the left proximal thigh to distal calf. Arterial studies including ankle brachial index and pulse volume returns revealed no significant

arterial occlusive disease. He had a past medical history of previous VLU as well as other comorbidities including obesity, diverticulosis, pulmonary embolism (2-years prior), and cardiomyopathy (NYHA Class I). His medications included warfarin, furosemide, lisinopril, carvedilol, pentoxifylline, and vitamin D supplementation throughout the course of healing. Wound treatments included debridement followed by application of absorptive dressing (collagen and oxidized regenerated cellulose) and multilayered compression wraps changed at least weekly (Unna boot or Coflex boot).

Sample collection

Samples were collected in accordance with the University Hospitals Case Medical Center IRB (protocol number 05-11-30) with informed consent from the patient. Samples were obtained after his dressing was removed and his wound was wiped with sterile gauze to remove excess drainage and loosely adherent slough. An Epicentre Catch-All Sample Collection Swab (Epicentre Cat # QEC89100) soaked in sterile collection buffer (0.1% Tween 20 in PBS) was then rolled over a 1 cm² area in the center of the wound for 10 seconds using sufficient pressure to extract wound tissue fluid (Levine technique). The same technique was used to collect samples from a 1 cm² area at the most distal wound edge. Debrided tissue was collected from same 1 cm² regions using a 4-mm curette after anaesthesia was obtained with 2% lidocaine gel to the wound under occlusion for approximately 5 minutes. After debridement, swabs were collected from the same 1 cm² regions using the same type of swab, collection buffer, and technique as the before debridement swab. Samples were stored in 1.5-mL microfuge tubes at -80 °C until further processing.

Bacterial culture

Samples for bacterial culture were obtained when the patient developed three or more clinical signs and symptoms of infection according to a previously described scoring system.⁶ Samples were collected for aerobic and anaerobic bacterial culture of the wound center after debridement by rotating a Copan Diagnostics ESwab (#480C) over a 1 cm² area in the center of the wound for 5–15 seconds using sufficient pressure to extract wound tissue fluid. The swab was placed in the ESwab transport media and immediately delivered for aerobic and anaerobic bacterial culture in the clinical laboratory improvement amendments certified clinical microbiology laboratory of University Hospitals Case Medical Center. Cultures were prepared by plating on BBL™ Trypticase Soy Agar with 5% Sheep Blood, MacConkey II, Chocolate II, and CDC Anaerobe 5% Sheep Blood Agar. Cultures were incubated at 37°C aerobically and anaerobically (Remel Anaero Pack System) and deemed negative after 48 hours of aerobic growth and 72 hours of anaerobic growth.

DNA extraction

Catch-All Swabs and accompanying tissue were transferred to 0.7 mm garnet bead tubes (MoBio # 13123-50) using sterile forceps, and total DNA was extracted using a modified protocol for the MoBioPowerMag Soil DNA Isolation Kit, optimized for use with the Eppendorf epMotion (MoBio # 27100-4-EP). Prior to bead beating, 750 µL of the RNase A/ Bead Solution and 60 µL of the lysis solution were added to tubes, which were then incubated at 70°C for 10–30 minutes. Cells were then lysed using a MP FastPrep-24 at 6.5

m/s for 60 seconds. Tubes were pelleted twice for 10 minutes at room temperature and $21 \times 10^3 \times g$ to ensure complete removal of inorganic swab material. The standard protocol utilizing an Eppendorf epMotion 5075 was then followed from the inhibitor removal technology step onward. DNA was eluted in MagnaClear Elution Buffer and stored at -20°C until library preparation.

Quantitative PCR

Bacterial load was determined by qPCR to calculate the overall copy number of 16S rRNA per extracted DNA sample. Standards were developed by transforming Stellar Competent cells (Clontech #636763) with pGBT9 plasmid (Clontech #K1605-A) digested with BamHI-HF (New England Biolabs #R3136S) and ligated with the Ion Plus Fragment Library Kit DNA Ligase and Buffer (Life Technologies #602-1152-01, #602-1150-01) to include an insert of 16S rRNA (23F/806R). Transformed cells were grown in Miller LB Broth (Fisher #BP1426) with $100\ \mu\text{g/mL}$ ampicillin (Roche #10-825-242-001) for 18-hours prior to plasmid harvest with the Qiagen Plasmid Midi Kit (Qiagen #12143). Standardized plasmid DNA was confirmed to be free of genomic contamination by gel electrophoresis, and then diluted from 1.4×10^{10} copies/ μL to 1.4×10^7 copies/ μL , the lower detection limit of the assay. Quantification of the DNA extract from the samples was performed by qPCR. Each $20\ \mu\text{L}$ reaction contained $10\ \mu\text{L}$ $2\times$ Bullseye TaqProbe qPCR Master Mix (Midsco #BEQPCR-P), $2\ \mu\text{L}$ template DNA diluted to $0.8\ \text{ng}/\mu\text{L}$, $6\ \mu\text{L}$ DNA grade filter sterilized water (Fisher #BP2470-1) and $2\ \mu\text{L}$ $10\times$ primer/probe assay mix.¹² DNA was denatured 10 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds in a StepOne Plus RT PCR System (Applied Biosystems). Bacterial load was reported as copies/ μL of extracted DNA sample. Results were compared by student's paired *t* test between different wound regions (wound center vs. edge) and depths (swab before debridement and after debridement).

Library preparation and high-throughput sequencing

PCR targeting the V4 region of the 16S rRNA (F515/R806) was performed on each sample in triplicate, according to established protocols.^{13,14} Briefly, the forward primer was composed of a region targeting 515F, primer linker and pad regions, and the 5'-Illumina adapter. The reverse primers were made of a region targeting 806R, primer linker and pad regions, and the reverse complement of the 3'-Illumina adapter. Each reverse primer also contained a unique 12-base error-correcting Golay barcode, enabling all of the samples to be multiplexed on a single MiSeq run.¹³

PCR reactions contained $16.25\ \mu\text{L}$ Molecular Biology Grade Water (Fisher BP2819-1), $0.25\ \mu\text{L}$ TaKaRa Hot Start DNA Polymerase (Clontech TaKaRa R007A) ($5\ \text{units}/\mu\text{L}$), $2.5\ \mu\text{L}$ $10\times$ Buffer with MgCl_2 ($100\ \text{mM}$ Tris-HCl ($\text{pH}\ 8.3$), $500\ \text{mM}$ KCl, $15\ \text{mM}$ MgCl_2), $2.0\ \mu\text{L}$ deoxynucleotide triphosphate ($2.5\ \text{mM}$ each), $1.0\ \mu\text{L}$ Primers 515F and 806R working solutions ($0.5\ \mu\text{M}$ final concentration), and $2.0\ \mu\text{L}$ of total genomic DNA. The DNA was denatured for at 98°C for 3 minutes, followed by 35 amplification cycles of 98°C for 10 seconds, 56°C for 30 seconds, 72°C for 60 seconds, and a final extension step of 72°C for 10 minutes. Triplicate samples were pooled and purified using the MoBio UltraClean-htp 96 Well PCR Clean-Up Kit (MoBio Cat# 12596-4). Purified PCR products were then quantified

using a Qubit and normalized to 1 $\mu\text{g/mL}$. Normalized samples were then pooled to create a sequencing library, and quantified via qPCR. A 50% spike of PhiX control was then added to the library during sample preparation prior to sequencing. All 93 samples were sequenced in single paired-end 2 $\times$ 251 bp run on an Illumina MiSeq (Illumina #MS-102-2002).

Data quality filtering

A total of 15,996,809 raw sequencing reads were produced on the MiSeq with a range of 12,391–445,616 sequences per sample. The QIIME software package version 1.6.0 was used to split the forward read into libraries for each sample, followed by length and quality filtering. Reads that mapped to the PhiX control genome commonly used on the MiSeq platform were removed. The UPARSE Pipeline was used to dereplicate, sort by abundance and discard singletons.¹⁵ Additional chimeras were removed using UCHIME. Quality filtered sequences are then clustered into operational taxonomic units (OTUs) at 97% identity, which is normally considered an appropriate proxy for species level identification. A total of 1,196 OTUs were identified from 10,279,341 high-quality sequences from 93 samples. OTUs were then identified using the ribosomal database project classifier, as implemented in QIIME. The Shannon diversity index of each sample was also computed using the alpha diversity function in QIIME after samples were rarefacted to 10,000 sequences/sample. Bacterial community differences were quantified using weighted UniFrac, a distance metric for comparison of complex communities.¹⁶

RESULTS

Patient presentation and prospective wound sampling of bacterial bioburden

The patient developed a new wound on the right medial calf 2-weeks prior to presentation at our clinic. At his first visit, the wound had not received other treatments, and he was started on standard therapy, including wound debridement, dressings, compression bandages, and weekly follow-up visits, as detailed further in Materials and Methods section. At each visit over the 15-week healing course, wound size was documented by planimetry, and clinical signs of infection were prospectively documented and treated according to a previously described scoring system specific to chronic wounds.⁶ Prior to each weekly treatment, wound samples were obtained from two wound regions (center and distal edge) at three depths (swab before debridement, debridement curettings, and swab after debridement) for the first 8 weeks of healing. After week 8, the wound did not require debridement, so swab samples were taken only from the center and distal wound regions on weeks 10–15.

Over the first 2 weeks following initial presentation, the patient had worsening signs of infection, including increasing pain, erythema and edema of the periwound skin, and expanding wound size. On week 2, conventional bacterial culture of the central wound was performed, and the patient was started empirically on systemic antibiotic treatment with doxycycline 100 mg twice daily due to its general gram-positive and gram-negative antibiotic coverage and anti-inflammatory capabilities.^{17,18} The wound bacterial culture was positive for a single species, *Enterobacter cloacae*, and the antibiotic was changed according to culture sensitivities to trimethoprim/sulfamethoxazole (TMP/SMX) 160/800 mg twice daily on week 3 and continued for 2 weeks. After antibiotic treatment concluded at week 5,

wound healing stalled for 2 weeks, and then progressed to heal after 15 weeks without further antimicrobial treatment. The change in the patient's wound area is summarized in Figure 1A, showing the different phases of healing, including wound expansion, antibiotic treatment, stalled healing, and resolution.

Qualitative and quantitative bacterial dynamics throughout healing

To determine the bacterial bioburden present in the wound, we performed qPCR of the 16S rRNA bacterial gene for analysis of bacterial load (Figure 1B) and 16S rRNA sequencing, which identifies bacterial taxa and quantifies their relative abundances in each sample (Figure 1C).^{13,14} This demonstrated that the increase in wound area through week 3 was associated with both increased bacterial load and relative abundance of the family *Enterobacteriaceae*, of which *E. cloacae* is a member, though we were unable to identify the OTU with increased taxonomic resolution. (Figures 1B and C depict bacterial bioburden of the central wound before debridement; other samples are shown in Figure 2 and Supporting Information Figure 1.) This dominance of *Enterobacteriaceae* decreased only minimally with doxycycline treatment (weeks 2–3). However, switching to an antibiotic that better targeted *E. cloacae*, TMP/SMX, correlated with a decrease in wound size (Figure 1A), bacterial load (Figures 1B and 2), and relative abundance of *Enterobacteriaceae* (Figure 1C). Bacterial diversity overall decreased with initiation of TMP/SMX treatment, then increased during the 2-week stalled healing phase, then was lower as the wound healed (Figure 3). Interestingly, the decrease in bacterial community diversity during the resolution phase correlated with dominance of the genus *Staphylococcus*, even as the wound area steadily declined without clinical indications warranting further antibiotic treatment.

Biogeography of bacterial bioburden

In addition to longitudinal characterization of wound healing, we also performed detailed sampling of the different wound regions (wound center vs. edge) and depths (before debridement, debrided tissue, and after debridement) to examine intrawound variation in bacterial bioburden, including bacterial load and community structure. The bacterial load was significantly higher in samples from the wound center than the wound edge ($p = 0.049$ including only time points with samples at all depths, or $p = 0.035$ including all time points); however, the load at different wound depths was not significantly different (Figure 2). To quantify differences in bacterial community structure between the wound regions and depths, we calculated the UniFrac distance, a metric used in microbial ecology to quantify dissimilarity between bacterial communities (Figure 4).¹⁶ A lower UniFrac distance indicates more phylo-genetic relatedness between communities. The average weighted UniFrac distance between samples was significantly lower between wound regions than between wound depths or consecutive time points (at same region and depth). This indicates a higher level of similarity in bacterial community structure between different wound regions than between different wound depths sampled on the same day. Interestingly, changes in the before-debridement samples most strongly correlated with wound expansion, antibiotic therapy, and healing. Conversely, deeper samples (debrided tissue and postdebridement) did not experience such large shifts in microbial community structure (Supporting Information Figure 1).

DISCUSSION

This study demonstrates that the wound bacterial bioburden is dynamic and multidimensional throughout the different phases of healing in a chronic wound. In this case, wound expansion, increasing pain, erythema, and edema of the periwound skin were accompanied by increased bacterial load and relative abundance of *Enterobacteriaceae*. This combination of clinical signs and quantification of bacterial load and community structure more clearly defined a pathogenic infectious event. In general, the DNA-based methods provide an unbiased, quantifiable, and potentially expedited analysis of the predominant bacteria associated with worsening clinical symptoms, which could significantly improve clinical management beyond the current standard of care. Though currently primarily only available for research use, as next-generation sequencing becomes more affordable, it is likely that more comprehensive microbial genomic characterization will be more clinically accessible in the foreseeable future—including detailed taxonomic identification, antimicrobial susceptibility, as well as analysis of unique functional and virulence factors.

Once the infection was treated, this patient's wound experienced a transient bacterial community shift to decreased diversity, followed by increased diversity as wound healing stalled for a period of 2 weeks. As the wound transitioned to the resolution phase, *Staphylococcus* increased in relative abundance and the overall microbial diversity decreased. Though this clinical case is limited to description of associations rather than causation, this increase in *Staphylococcus* relative abundance as the wound improved likely represents a benign bacterial colonization not requiring antibiotic treatment. This illustrates the importance of combining clinical indicators of the wound healing trajectory with characterization of the microbial bioburden to determine pathogenicity and avoid inappropriate antibiotic administration. Determining microbial pathogenicity by comparing wound microbial bioburden to intact periwound skin is less likely to be useful due to the wide variability in skin inflammation throughout the healing process that is difficult to consistently clinically characterize.

Notably, the swabs collected prior to debridement could be used to quantify changes in the bacterial bioburden that correlated with the different wound phases. This latter finding is novel and clinically meaningful because these superficial samples are easier to collect, less costly, and involve less risk to the patient than either debridement or biopsy samples. Thus, it will likely be more informative and efficient in future studies to use simplified superficial sampling schemes to determine longitudinal changes in microbial bioburden associated with clinical diagnosis and treatment of wound infection rather than further emphasis on the need for detailed and deep wound sampling methods.

In summary, DNA-based microbial identification methods can readily quantify the multidimensional aspects of microbial bioburden—including microbial load, community structure, and diversity—increasing the potential for clinically relevant breakthroughs in the diagnosis and treatment of chronic wound infections, particularly as these technologies become progressively more affordable and clinically accessible. However, these advances are only possible if guided by studies that include detailed clinical indicators to determine the pathogenic significance of patterns of microbial dynamics. Thus, a combination of

improved study design and use of molecular methods has potential to lead to more targeted therapeutics, thereby decreasing wound infection rates while reducing the risk of antimicrobial resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Weekly change in wound area and microbial bioburden in a VLU. (A) Wound area (cm²) at each weekly time point, showing phases of wound expansion, antibiotic treatment, stalled healing, and resolution. (B) Bacterial load (16S copies per μL extracted DNA sample) at each weekly time point from swab samples collected from the wound center before debridement. The bacterial load of other sampling depths and regions are shown in Figure 2. (C) Stacked graphs showing the relative abundance of the bacterial taxa at each weekly time point. Other low abundance bacteria representing <5% and >5% relative abundance of any sample are grouped together and labelled accordingly. As with 1B, samples shown here are from the wound center before debridement. The stacked graphs of other sampling depths and regions are shown in Supplemental Figure S1.

Figure 2.

Bacterial load each week at different sampling locations. Bacterial load as determined by qPCR of 16S rRNA (copies per μL extracted DNA sample) at each weekly time point at the different sampling regions, including (A) central wound and (B) distal wound at the different sampling depths (before debridement, debrided tissue, and after debridement).

Figure 3.

Bacterial diversity of different wound depths at weekly sampling time points. Average Shannon diversity index (SDI) at each weekly sampling time point at the different sampling depths (before debridement, debrided tissue, and after debridement). Each time point is an average of the different sampling regions (center wound and distal wound), which were similar as demonstrated by the standard error bars. The SDI accounts for the relative abundance of each species within a sample. The SDI in this patient was highly variable among the samples during the initial infectious event. After antibiotic treatment with TMP/SMX, the overall sample diversity transiently decreased (week 6), then increased with stalled healing, and then decreased again as the wound resolved.

Figure 4.

Bacterial community average weighted UniFrac distances. Bacterial community distance calculated using weighted UniFrac, a distance metric used in ecology to quantify the dissimilarity of microbial communities that is weighted by abundance. A UniFrac distance value of 0 describes identical communities, while a value of 1 means that no community members are shared. By this metric, samples taken from different regions (wound center vs. distal wound edge at the same depth) have microbial communities that are more similar than samples of different depths (before debridement, debridement, and after debridement) or consecutive time points at a single wound region and depth. Error bars indicate standard error of the average.