Temporal changes in the diversity and composition of the bed microbiota of patients with pressure ulcers

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Abstract

Wound infection is a life-threatening problem in pressure ulcers and still develops despite interventions to reduce bioburden on wounds and periwound skin. We focused on the bed environment as the hotbed for bacteria on the wound because many patients with pressure ulcers are immobile. However, the formation process of the bed microbiota remains unclear. This study aimed to investigate the temporal changes in the diversity and composition of the bed microbiota of one patient each with an infected pressure ulcer, non-infected pressure ulcer, and without a pressure ulcer. Microbiome samples were obtained from three areas, namely the bed sheet area around the buttocks, center of the wound, and periwound skin for 7 consecutive days. The microbiota were determined by 16S ribosomal RNA gene sequencing. Sequences were clustered into operational taxonomic units, and diversity indices were calculated. Regarding the evaluation of temporal changes, the day following the changing of bed sheets was designated as the starting point. The analysis of alpha diversity showed no significant changes in bed environment over time, and this was evident in all patients. In addition, the temporal change in the composition of bed microbiota for the patient with a pressure ulcer was greater than for the patient without a pressure ulcer. This finding suggests that the bed environment is a source of wound microbiota, and the presence of a pressure
Infection is a common problem in chronic wounds, including pressure ulcers, and causes delayed healing, leading to a reduced quality of life and significant economic burden. Furthermore, wound infection increases the risk of death for patients with pressure ulcers. The development of infection requires three elements: 1) infectious host, 2) source of infection, and 3) route of transmission. Therefore, a variety of care options have been implemented in clinical practice depending on these elements, including nutritional interventions to improve host immunity and aid in the treatment of underlying diseases, wound cleaning and debridement to reduce bioburden (number of bacteria living on a surface), and use of appropriate dressings to prevent bacterial contamination of the wound. However, despite these interventions, wound infection still develops in up to 30% of pressure ulcers. These results indicate that current interventions are insufficient; therefore, a new approach is required to prevent infection of pressure ulcers.

Pressure ulcers are open wounds that are constantly exposed to external bacteria, especially from the bed environment because many patients with pressure ulcers are immobile. Moreover, the patient’s bed linens are known to be a potential cause of infection, as body temperature and sweat create the right environment for bacterial growth. This highlights that the bed environment of patients with pressure ulcers should be targeted in the establishment of prevention strategies for wound infections as the source of infection. Although antimicrobial linens have been developed, these have failed to prevent wound infections. In addition, the danger of oral and dermal exposure to nanosilver, which is widely used for antibacterial applications in linens, has been pointed out because it may cause damage to the liver and kidney and irritation of the eyes, skin, and respiratory tract. Therefore, it is necessary to develop novel interventions for the bed environment that are appropriate for patients with pressure ulcers.

Traditionally, infection control studies have relied on microbiological culture methods in assessing bioburden. However, this method is unable to characterize the microbial diversity of abiotic hospital surfaces because 99% of microbes in the environment cannot be cultivated. As a consequence, an increasing number of studies have utilized culture-independent, high-throughput sequencing technologies from recent advancements in gene sequencing because they allow researchers to sequence metagenomes that cannot be cultured in laboratories. For example, studies have reported the investigation of microbiota on hospital surfaces, mainly in intensive care units and neonatal intensive care units. In this respect, in our previous study, we investigated the composition and diversity of microbiota in pressure ulcers and the patient’s bed environment using the culture-independent method in an attempt to establish a wound infection prevention care strategy targeting the bed environment of patients with pressure ulcers. Results demonstrated that bacterial dissemination occurs between both the wound surface and bed environment in all patients with pressure ulcers. Particularly, findings suggest that the bed environment may be a source of bacteria present in the wound. However, the formation process of the bed microbiota and timing of nursing interventions to prevent wound infections remain unclear. In addition, in our previous study, we did not include any patients without pressure ulcers or patients with severely infected wounds; therefore, the impact of pressure ulcers and wound infection on the bed environment is still unclear and needs to be determined. Investigating the differences in bed microbiota due to these factors may provide further support for the hypothesis that the bed environment is a reservoir for wound microbiota, and consequently, represents a source of wound infection. Accordingly, this study aimed to investigate the temporal changes...
in the diversity and composition of the bed microbiota in a patient with an infected pressure ulcer, non-infected pressure ulcer, and without a pressure ulcer.

**Methods**

1. **Participants**

Three immobile patients in a long-term care hospital in Ishikawa Prefecture, Japan, were included in the study. Of these patients, one had an infected pressure ulcer, one had a non-infected pressure ulcer, and one did not have a pressure ulcer. In this specific hospital, bathing of patients and changing of bed sheets are performed once a week, and clothes are changed twice a week. With regard to the management of pressure ulcers, standard wound care is provided according to the national guidelines. Patient characteristics and wound-related information were collected from the medical records and are summarized in Table 1.

Monitoring and assessment of pressure ulcers were performed by an expert wound, ostomy, and continence nurse (HS) using the DESIGN-R® instrument.

Patient 1 was a 93-year-old woman with an infected pressure ulcer at the sacral region. The total score on the Braden scale was 9 points, with each item scored as follows: sensory perception, 1; moisture, 4; activity, 1; mobility, 1; nutrition, 1; and friction and shear, 1. She was immobile due to cerebral infarction sequelae and always used a diaper. There was no urine exposure on the skin or wound site due to the use of a urinary catheter. Although fecal incontinence occurred once during the investigation period, there was no wound contamination. Her serum albumin level was 2.2 g/dL, and the patient was undernourished. Repositioning was performed every 2 h for pressure relief, and an air mattress was used for pressure redistribution. Ten days after pressure ulcer development, the wound was completely covered by eschar. After approximately 3 weeks, the eschar was debrided, and undermining was identified. The wound exhibited signs of inflammation throughout the study period. Furthermore, biofilm was detected by noninvasive wound blotting as previously described, and wound inflammation was assessed by thermography (Thermo Shot F30: Nippon Aviotronics Co., Ltd., Yokohama, Japan). The total DESIGN-R® score was 30 points, and the scores for each item were as follows: depth, 4; exudate, 3; size, 8; inflammation, 1; granulation tissue, 6; necrotic tissue, 3; and pocket, 9. The wound and periwound skin were cleaned daily with a cleansing agent. The wound was treated with cadexomer iodine, and the gauze and exudate-

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Wound characteristics data were collected at the first sampling day.
absorbent pad were changed daily. The wound data of patient 1, including photographs, thermographic images, and distribution of biofilm detected by wound blotting, are shown in Figure 1.

Patient 2 was a 90-year-old woman with a non-infected pressure ulcer. The total score on the Braden scale was 11 points, with each item scored as follows: sensory perception, 3; moisture, 2; activity, 1; mobility, 1; nutrition, 3; and friction and shear, 1. The patient was immobile due to cerebral infarction. She was using diapers but not a urinary catheter. She had fecal incontinence; therefore, cotton was applied on the buttocks to prevent contamination of the wound. The serum albumin level was 3.2 g/dL, and she had moderate malnutrition. An air mattress was used, with repositioning every 2 h for decompression. Similar to that seen in patient 1, her pressure ulcer was in the sacral region. Neither biofilm nor wound inflammation was detected throughout the investigation period. The DESIGN-R® total score was 7 points, and the scores for each item were as follows: depth, 2; exudate, 1; size, 3; inflammation, 0; granulation tissue, 0; necrotic tissue, 3; and pocket, 0. The DESIGN-R® score did not change throughout the study period. The wound and periwound skin were cleaned daily with a cleanser. Wound care consisted of the use of antimicrobial dressings, but no topical medication was administered. Figure 2 shows the wound data of patient 2.

Patient 3 was a 93-year-old woman without a pressure ulcer. The total score on the Braden scale was 9 points, with each item scored as follows: sensory perception, 1; moisture, 4; activity, 1; mobility, 1; nutrition, 1; and friction and shear, 1. She was immobile due to cerebral infarction and required assistance in changing the extremity position. She used a diaper and urinary catheter. The serum albumin level was 3.0 g/dL, and she had poor nutritional status. An air mattress was used, with repositioning every 2 h to prevent pressure ulcers. No dressings, such as polyurethane film dressing, dressings with sliding function, and polyurethane foam/soft silicone dressings, were applied as preventive skin care. The sacral skin did not exhibit dryness and was not contaminated by urine or fecal material.

2. Sample collection

Microbiome samples were obtained using a moistened cotton swab (saline containing 0.1% Tween-20) for 7 consecutive days from February 15 to 21, 2018. In patient 1, sampling started 3 days after wound debridement, and in patient 2, sampling started 3 weeks after pressure ulcer development, when slough was present on the wound bed. In patient 3, sampling started on the same day as for other patients, after confirming the absence of skin abnormalities. Swab samples were obtained from three areas, namely the bed sheet area around the buttocks, center of the wound, and periwound skin (1 cm away from the wound edge) representing the bed environment,
wound, and normal skin, respectively. The wound was swabbed over a square area of 1 × 1 cm according to the Levine technique. Samples for sites other than the wound were collected from a square area of 4.4 × 4.4 cm by swabbing twice using the Z-stroke technique. On the day in which changing of bed sheets was scheduled, samples were collected before this procedure.

3. Sample processing

DNA extraction and amplification for amplicon sequencing were conducted as described in a previous study with slight modifications. The V3-V4 region in the 16S rRNA gene was amplified using the first PCR primers (F: 5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-CCTACGGGNGGCWGCAG-3′; R: 5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGA TCT-ACTACHVGGGTATCTAAKCC-3′). Cycling conditions were 94℃ for 2 min; then 30 cycles of 94℃ for 30 s, 50℃ for 30 s, and 72℃ for 60 s; and finally, 72℃ for 5 min. An equimolar mixture of all amplicons was outsourced (FASMAC Co., Ltd., Kanagawa, Japan) for Illumina MiSeq 16S amplicon sequencing. The non-chimera sequences were obtained following the methods described previously using QIIME (version 1.9.1). Sequences were clustered into operational taxonomic units (OTUs) using “pick_de_novo_otus.py” command at 97% similarity, and OTUs corresponded to the genus level. The microbiota composition was investigated using OTUs, and the dominant genus was calculated by the median relative abundance of OTUs in 7 days. The temporal changes in alpha and beta diversity are shown in Figure 3. Alpha diversity is species richness within a single microbiota. In the alpha diversity analysis, the samples were rarefied at 34,000 depth (minimum read number among all samples) to compare the diversity under the same conditions followed by a calculation of the phylogenetic diversity index. A higher value of the phylogenetic diversity index indicates a higher diversity of the microbiota. Beta diversity shows the differences in the microbiota between different environments. The weighted UniFrac dissimilarity index was calculated using “beta_diversity.py” command to assess beta diversity. Index values range between 0 and 1; the closer the value is to 1, the higher the differences between the microbiota. Regarding the evaluation of temporal changes, the day following the changing of bed sheets was designated as the starting point (Day 1). The temporal changes in alpha diversity were investigated by tracking values over a 7-day period. The temporal changes in beta diversity were confirmed by tracking values from the microbiota between Day 1 and each sampling day.

4. Statistical analysis

Differences in alpha diversity between patients were analyzed using the Kruskal-Wallis test followed by the Steel-Dwass test for multiple comparisons. These tests were also performed to compare the size of temporal changes in the diversity between patients. The beta
diversity index and all combinations of sampling points were used in the statistical analysis. The P-values <0.05 were considered statistically significant. All statistical analyses were conducted using the R software package, version 3.6.3 (http://www.rstudio.com/).

5. Ethical considerations

The study protocol was approved by the ethics committee of the Graduate School of Medicine, The University of Tokyo (Approval No. 11812). Informed consent was obtained from all patients before commencement of the study.

Results

1. Microbiota composition

The relative abundance of OTUs and microbiota composition in the environment of each patient are shown in Figure 4. The dominant genera (median relative abundance) in the wound, skin, and bed samples of patient 1 were Finegoldia (36.0%), Arcanobacterium (20.6%), and Corynebacterium (24.2%), respectively. In terms of temporal changes, the relative abundance of Proteus in the wound and skin was higher on Day 1 and Day 7. From Day 5, Staphylococcus and Corynebacterium decreased and Finegoldia, which was the most dominant genus in wound samples, increased in the skin microbiota. Although there were no considerable differences in the bacteria composing the bed microbiota across the 7 days, Finegoldia was detected with 1.0% for a median of 7 days in the bed samples. Likewise, Corynebacterium was detected in wound samples throughout the study period. [Ruminococcus] (25.0%) was the most dominant genus in bed samples of patient 2 and also found in wound samples. Staphylococcus was dominant in both wound and skin samples (67.7% and 23.4%, respectively) and the second most dominant genus in bed samples (9.7%). Throughout the 7 days, Staphylococcus and Corynebacterium accounted for the majority of bacteria in the wound, and the bacteria composing the microbiota remained largely unchanged. Moreover, the bacterial genera composing the microbiota of the skin and bed environment were more than those in the wound, and the members remained unchanged for 7 days. The dominant genera in the bed and skin samples of patient 3 were Staphylococcus (17.0%) and Corynebacterium (19.4%), respectively. The relative abundance of Akkermansia was higher in the skin after Day 6 and correspondingly increased in the bed environment. In addition, the bacteria in the skin and bed microbiota remained largely unchanged.

2. Alpha diversity

The value of alpha diversity of bed samples was compared among patients to identify differences in microbial diversity. The Steel-Dwass test showed no significant differences in phylogenetic diversity index (patients 1 and 2, p=0.14; patients 1 and 3, p=0.44; patients 2 and 3, p=0.28). Furthermore, the temporal changes in alpha diversity are shown in Figure 5. In both patients with pressure ulcers, the wound microbiota had the lowest diversity among the three sites. In patient 1, the 7-day maximum and
minimum values in the skin samples were 388 and 184, respectively. In the bed environment, the maximum and minimum values were 340 and 264, respectively, with the variation being greater for the skin. In patient 2, the maximum and minimum values in skin and bed were 336, 270, 325, and 255, respectively, and the diversity of their microbiota remained stable. In patient 3, the maximum and minimum values in skin and bed were 436, 278, 332, and 243, respectively, and the diversity in the bed microbiota was lower after Day 2.
3. Beta diversity

Following the start of the collection period, changing of bed sheets for patient 1 was performed on Day 6, patient 2 on Day 2, and patient 3 on Day 3. The temporal changes over a 7-day period in beta diversity of the microbiota are shown in Figure 6. In patient 1, the wound values were around 0.7 except on Day 7. Besides, up to Day 6, the skin and bed environment values were smaller than the wound. In patient 2, the maximum value of the wound was 0.2, and there were many days with the lowest value in the wound throughout the 7 days. Patient 3 showed stable values for both the skin and bed environment up to Day 4, but the bed environment showed smaller values after Day 5. As demonstrated in Figure 7, beta diversity of patient 3 was significantly lower compared to those of patients 1 and 2 (p<0.01 and p<0.01, respectively), and this was evident throughout the study period.

Discussion

To the best of our knowledge, this is the first study to reveal temporal changes in the microbial diversity of the bed environment over a 7-day period using 16S rRNA gene analysis. Analysis of alpha diversity, which indicates species richness within a single microbiota, showed no significant changes in the bed environment over time, and this was evident in all patients. Analysis of beta diversity, which shows the differences between microbiota samples, revealed that the patient without a pressure ulcer presented a smaller change in bed microbiota than the other patients. These findings suggest that the presence of pressure ulcers may affect the microbiota in the bed environment and that interventions in the bed environment may be effective in preventing wound infection.

In terms of comparison of alpha diversity using the phylogenetic diversity index, there was no significant
difference in the diversity of the bed microbiota among patients. In accordance with our previous study\textsuperscript{13}, Corynebacterium and Staphylococcus, which are known commensal skin bacteria\textsuperscript{21}, were also found in abundance in the bed microbiota in the present study. Moreover, the dominant genera in the bed and wound were detected in the microbiota of the skin samples. Furthermore, as a result of the assessment of relative abundance, the dominant bacteria in the wound were also detected in the skin and bed environment throughout the 7 days. Furthermore, when the relative abundance of bacteria in the wound increased, its relative abundance also increased in the bed environment. The changes in relative abundance and members of the microbiota are also reflected in alpha diversity, and these findings suggest that bacterial dissemination occurs between the wound and bed environment. In the 7-day alpha diversity assessment, no significant changes in diversity due to the duration of use of the sheets were observed in all cases. The bed environment is an excellent substrate for bacterial growth under appropriate moisture and temperature
conditions. Hence, despite changing the bed sheets with new ones, the microbiota in the bed environment is expected to form immediately or at least within a day, suggesting that the bed linen can be a possible reservoir of bacteria contributing to the maintenance of specific wound microbiota. Further study is also required to investigate the effects of care other than changing of bed sheets (e.g., changing of diaper and clothes).

In this study, the patient without a pressure ulcer demonstrated the smallest change in beta diversity of bed microbiota compared to the other patients, indicating that the presence of pressure ulcers affects the microbiota in the bed environment. The study on patients with burns has demonstrated that dressing change increases the airborne bacteria level. This result suggests that dressing change may contribute to bacterial dissemination. Considering this, bacteria may have contaminated the bed sheets through daily wound care, influencing the microbial diversity of the bed environment. Furthermore, even if the bacteria on the wound bed are reduced by cleansing and debridement, the same wound microbiota may form again through bacterial dissemination from the bed environment. Therefore, interventions that take into account the patient’s bed environment as well as the wound site are necessary to prevent wound infection.

The number of patients surveyed in this study was small. This precludes robust conclusions. However, our results demonstrated that alpha and beta diversity of bed microbiota did not depend on the infection status of the wound. However, ulcer depth, surface area, and wound infection have been reported to influence the composition and diversity of chronic wound microbiota. Considering this, the present study did not reveal any difference in microbial diversity depending on wound severity, but it does not mean that wound severity does not affect the diversity of bed microbiota because bacterial dissemination occurs between the wound and bed environment. Therefore, in future studies, it is necessary to increase the sample size for more robust conclusions and examine the impact of wound severity on the bed microbiota.

We recognize several limitations of this study. The two pressure ulcers investigated in the present study differed in depth. Particularly, we examined a patient with an infected wound that penetrated the subcutaneous tissue (i.e., category III, according to the international pressure ulcer classification system) and a patient with a non-infected wound with partial thickness loss of dermis (i.e., category II). Therefore, it is not possible to distinguish whether the results obtained in this study are due to the differences in wound depth or presence of wound infection. A study on pressure ulcers with the same depth is required to determine the contribution of the bed environment to wound infection. Besides, swabs have been used to detect bacteria in hospital curtains and sheets, but no swabbing method was established to collect samples to identify the microbiota. In this study, the Z-stroke method which is established as the collecting method of the skin microbiota was used to collect samples to allow comparison with the skin microbiota, but it may underestimate the microbiota in the bed environment.

**Conclusion**

This is the first study to quantitatively assess the temporal changes in the composition and diversity of bed microbiota in patients with infected and non-infected pressure ulcers. During the 7-day period, no significant changes were found in the diversity according to the duration of use of the sheets in all cases. The temporal change in the composition of bed microbiota was greater in the patient with a pressure ulcer than in the patient without a pressure ulcer. This clearly suggests that the presence of a pressure ulcer is likely to affect the patient’s bed environment.

**Acknowledgments**

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褥瘡患者における寝床環境の細菌叢多様性の細時的変化

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要 旨

褥瘡患者において創傷感染は生命にかかわる問題であり、創部や創周囲皮膚の細菌負荷を軽減するケアを実施しているにもかかわらず、いまだに制御することができていない。褥瘡患者の多くは寝たきりであることから、創部上での細菌の供給源として寝床環境に着目した。しかし、寝床環境の細菌叢の形成過程はこれまでに明らかになっていない。そこで本研究では、感染褥瘡患者、非感染褥瘡患者、非褥瘡保有者患者的寝床環境の細菌叢を調査し、多様性と組成の細時的変化を検討した。細菌叢サンプルは臀部付近のシーツ、創部の中心、創周囲皮膚の3部位から7日間連続して採取された。細菌叢は16S ribosomal RNA遺伝子解析を用いて特定した。得られたシーケンスをoperational taxonomic unitsに分類し、細菌叢の多様性を評価した。細時的変化の評価はシーツ交換翌日を起点とした。α多様性の解析では、全患者においてシーツの使用期間に伴う有意な細時的変化は認められなかった。またβ多様性の解析では、非褥瘡保有者と比較し、褥瘡保有者のほうが有意に寝床環境の細菌叢の細時的変化が大きかった。これらの結果は、寝床環境が創部細菌叢の供給源となっており、褥瘡の存在が寝床環境の細菌叢に影響を与えている可能性が高いことを示している。創傷感染予防において寝床環境を考慮した介入を実施することが効果的だと考えられる。

キーワード：リネン、シーツ、微生物多様性、細菌伝播、慢性創傷