Comparative Analysis Between 16S rRNA NGS vs Conventional Culture associated with the Treatment Outcome of Diabetic Foot Ulcers

Sik Namgoong, MD, PhD[1,2,*], Yewon Choi, MD[1], Hyun Jyung Oh, MS[1], Seung-Kyu Han, MD, PhD[1], Gordon K. Lee, MD[2]

[1]Department of Plastic Surgery, Korea University Guro Hospital, Korea University College of Medicine, Seoul, Korea

[2]Division of Plastic and Reconstructive Surgery, Department of Surgery, Stanford University School of Medicine, Palo Alto, CA

Abstract

Background: Effective treatment of wound-site infections in diabetic foot ulcer (DFU) patients is crucial for a good prognosis. Recently, 16S rRNA next-generation sequencing (NGS) has been the main focus of research for accurately detecting wound-site microbes, which is vital in optimal antibiotic treatment. We compared the conventional culture-based detection method to the 16S rRNA NGS method to predict the DFU treatment outcomes.

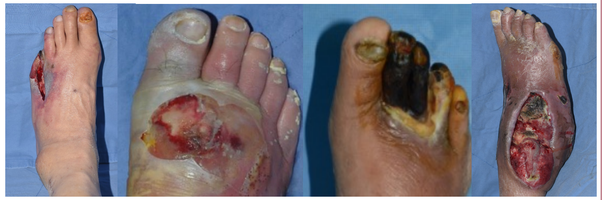
Methods: Wound-site samples from 47 DFU patients who were treated at Korea University Guro Hospital from February 2021 to November 2021 were analyzed with both conventional culture and NGS methods. We set the primary outcome as the healing status of each patient, which was assessed using the SINBAD score and amputation status, and the secondary outcomes as wound-site ischemia and infection control.

Results: The NGS method detected a broader range of microbial species (Shannon index=1.369 ± 0.755, Simpson index=2.987 ± 1.383) compared to the conventional culture method (Shannon index=0.693, Simpson index=1.269). Sixteen species were found using the two methods, which were all anaerobes. The most significant discordance of detected species was found in the SINBAD≥3 group (40.79%), and within that group, the patients with an absence of ischemia but poor infection control had the largest discordance (85.22%). Among the microbes detected significantly different between the two methods, *B.fragilis, S.agalactiae, S.aureus*, and *S.constellatus* were associated with poor prognosis, which were mainly detected in NGS than culture.

Conclusion: Early studies now suggest that 16S rRNA NGS may be an effective diagnostic tool for treating diabetic foot infection. We look forward to larger pivotal studies to confirm these initially promising findings.

Backgrounds

Diabetic foot ulcer (DFU) is one of the largest growing complications in patients with diabetes mellitus. The lifetime incidence of DFU is as high as 25% in diabetic patients, and the 5-year mortality rate in DFU patients is reported to be at least 30%. Along with the main causes of DFUs such as primary vascular diseases, peripheral neuropathy, and external trauma, controlling the site of infection in DFU patients is of utmost importance, as untreated wounds can frequently lead to amputations and even death.



For the treatment of DFU-site infections, conventional culture-based methods are currently the main protocol for optimal antibiotic selection. In most clinical setting however, most microorganisms in DFU-site infection wounds are difficult to culture accurately because of the physical barrier of biofilms and limiting conditions of artificial culture media, which frequently lead to biased results.

For the past few years, the application of the 16S rRNA next generation sequencing (NGS) technology has allowed numerous studies to gain insight into the pathophysiological nature of DFU-site microbiomes and to present novel methodological approaches for more accurate diagnosis. Currently however, there are few evidence of the validity of NGS methods compared to culture-based methods in DFUs regarding treatment prognosis.

In this study, we have analyzed the microbiota of DFU-site infections using both NGS and culture methods. Our purpose was to investigate the differences of the detected microbes between the two methods and associate them with DFU treatment outcomes.

Methods

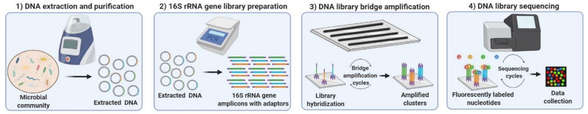
Patient Enrollment

We prospectively enrolled 47 patients with DFUs over a 12-month period at Korea University Guro Hospital, a tertiary teaching hospital in Seoul, Republic of Korea from February 2021 to June 2021. The patients were definitively diagnosed with DFU according to the 2019 International Working Group on the Diabetic Foot (IWGDF) guidelines, primarily using the site, infection, neuropathy, bacterial infection, and depth (SINBAD) classification and the score greater than or equal to three indicating positive. The patients who had a history of DFU in the past, or who had already received any other antimicrobial treatment or surgical debridement prior to this study were excluded from our analyses. All patients have agreed to provide their own samples and their clinical data with an informed consent. This study was approved by the institutional review board (IRB) of Korea University Guro Hospital (2019GR0425).

Sample Collection and Treatment Protocols

All specimens were collected before the initial treatment. The wounds were cleansed with sterile saline and hydrogen peroxide before each sample extraction, and the samples were collected from the near-bone areas deep within the necrotic tissue in order to avoid any hinderance of measurements due to microbial biofilms. The collected samples were immediately placed in the aerobic and anaerobic blood culture bottles (thioglycollate broth was used for exact differentiation between aerobic and anaerobic microbes) and sent to the microbiology laboratory. Samples for the NGS were kept separately in a sterile vial near zero degrees celcius and was sent to the research laboratory.

Either surgical debridement or antibiotics were applied to the patients according to their wound status and the culture results. The treatment outcomes were evaluated by an experienced plastic surgeon in terms of healing status, ischemia, and infection control. Healing status were categorized into either amputation below the knee, SINBAD score ≥ 3, or SINBAD score < 3. The patients with SINBAD score greater or equal to three were regarded as patients whose healing status showed wax-and-wane patterns, and patients with SINBAD score below three were regarded as patients who had significant remission. The evaluation of ischemia and infection control status were done using the classification system of the society for vascular surgery wound, infection, and foot infection (WIfI)[22]. Following the criteria from the American Diabetes Association (ADA), the ischemic status of the patient was considered as present if the transcutaneous oxygen pressure (TcPO2) was below 40 mmHg.



16S rRNA Sequencing

DNA was extracted from the skin and bone samples and swab samples from sterile swab kit using the DNeasy Blood & Tissue kit (Qiagen, Germany). The bacterial 16S rRNA gene V3-V4 region was used for PCR amplification. The primers used were 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR process of amplification was initial denaturation at 95C for 5 minutes, 28 cycles consisting of 15 seconds denaturation at 95C, 30 seconds annealing at 55C, and 30 seconds extension at 72C, with a final extension at 72C for 10 minutes. Amplicons of 16S rRNA V3-V4 region were maintained in equal amounts, and pair-end 2x300 base pairs were sequenced by the Illumina MiSeq platform in conjunction with the MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China). The raw pair-ended amplicon sequence reads were retrieved. The raw 16S amplicon sequences were processed by QIIME2 (version 2019.4). Preprocessing of the sequence reads was performed by the DADA2 plugin for the removal of the adapter, filtering quality (Q-score cutoff at 25), denoising, merging, and removal of chimeric-sequences. Then, high-quality sequences were retrieved as amplicon sequence variants (ASVs), and we used ASVs for diversity and taxonomic analyses. The Greengenes (version 13_8), a taxonomy classifier, which was used to pretrain on the primer set, was used to generate phylogenetic amplicons, cluster at 99% similarity, and taxonomically classify ASVs. Alpha diversity measurements, including richness, evenness, Shannon index, and Simpson index, were calculated.

Statistical Analyses

The software R version 4.1.2 was used for the statistical analyses and graphical visualization of the results. The alpha diversity of each method was calculated via Shannon and Simpson index. Fisher's exact test was performed to test the independence between categorial variables. P-values under 0.05 were considered as statistically significant.

Results

Patient Demographics

A total of 47 patients have enrolled in the study, and the samples from each patient were collected before the start of the treatment and have been analyzed via both culture and NGS. The baseline characteristics of the patients are presented in Table 1. The mean age of the patients was 65.18 ± 10.47 years, and 34 patients (70.83%) were male. Since the enrolled patients have all been diagnosed with diabetes mellitus, the average glycated hemoglobin level (HbA1c) was 8.44 ± 2.26 %, and the average serum glucose level was 204.5 (IQR=154.5-256.0) mg/dL. Most patients had definitive signs of inflammation with the mean C-reactive protein levels being 15.39 (IQR=2.28-37.94) mg/L and mean erythrocyte sedimentation rate (ESR) being 74.42 ± 36.72 mm/h.

Characteristics	Values (N=47)
Age, yrs	65.18 ± 10.47
Male, n	34 (72.34%)
BMI, kg/m²	22.80 ± 3.19
Hemoglobin, mg/dL	8.44 ± 2.26
WBC (x10³/L)	8.25 (6.67-10.50)
Neutrophil (x10³/L)	5.53 (4.55-7.66)
Procalcitonin, mg/dL	0.11 (0.06-0.35)
C-reactive protein (CRP), mg/L	15.38 (2.28-37.94)
Erythrocyte Sedimentation Rate (ESR), mm/h	74.42 ± 36.72
Serum Creatinine, mg/dL	3.53 ± 3.54
Glucose, mg/dL	204.5 (154.5-256.0)
HbA1c, %	8.44 ± 2.26

Table 1. Patient Demographics

Oragnism	Gram	Genus	Species	Culture (N=47)	NGS (N=47)	P value
Aerobic	+	Corynebacterium	C. striatum	1	0	1.0000
	-	Pseudomonas	P. aeruginosa	3	2	0.1249
		Proteus	P. vulgaris	1	0	1.0000
Anaerobic	+	Enterococcus	E. avium	1	0	1.0000
			E. faecalis	3	5	0.0006*
		Staphylococcus	S. aureus	11	12	0.0002*
			S. epidermidis	1	0	1.0000
			S. lugdunensis	1	3	0.0638
		Streptococcus	S. agalactiae	4	8	0.0004*
			S. constellatus	2	1	0.0426*
	-	Klebsiella	K. oxytoca	2	3	0.0028*
		Citrobacter	C. freundii	2	7	0.0213*
		Escherichia	E. coli	3	0	1.0000
		Serratia	S. marcescens	3	0	1.0000
		Bacteroides	B. fragilis	2	7	0.0194*
		Enterobacter	E. cloacae	1	2	0.0426*

Table 2. Detected Microbial Clades using Culture and NGS

Detected Microbes using Culture and NGS

Among the enrolled patients, a total of 47 samples were collected. The alpha diversity indexes of the culture and NGS methods are graphically presented in Figure 1. Compared to the culture method (Shannon index=0.693, Simpson index=1.269), NGS detected much wider variety of microbial species (Shannon index=1.369 ± 0.755, Simpson index=2.987 ± 1.383). The indexes of the culture-based method were given as a mean estimate since only few microbes were detected in the culture.

The number of detected microbial species and their heatmap are each presented in Table 2 and Figure 2. In total, 16 species have been found using the two methods. After quantitative correction, only 9 species were found in NGS. The microbial species which were differentially detected by the two methods were all anaerobic bacteria: E. faecalis, S. aureus, S. agalactiae, S. constellatus, K. oxytoca, C. freundii, B. fragilis, and E. cloacae. More specific data of detection results are in Supplementary Figure 1 and 2.

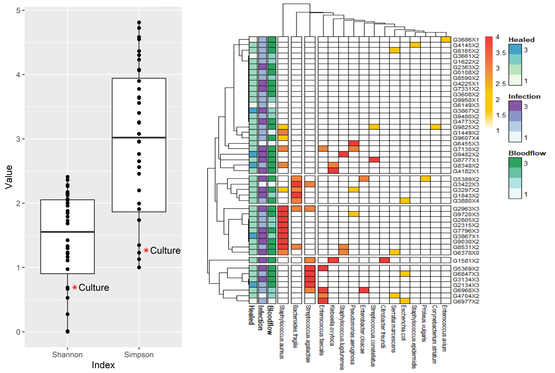


Figure 1. Alpha diversity index (Shannon and Simpson) of the NGS method compared to the culture-based method.

Figure 2. Heatmap of detected microbes via NGS and culture.

Different Findings between Culture and NGS regarding Post-Treatment Healing Status

Among of all 47 patients after the treatment, 5 patients (10.64%) had SINBAD score under 3, 38 patients (80.85%) had SINBAD score greater than or equal to 3, and 4 patients (8.51%) eventually had below-the-knee amputation. The microbial species detected with culture and NGS in each group according to their healing status are respectively in Figure 3A, 3B, and 3C, along with species detected exclusively in only one of the two methods presented in Table 3. The group with the most difference of results was the group with SINBAD ≥ 3, having 59.21% of concordance between culture and NGS. In SINBAD ≥ 3 healing group, B. fragilis and S. agalactiae were detected more in NGS than in culture (Figure 3B).

Different Findings between Culture and NGS regarding Post-Treatment Wound-site Ischemia and Infection Control in SINBAD ≥ 3 Group

Figure 4A and 4B shows the relative amount of detected microbial species which are grouped again with either (A) Absence of ischemia with good infection control, or (B) Presence of ischemia or bad infection control, meaning a patient was assigned to the latter if any one of the two complications were present. B. fragilis and S. agalactiae were prominently detected in the NGS compared to culture in the former group (Figure 4A), and S. agalactiae and S. constellatus in the latter group (Figure 4B) which were all the species that were detected significantly different (Table 2).

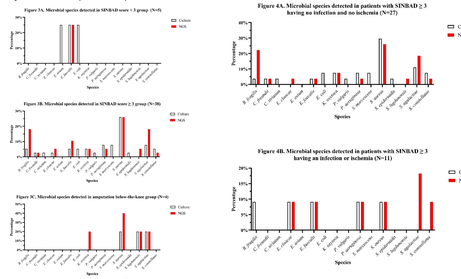


Figure 3. Relative amount of detected microbes associated with post-treatment healing status. (A) SINBAD score < 3, (B) SINBAD score ≥ 3, (C) Amputation below the knee.

Figure 4. Relative amount of detected microbes associated with post-treatment blood flow and infection control outcomes in patients with SINBAD score ≥ 3.

Conclusions

In conclusion, the 16S rRNA NGS method produces more refined results compared to the original culture-based method in terms of predicting the DFU treatment outcome. We believe that our study can be a strong motive for forthcoming studies to prove the superiority of NGS to the current culture-based detection method, or even replace the latter in future wound infection treatment guidelines.

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