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Original Article

Microbiome of limb-threatening diabetic foot ulcers indicates the association of fastidious *Stenotrophomonas* and major amputation

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Stenotrophomonas spp

Abstract *Background:* Proper identification of the polymicrobial microorganisms in patients with limb-threatening diabetic foot ulcers (LTDFUs) using conventional culture is insufficient. This prospective study evaluates the potential value of adjuvant molecular testing assisting in identify fastidious micro-organisms in LTDFUs compared to standard treatment alone.
Methods: Ninety patients with LTDFUs received interdisciplinary and standard antibiotic treatment in a referral diabetic foot center. A simultaneous 16S amplicon sequencing (16S AS) specimen along with conventional culture collected at admission was used to retrospectively evaluate the microbiological findings and its association with amputation outcomes.
Results: The microorganism count revealed by 16S AS overwhelmed that of conventional culturing (17 vs. 3 bacteria/ulcer respectively). The *Stenotrophomonas* spp. revealed in 29 patients were highly correlated with major (above ankle) amputation (OR: 4.76, 95% CI 1.01—22.56), while only one had been concomitantly identified by conventional culturing. Thus, there were 27 cases without proper antibiotics coverage during treatment.

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Conclusions: Adjuvant molecular testing assisted identification of fastidious pathogens such as *Stenotrophomonas* infection and might be associated with major amputation in patients with LTDFUs.

Introduction

Diabetic foot ulcer (DFU) is a common and complex complication of diabetes and accounts for the majority of non-traumatic lower-extremity amputations (LEA) and hospitalizations due to diabetes complications.[1—4] Through the rigors of interdisciplinary treatment for limb-threatening diabetic foot ulcers (LTDFUs), limb-saving results still remain unsatisfactory,[5—7] the failure of which is mainly related to the complex wound environment, peripheral circulation,[8,9] and infection.[10,11] Standard treatment of diabetic foot infection involves administration of broad-spectrum antibiotics until the specific bacteria is revealed from wound culturing.[10,12] Conventional culture-dependent methods are usually time-consuming. Additionally, quantitative culture-based techniques[13] select for species that flourish under typical conditions of the microbiology laboratory, but these may not be the most abundant or clinically important pathogens.[14] As the results, conventional wound culturing methods may fail to identify slow growing, fastidious or anaerobic organisms.[15]

When a foot infection progresses to becoming limb threatening, the nature of multiple bacteria growth in the long-standing ulcer and frequently encountered antibiotic (topical, oral, or parental) pretreatment make it problematic for pathogenic microorganisms to be cultivated by conventional culturing methods.[16] However, early and accurate detection of causative pathogens is likely important to enabling appropriate species-specific antimicrobial treatment in a timely manner to reduce risk for major amputation.

Molecular methods are advancing and becoming more accessible and affordable to identify the pathogens present.[16] Following genomic amplification, 16S rRNA gene fragments are sequenced and can further identify probable pathogens in patients with culture-negative infections.[17] This study aimed to compare the microorganisms identified by cultures and 16S amplicon sequencing (16S AS) and evaluate the potential clinical application in reducing LEA outcome.

Materials and methods

Patient enrollment

This prospective observational study was conducted in an interdisciplinary diabetic foot care center accredited by the International Diabetes Federation West-Pacific Region in Taiwan. All patients aged over 30 years admitted from July 1, 2020 to November 30, 2020 presenting with LTDFUs (defined as PEDIS infection grade $\geq$3 or infection grade 2 with perfusion grade $\geq$2) were included. The study was carried out in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Chang Gung Memorial Hospital (No. CMRPG3L1221 and No. NMRPG3K0391). Written informed consent was obtained from all participants.

This report follows the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines for cohort studies.[18]

Wound recording

According to the Infectious Diseases Society of America and the International Working Group on Diabetic Foot guidelines, all wounds were recorded as PEDIS describing the perfusion, extent size, depth, infection and sensation of the wounds.[19] The perfusion status was categorized into three grades, with grade 3 perfusion status representing critical limb ischemia as defined by the presence of gangrene or ulcers with an ankle pressure $<$70 mmHg, or monophasic wave form of distal segment of the posterior tibial artery and dorsalis pedis artery. Adjunct angiography was performed for confirmation. Osteomyelitis was defined in patients with a positive probe-to-bone test in combination with abnormalities on plain X-ray.[19]

Sample collection and processing

Following the guidelines of International Working Group on the Diabetic Foot (IWGDF),[20] all limb-threatening DFUs received sharp debridement to remove the necrotic tissue or surrounding callus before the sample collection. Sterile cotton swabs (Transystem, COPAN, Italia) were rotated with slight pressure on a 1 cm^2 area of viable tissue to take wound swabs for conventional microbiological culture testing and 16S AS. The swabs were transported to the Chang Gung Memorial Hospital Bacteriology Laboratory and placed on blood agar plates (tryptic soy agar, 5% sheep's blood, 10 μg/mL neomycin; BD Biosciences, Bedford, Massachusetts, USA). The plates were then cultured at 37 °C with 5% CO_2 atmosphere, and normal atmosphere for 48 h. Bacterial identification was determined using the Bruker LT microflex MALDI-TOF MS with Bruker BioTyper 3.1 system software (Bruker Daltonics, Bremen, Germany) and Bruker Biotyper database (DB5989MSP).[21,22] Furthermore, FlexAnalysis 3.3 (Bruker Daltonik GmbH, Bremen, Germany) was also implemented to acquire the numerical spectra data which derived from MALDI-TOF MS. For another part, according to the manufacturer's instructions, the swab was performed using QIAamp PowerFecal DNA Kit (Qiagen, United States) to extract genomic DNA, and the extracted

DNA was stored at −80 °C. The bacterial 16S AS was then constructed based on the hypervariable region V3−V4 [23] and sequenced using MiSeq System with MiSeq Reagent Kit v3 (600 cycles) (Illumina, United States). Next, the sequencing reads were de-multiplexed using MiSeq Reporter v2.6 according to sample barcodes and following this, merged paired reads, quality filtering and clustering into a zero-radius operational taxonomic unit (zOTU) were performed using USEARCH (v11, https://drive5.com). Final taxonomic assignments were completed using the SINTAX algorithm[24] with the ribosomal database project (RDP training set v16) serving as a species reference database. Alpha-diversity (e.g., Chao1 index and Shannon index) and Beta-diversity (e.g., Bray−Curtis dissimilarity) normalized by DESeq2[25] were both calculated and visualized in R (version 4.1.1).

Microorganism, antibiotics strategy, and consensus of managements

Ninety-five percent of specimens in this study were obtained from deep ulcers. Positive microbial cultures were defined as growth of the same pathogen on two or more culturing media. Broad-spectrum antibiotics were prescribed promptly for these patients initially, including third and fourth generation cephalosporin, extended-spectrum penicillin, fluoroquinolones, aminoglycosides and carbapenems. Empiric antibiotics were subsequently modified according to the results of wound cultures. Surgical debridement was performed in a timely manner by plastic surgeon, while major procedures such as endovascular therapy or LEA were scheduled immediately after the diabetic foot team reached consensus. Minor (below the ankle) or major (above the ankle) LEA outcomes were further analyzed.

Statistical analyses

Clinical demographics and PEDIS wound-grading were registered from the patient's first visit at admission, and routine laboratory data at enrollment were analyzed. Categorical variables were reported as numbers and percentages, and continuous variables were reported as medians and interquartile range. Comparisons between each microorganism group were performed using Pearson's chi-square test for categorical variables. The unadjusted odds

Table 1 Descriptive characteristics.

	Patient numbers				
	n = 90				
Age (years)	59.9 (51.8, 68.9)	**Wound assessment**		**Treatment**	
Male gender (n, %)	69 (76.7)	Wound duration (days)	80.0 (16.0, 201.3)	Endovascular therapy (n, %)	22 (24.4)
BMI (kg/m^2)	25.0 (22.3, 29.0)	Osteomyelitis (n, %)	35 (38.9)	Discharge status (n, %)	
Comorbidities		Necrotizing fasciitis (n, %)	16 (17.8)	No amputation	54 (60.0)
DM duration (years)	10.5 (5.0, 20.0)	**PEDIS classification at admission**		Minor amputation	26 (28.9)
Retinopathy (n, %)	51 (56.7)	Perfusion (n, %)		Major amputation	8 (8.9)
Neuropathy (n, %)	69 (76.7)	Grade 1	10 (11.1)	Mortality	2 (2.2)
Hypertension (n, %)	72 (80.0)	Grade 2	30 (33.3)		
Stroke (n, %)	18 (20.0)	Grade 3	50 (55.6)		
Coronary heart disease (n, %)	25 (27.8)	TcPO2 (mmHg)	40.0 (28.3, 49.0)		
Heart failure (n, %)	13 (14.4)	Wound size (cm2)	12.0 (4.0, 28.9)		
LVEF (n, %)	64.0 (57.5, 70.8)	Depth (n, %)			
ESRD (n, %)	30 (33.3)	Grade 1	4 (4.4)		
Previous DFU (n, %)	76 (84.4)	Grade 2	32 (35.6)		
Previous minor LEA (n, %)	65 (72.2)	Grade 3	54 (60.0)		
Lab data		Infection (n, %)			
Hemoglobin (mg/dL)	10.5 (9.5, 12.7)	Grade 2	21 (23.3)		
Leukocyte count (10^3/mL)	11.0 (7.4, 15.7)	Grade 3	45 (50.0)		
C-reactive protein (mg/dL)	65.5 (16.4, 188.0)	Grade 4	24 (26.7)		
Albumin (g/dL)	3.4 (3.0, 3.9)	Sensation (n, %)			
eGFR (ml/min/1.73 m^2)	65.9 (39.5, 95.0)	Grade 1	48 (53.9)		
HbA1c (%)	7.6 (6.8, 9.4)	Grade 2	41 (46.1)		
Triglyceride (mg/dL)	122.5 (98.3, 153.5)				
HDL (mg/dL)	28.5 (22.8, 34.5)				
LDL (mg/dL)	79.0 (63.0, 112.0)				

BMI, body mass index; LVEF, left ventricular ejection fraction; ESRD, end stage renal disease; DFU, diabetic foot ulcer; LEA, lower extremities amputation; eGFR, estimated glomerular filtration rate.

ratio (OR) and 95% confidence interval (95% CI) for amputation were calculated. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS for Mac, version 26.0, IBM Corp., Armonk, NY, USA) data analysis software.

Results

Characteristics of study population

As shown in Table 1 the median age of 90 patients was 59.9 (51.8, 68.9) years, with male gender predominating (76.1%). Twenty percent of them had prior stroke, 27.8% had prior coronary heart disease, 14.4% had heart failure, and 33.3% of them received regular dialysis while seventy-two percent had already experienced DFU before admission.

The characteristics of LTDFUs showed most of them were deep ulcers, 55.6% were in worst grade of perfusion, and seventy-six percent had an infection score greater than 3.

Following intensive limb-salvage treatment, 28.9% and 8.9% of patients still received minor and major LEA respectively.

Conventional culture vs. 16S AS analysis

The medians of bacteria per ulcer were 3 (2, 3) and 17 (14, 24) by conventional culture and 16S AS respectively. Based on presence or absence in each sample, the percentage of specific pathogens is illustrated in Fig. 1. All pathogens detected by using conventional culturing technique were identified by 16S AS.

Study the specific pathogens associated with amputation outcomes

Forest plot analysis of specific pathogens associated with amputation outcomes in patients with LTDFUs as demonstrated in Fig. 2 and the Supplemental Fig. S1. In analysis of the pathogens from conventional culture, only *Bacteroides* spp. (OR: 4.22, 95% CI 1.43–12.43) could predict minor LEA while no significant spp. was found to associate with major LEA.

By using 16S AS, *Stenotrophomonas* spp. had predictive value of major LEA (OR: 4.76, 95% CI 1.01–22.56), while *Actinomyces* spp. (OR: 4.20, 95% CI 1.37–12.86) and

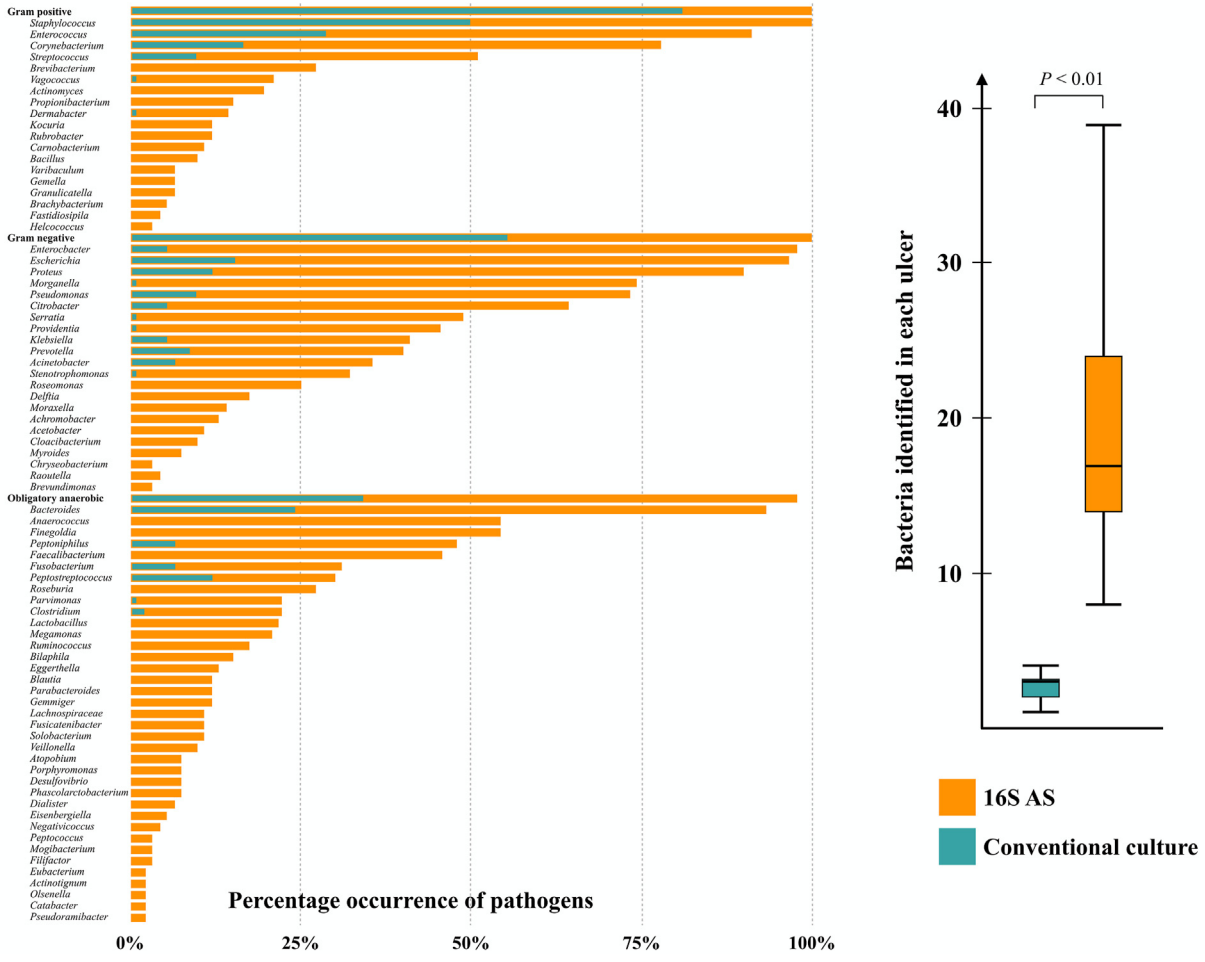

Figure 1. Commonly isolated bacteria from foot ulcer by using 16S amplicon sequencing analysis and conventional culturing. The bar in the middle of the boxes represents the median; the upper and lower hinges of the box represent the first and third quartiles.

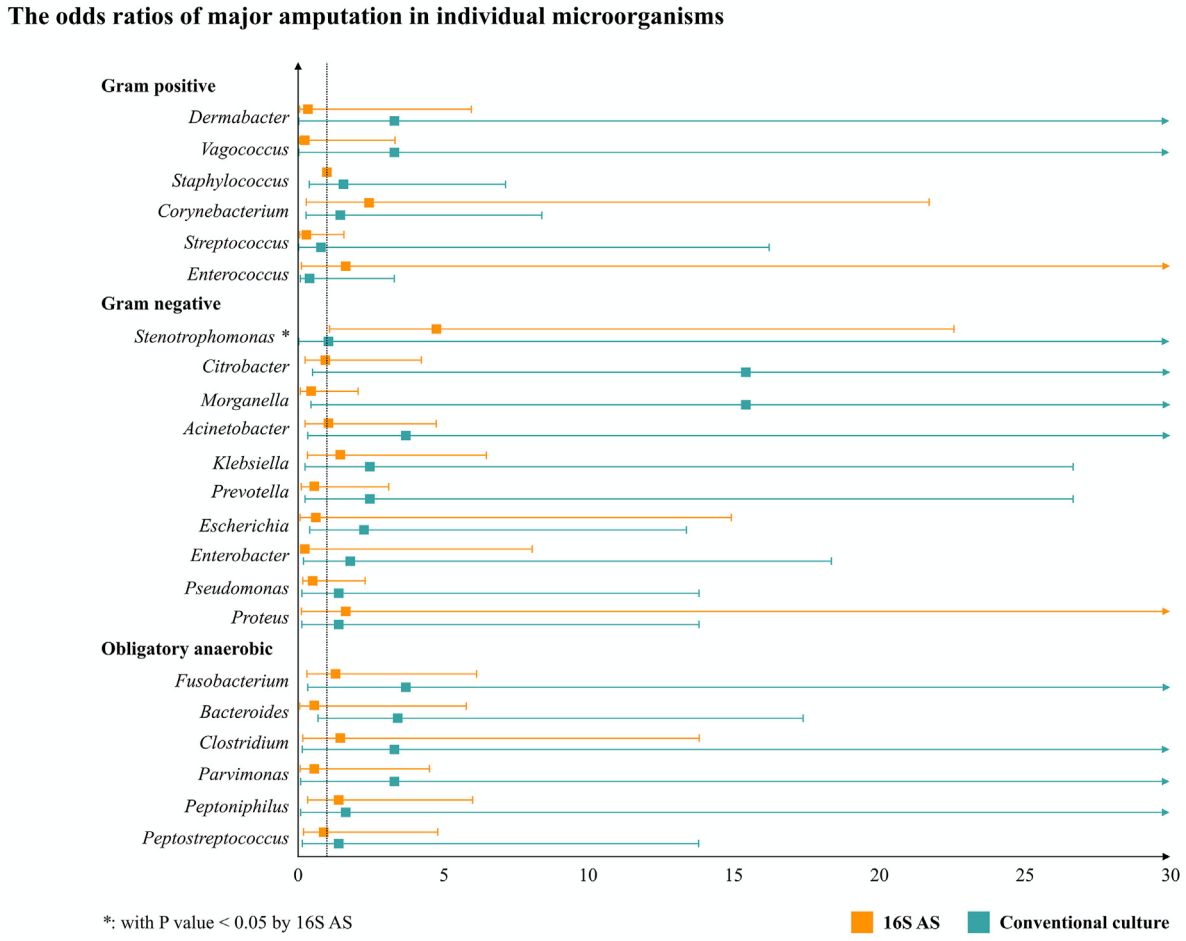

Figure 2. The odds ratios of major LEA in individual pathogens. By using 16S AS, *Stenotrophomonas* were associated with major amputation.

Varibaculum spp. (OR: 25.71, 95% CI 1.35—491.53) were associated with minor LEA.

Clinical characteristics of patients with stenotrophomonas infection

Table 2 demonstrated the clinical characteristics and treatment of 29 patients with *Stenotrophomonas* infection documented by 16S AS. All the 29 patients were in the most severe infection grade (3 or 4). Of note, only one case (case#1) had positive *Stenotrophomonas* infection detected by conventional culturing while only two cases (case#1 and 2) had received proper antibiotics (fluoroquinolone) to cover the *Stenotrophomonas* spp. None of these 29 patients had received TMP-SMX.

Discussion

In this study, 16S AS identified more genera than conventional culturing in LTDFU, especially for gram-negative and obligatory anaerobic pathogens. Further analysis of specific microorganism and amputation outcome reported the association between *Stenotrophomonas* spp. and major LEA. Of note, among the 29 patients with *Stenotrophomonas* infection detected using 16S AS, only one case was concomitantly identified by conventional culture.

Stenotrophomonas is a gram-negative obligatory aerobic bacterium that occurs in patients with prolonged hospitalization, prior exposure to broad-spectrum antibiotics treatment and intravascular catheterization,[26] and is usually resistant to several antibiotics because it confers various mechanisms of drug resistance such as decreased permeability, the production of beta-lactamase and carbapenemase enzymes, the production of aminoglycoside-modifying enzymes, and the presence of multidrug efflux pumps.[26,27] In our study, although broad-spectrum antibiotics were promptly prescribed after referral, only two of the 29 patients received fluoroquinolone with *Stenotrophomonas* infection.

Varibaculum and *Actinomyces* are both gram-positive and facultatively anaerobic bacteria from the family Actinomycetaceae, and prolonged bacterial cultures are necessary to identify these bacteria.[28] Both have been reported as potential pathogens in soft tissue infections resulting from trauma or foreign bodies.[29] In this study, it's difficult to conclude whether *Varibaculum* or *Actinomyces* were misidentified or unidentifiable by conventional

Table 2 Characteristics of patients with *Stenotrophomonas* infection.

No.	Age	Gender	CAD/HF	CVA	ESRD	OM	P	E (cm^2)	D	I	S	Number of spp. (culture/16S AS)	Extended-spectrum penicillin	3rd and 4th generation cephalosporin	Carbapenem	EVT	Outcome (Days§)
1*‡	62.3	M	◉/				2	93.5	2	4	1	1/30					No LEA
2‡	72.0	M	◉/◉		◉		2	6	3	3	2	5/30	◉				No LEA
3	31.8	M					2	100	1	3	2	1/46		◉			No LEA
4	35.6	M					1	2	2	4	1	3/19				◉	No LEA
5	39.5	M					2	5	2	3	1	1/26		◉			No LEA
6	42.2	M					1	4	3	4	2	3/16	◉	◉			No LEA
7	54.8	M				◉	3	8	3	3	1	1/10	◉				No LEA
8	56.6	M	◉/◉	◉			3	1	3	3	2	4/17	◉				No LEA
9	57.8	F			◉	◉	2	1	3	3	2	2/27					No LEA
10	59.1	M					3	14	2	3	1	1/12	◉				No LEA
11	59.9	M	◉/	◉	◉		3	233	3	3	2	2/16	◉				No LEA
12	60.7	M					3	117	2	4	1	2/18			◉		No LEA
13	62.1	M	◉/			◉	1	0.3	2	4	1	4/24		◉			No LEA
14	68.8	M	◉/				2	6	2	3	1	1/30		◉			No LEA
15	51.4	M		◉	◉	◉	2	3	3	3	2	0/43					Minor LEA (5)
16	53.2	M			◉	◉	3	10.5	3	3	1	5/26		◉	◉		Minor LEA (9)
17	60.1	M	◉/		◉	◉	2	12	3	3	2	2/20	◉	◉			Minor LEA (1)
18	60.7	F					3	84	2	4	1	2/21		◉		◉	Minor LEA (27)
19	67.5	F	◉/		◉		3	4	3	3	2	3/14	◉	◉		◉	Minor LEA (6)
20	67.7	F	◉/			◉	3	0.6	2	4	1	5/17	◉				Minor LEA (15)
21	72.2	M				◉	3	12.5	3	3	2	2/20	◉	◉		◉	Minor LEA (21)
22	75.1	F			◉		3	0.2	3	3	2	4/21		◉		◉	Minor LEA (13)
23	88.2	F	◉/◉				2	18	3	3	1	1/23	◉	◉		◉	Minor LEA (2)
24	59.9	F			◉		3	20	2	4	1	3/13	◉	◉			Major LEA (16)
25	62.7	M	◉/◉		◉		3	16	3	4	2	3/16	◉	◉	◉	◉	Major LEA (14)
26	69.8	M		◉	◉		3	35	3	4	1	5/14			◉		Major LEA (3)
27	71.3	F	◉/	◉		◉	3	96	3	4	2	4/19	◉				Major LEA (14)
28	75.7	M	◉/◉	◉			2	3	2	3	2	3/16	◉	◉			Major LEA (8)
29†	50.3	M	◉/			◉	2	18	3	4	2	2/15	◉	◉	◉		Mortality (86)

*: *Stenotrophomonas* spp. was documented by both 16S rRNA sequencing and conventional wound culture.
‡: Patients who received Fluoroquinolone treatment for *Stenotrophomonas*.
†: Mortality due to infective endocarditis (tissue culture: MRSA).
§: Time interval between sampling and outcome event.
Extended-spectrum penicillin: Including ampicillin, amoxicillin/clavulanic acid, ampicillin-sulbactam, and piperacillin-tazobactam.
3rd and 4th generation cephalosporin: Including ceftazidime, cefoperazone-sulbactam, ceftriaxone, and cefepime.
Aminoglycosides: Including amikacin and gentamicin.
Carbapenem: Including doripenem, ertapenem, imipenem, and meropenem.
Abbreviations: CAD, coronary artery disease; HF, heart failure; CVA, cerebrovascular accident; ESRD, end stage renal disease; OM, osteomyelitis; P, perfusion; E, extension.
D, depth; I, infection; S, sensation; EVT, endovascular therapy.

culture, while l6S AS assisted in identifying these microorganisms. *Varibaculum* and *Actinomyces* were prone to having minor LEA in this study. Fortunately, they are usually susceptible to beta-lactam antibiotics[28]; therefore, *Varibaculum* and *Actinomyces* appear unrelated to major LEA.

Clinical studies using next generation molecular sequencing for diabetic foot diseases are still rare. In a study of 31 patients with DFU without surgical intervention (94% of whom had osteomyelitis), Mudrik-Zohar et al.[30] reported that 7 times more genera were identified by 16S AS compared to conventional culture, which is similar to our study. They reported that *Bacteroides* was prevalent among patients who underwent amputation. In patients with LTDFU, similar to our study, deep surgical debridement may reduce the abundance of anaerobic bacteria[31] and therefore reduce the impact of *Bacteroides* on amputation.

Our recent study reported that *Proteus* spp. and *E. coli* were associated with minor LEA, whereas *Klebsiella* spp., *E. coli*, and *Morganella morganii* were associated with major LEA in patients with LTDFU by conventional culturing.[32] Compared with 16S AS, conventional culturing methods might result in bacteria of interest in patients with LTDFU remaining unidentified. False-negative culture results make it problematic to identify slow-growing or fastidious pathogens,[16] resulting in a delay in the appropriate treatment.

This study has been limited to one center and limited sample size. The efficacy of empirical antibiotics treatment against pathogens have not presented. The application of 16S AS was limited by the short read lengths obtained and sequencing errors and do not allow strain-level description of the community.[33] Therefore, the results of 16S AS study may only confirmed colonization of microorganisms. However, compared with advanced molecular methods such as metagenomic next-generation sequencing, 16S AS is currently less costly and more practical for clinical approach. Though tissue biopsy remains the gold standard collection method, the deep swab remains more practical used in the real-world. Moreover, study has been shown 16S AS provides potential for using less invasive swabs to improve bacterial identification for DFUs.[34] A larger sample investigation is needed to clarify the microbiological impact.

Conclusion

Previously unidentified fastidious pathogens such as *Stenotrophomonas* spp. might be associated with major amputation outcomes during treatment of LTDFU. Further large-scale studies are needed to clarify the value of clinical application of molecular testing for reducing amputation.

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Declaration of competing interest

No potential conflicts of interest relevant to this article were reported.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2023.10.007.