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Analysis of Lower Leg Ulcer Pathogens Using 16S rRNA Gene Based Method

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Authors' contributions

This work was carried out in collaboration among all authors. Author ECO designed the study and wrote the first draft of the manuscript in collaboration with author OOO. Author UGE sourced for the literature materials used in the study and articulated the final write up. Author OID managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

The study was carried out to determine the bacterial pathogens associated with lower leg ulcers in Ebonyi State from July, 2016 to July of 2017, using wound swabs from eligible patients with lower leg ulcers. The swabs were processed and analysed using standard microbiological methods, isolated microbial pathogens were identified by employing standard biochemical test, microbial identification tests and standard molecular methods for DNA extraction. Pressure ulcers 450 (37.2%) was found to be the most commonly infected, closely followed by diabetic foot ulcers 300 (24.8%) and non-healing surgical ulcers 210 (17.4%). *Staphylococcus aureus* strain ATCC 12600 (12.0%, 6.7% and 16.7%) was the most predominant in venous, diabetic and non-healing surgical ulcers respectively, while pressure ulcer had *Pseudomonas aeruginosa* strain M37351 (8.2%). Out of 1500 specimens examined, 1210 (80.7%) showed positive microbial growth, while 290 (19.3%)

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were not infected. Age group of 31-40 years had the highest prevalence rate of 20.7%, followed by 41-50 years (20.5%) while the least was 0-10 years (0.1%). The males were mostly affected than females. This study has revealed a high index of microbial involvement in lower leg ulcer in Ebonyi State. We recommend a multidisciplinary approach to leg ulcer management and specific intervention strategies, not only to treat but also to reduce and subsequently prevent their spread in rural communities. This results and findings will hopefully help to create awareness on the imperative to improve the quality of the treatment regime employed. Thus, each health institution should carry out a survey to determine the common microbial wound pathogens among their patients.

Keywords: Lower leg ulcer; bacteria; 16S rRNA and pathogens.

1. INTRODUCTION

The term ulcer refers to a serious, long-lasting wound, and is described as chronic if it does not show any healing tendency within six weeks. Ulcers (wounds) may arise post-operatively, following farm injuries, scratches, hoe cuts, thorns cuts, burns, or in association with certain medical conditions such as diabetes mellitus, haemoglobinopathy, lower extremities arterial disease, vasculitis, ulcerative skin diseases and malignancies [1].

Leg ulcer is known as lower limb ulcer, is defined as a defect in the skin below the level of knee persisting for more than six weeks and shows no tendency to heal after three or more months [2]. Ulceration of the lower legs is a relatively common condition amongst adults, and it causes pain and social distress [3]. Ulcers of skin can result in complete loss of the epidermis and often portions of the dermis and even the subcutaneous fat [4]. The incidence of ulceration is rising as a result of the aging population and increased risk factors for atherosclerotic occlusion such as smoking, obesity, and diabetes. In the course of a lifetime, almost 10% of the population will develop a chronic wound, with a wound-related mortality rate of 2.5%. Shukla, et al. [5] reported that inappropriate treatment of acute traumatic wounds was the most common cause of the chronic wound. A study carried out by [6] showed that the principal etiology (67%) of ulceration is trauma or traumatic wounds compounded by infection, while diabetic ulcers, venous ulcers, and pressure ulcers accounted for 4.9%, 6.5%, and 9.2%, respectively. The majority of these wounds were seen in farmers and other agricultural workers [6,7]. The current spread of multi-drug resistant bacteria pathogens has added a new dimension to the problem of wound infections. This is particularly worse in resource-poor countries where the sale of antibiotics is under poor control [8]. Leg ulcers are debilitating and they greatly reduce the patients' quality of life.

The traditional identification of bacteria on the basis of phenotypic characteristics is not as reliable as recent identification which is based on genotypic methods. The ribosomal RNA sequence based analysis is an implicit and unique method to understand microbial diversity within and across a group and also to identify new strains [9].

In this study, we employed 16S ribosomal RNA gene based method to investigate pathogens associated with lower leg ulcers in Ebonyi State, Nigeria. Through this approach, we identified the predominant bacterial species present within the ulcer types and assessed whether differences in patient demographics affected the composition of the microbes in these samples.

2. MATERIALS AND METHODS

2.1 Study Area

The study was carried out at the Federal Teaching Hospital Abakaliki (FETHA) and private hospitals in the three Senatorial Districts of Ebonyi State.

2.2 Study Design

A total of 1500 specimens were collected from patients of lower leg ulcers in the three Senatorial Districts of Ebonyi State.

2.3 Ethical Consideration

Prior to the commencement of the study, ethical clearance was sought from the Federal Teaching Hospital Abakaliki (FETHA) ethical committees. In addition, letters requesting for collaboration was written to the management of all the private

hospitals from which specimens were collected and consent was obtained from parents or close relatives.

2.4 Collection of Specimens

The specimens were collected with sterile swab sticks in accordance with standard routine procedure. If delay is unavoidable (more than 2 hours), specimens were placed in Stuarts transport medium and refrigerated immediately [1]. Specimens were analyzed on the same day of collection. A structurally designed questionnaire was used for obtaining information concerning each patient. Specimens were completely and properly labeled as well as from the hospital records. Patients on antimicrobial therapy within 72 hr of presentation were included in the study.

2.5 Culture of Specimens on Media

Cultures of the specimens were made on nutrient agar, Mannitol Salt Agar, EMB, blood and MacConkey agar for the isolation of the bacterial. After incubation at 37℃ for 24 hrs, the plates were observed; carefully examined and distinct growths were sub-cultured on fresh medium for purity. The bacterial isolates were identified using their cultural identification, morphological and other biochemical characteristics as described by Bergey's Manual of Determinative Bacteriology [10,11].

2.6 Identification of isolates by DNA Sequencing

The Methods employed were the following.

1: Culture on Nutrient Broth

2: DNA extraction: ZR D3024 Quick-gDNA™ MiniPrep (50 Preps)

3: PCR amplification and DNA sequencing by Sanger sequencing method

4: Blasting analysis: CLC BIO AND NCBI BLAST ONLINE

SEQUENCING: Sanger sequencing

Primer that was used: Bacteria: 16S, 27-F

2.7 Molecular Analysis

DNA Extraction was performed at the Anaerobe Laboratory, Molecular Biology and biotechnology Division, Nigerian Institute of Medical Research, Yaba, Lagos. Methodology was based on PCR and metagenomics analysis. While sequencing analysis was done at Inqaba Biotechnology Pty South Africa.

2.8 DNA extraction ZR D3024 Quick-gDNA™ MiniPrep (50 Preps)

DNA extraction was from a 24 hours growth of microbial isolates in BHI broth harvested by centrifugation at 14, 000 x g for 10 minutes. The cells was washed three times in 1 ml of ultra-pure water by centrifuging at 12,000 rpm for 5 min. DNA extraction and purification was done using ZR Fungal/Bacterial DNA MiniPrep™50 Preps Model D6005 (Zymo Research, California, USA). 50-100 mg of bacterial cells was resuspended in 200 µl of sterile water. This was transferred into a ZR Bashing Bead™ Lysis Tube. Exactly 750 µl lysis solution was added to the tube. The bead containing the solution was secured in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for 5 minutes. The ZR Bashing Bead™ Lysis Tube was centrifuged in a microcentrifuge at 10,000 x g for 1 minute. 400 µl of the supernatant was pipetted into a Zymo-Spin™ IV Spin Filter in a Collection Tube and centrifuged at 7,000 x g for 1 minute. This was followed by the addition of 1,200 µl of Bacterial DNA Binding Buffer into the filtrate in the Collection Tube. After this, 800µl of the mixture was transferred into a Zymo-Spin™ IIC Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute.

The flow through was discarded from the Collection Tube and the process was repeated to obtain the remaining products. The 200 µl DNA Pre-Wash Buffer was added into the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute. This was followed by the addition of 500µl Bacterial DNA Wash Buffer into the Zymo-Spin™ IIC Column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IIC Column was transferred into a clean 1.5 ml micro-centrifuge tube and 100 µl of DNA Elution Buffer was then added directly to the column matrix. This was centrifuged at 10,000 x g for 30 seconds to elute the DNA. The Ultra-pure resulting filtrate (DNA) obtained was used as a template during the assay. This was transported in ice to the laboratory for sequencing.

2.9 Gel Electrophoresis of DNA

For the electrophoresis, 0.8% agarose was prepared by weighing 0.8 g of agarose powder with a weighing balance. The powder was mixed

with 100 ml of electrophoresis buffer and then heated in a microwave oven until completely melted. Ethidium bromide was added to the gel at a final concentration 0.5 ug/ml to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60℃, it was poured into a casting tray with a comb placed across its rim to form wells. The gel was allowed to set for 30 minutes and the comb was removed. 20 µl of the DNA samples was then loaded into the wells after mixing with 2 µl of bromophenol blue. A DNA molecular weight marker was loaded into one of the wells. The plastic tray with the gel was inserted horizontally into the electrophoresis chamber and covered with buffer. 70 V was applied for 1 hr 30 min. The distance DNA migrated in the gel was judged by visually monitoring migration of the tracking dyes. The DNA was visualized by placing the gel in an ultraviolet transilluminator in a photo documentation system (Clinix Japan, Model 1570). The size of the visible bands obtained was calculated by matching that of the isolates with the standard bands produced by HIND III marker.

2.10 PCR Amplification and DNA Sequencing by Sanger Sequencing Method and Blast Analysis (CLC BIO and NCBI blast online)

DNA sequencing was performed by Sanger (dideoxy) Sequencing Technique to determine the nucleotide sequence of the specific microorganism isolated using automated PCR cycle- Sanger Sequencer™ 3730/3730XL DNA Analyzers from Applied Biosystems [12,13]. This result was obtained as nucleotides. Sequence analysis from resultant nucleotides base-pairs was performed by BLAST analysis by using CLO Bio software and by direct blasting on http://blast.ncbi.nlm.nih.gov

For every set of isolate, a read was BLASTED and the resultant top hits for every BLAST result showing species name was used to name the specific organism.

3. RESULTS

The bacteria associated with lower leg ulcers of patients in Ebonyi State were studied.

Fig. 1 depicted the PCR amplification product of the 16S rRNA gene using the primer 27F (5'AGA GTT TGA TCC TGG CTC AG-3'). This was separated on a 1.5% agarose gel electrophoresis. The DNA bands were visualized after ethidium bromide staining using 100bp DNA ladder as DNA molecular weight standard.

The gene sequences of the various bacterial isolates are presented in Tables 1-13. The result shows the nucleotide sequence of the isolated bacteria based on 16S rRNA. The blasting of the sequence results was done using the online blast software at http://blast.ncbi:nlm.nih.gov/blast.cgi. The result for every set of isolate was taken from the top hit of the blast showing species name and the strain number. Following this, the bacteria identified were *Staphylococcus aureus* strain ATCC 12600, *Enterococcus faecalis* strain ATCC 19433, *Proteus mirabilis* strain ATCC 29906, *Proteus vulgaris* strain ATCC 29905, *Klebsiella aerogenes* strain KCTC 2190, *Escherichia coli* strain U 5/41, *Serratia marcescens* strain NBRC 102204, *Pseudomonas aeruginosa* strain M37351, *Streptococcus pyogenes* KS030, *Enterobacter cloacae* strain NBRC 13535, *Peptostreptococcus* strain *anaerobius* DSM 2949, *Clostridium tetani* strain E88 and *Corynebacterium ulcerans* strain 0102.

Fig. 1. Amplification of PCR products from isolates

Lane 1 was 100 bp DNA ladder. Lanes 2–13 were PCR amplified from different isolates.

Table 1. Nucleotide sequence of the isolate code LU10 based on 16S ribosomal RNA

Specimen code	Isolation code	Gene sequence	Microbe/strain code	GeneBank/ Accession number
U5	LU 10	AGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACGGACGAG AAGCTTGCTTCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGATAACCT ACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATATTT TGAACCGCATGGTTCAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGATGGAT CCGCGCTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCAT AGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACT CCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGG AGCAACGCCGCGTGAGTGATGAAGG	*Staphylococcus aureus* strain ATCC 12600, 16S rRNA gene	NR 115606.1

Table 2. Nucleotide sequence of the isolate code LU14 based on 16S ribosomal RNA

Specimen code	Isolation code	Gene sequence	Microbe /strain code	Gene Bank/ Accession number
U9	LU14	GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGCTTCTTTCCTCCCGAGTGC TTGCACTCAATTGGAAAGAGGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTACCC ATCAGAGGGGGATAACACTTGGAAACAGGTGCTAATACCGCATAACAGTTTATGCCGCAT GGCATAAGAGTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAG CTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGA TCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT CTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGG ATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGACGTTAGTAACTGAACGTCCCCTGACG GTATCTAACCAGAAAGCCACGGC	*Enterococcus faecalis* strain ATCC 19433, 16S rRNA gene	NR 115765.1

Table 3. Nucleotide sequence of the isolate code LU15 based on 16S ribosomal RNA

Specimen code	Isolation code	Gene sequence	Microbe /strain code	GeneBank / Accession number
U10	LU15	TGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGAGAAAGCTTGCTTTCTTGCTGACGAGCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAATGTCTACGGACCAAAGCAGGGGCTCTTCGGACCTTGCACTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCTCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGATAAGG	*Proteus mirabilis* strain ATCC 29906, 16S rRNA gene	NR 114419.1

Table 4. Nucleotide sequence of the isolate code LU16 based on 16S ribosomal RNA

Specimen code	Isolation code	Gene sequence	Microbe /strain code	GeneBank/ Accession number
U11	LU16	AAATTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAG	*Klebsiella aerogenes* strain KCTC 2190, 16S rRNA	NR 102493.2

Table 5. Nucleotide sequence of the isolate code LU17 based on 16S ribosomal RNA

Specimen code	Isolation code	Gene sequence	Microbe /strain code	GeneBank/ Accession number
U12	LU17	TCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAATCGAGCGGTAACAGAAGAAAGCT TGCTTTCTTGCTGACGAGCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAG GGGGATAACTACTGGAAACGGTGGCTAATACCGCATGACGTCTACGGACCAAAGCAGGGG CTCTTCGGACCTTGCGCTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGAGGTAATG GCTCACCTAGGCAACGATCTCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGA GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAG CCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAGCGG GGAGGAAGGTGATAAAGTTAATACCTTT	*Proteus vulgaris* strain ATCC 29905, 16S rRNA gene	NR 115878 .1

Table 6. Nucleotide sequence of the isolate code LU18 based on 16S ribosomal RNA

Specimen code	Isolation code	Gene sequence	Microbe /strain code	Gene Bank/ Accession number
U13	LU18	AGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAAC ACATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTTGCT GACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGA TGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAA CGTCGCAAGCACAAAGAGGGGGACCTTAGGGCCTCTTGCCATC GGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCT CACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC AACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCA TGCNGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCA G	*Escherichia coli* strain U 5/41, 16S rRNA gene.	NR 024570.1

Table 7. Nucleotide sequence of the isolate code LU19 based on 16S ribosomal RNA

Specimen code	Isolation code	Gene sequence	Microbe /strain code	GeneBank/ Accession number
U14	LU19	ATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGGTAGCACAGGGG AGCTTGCTCCCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACT GCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTC GCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGAT GGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTG GTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTAC GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCAT GCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGA AGGTGGTGAGCTTAATACGTTCATCAATTGACGTTACTCGCAGAAGAAGCACCG GCTAACTCCGTGCCAGCAGCCG	*Serratia marcescens* strain NBRC 102204, 16S rRNA gene	NR 114043.1

Table 8. Nucleotide sequence of the isolate code LU20 based on 16S ribosomal RNA

Specimen code	Isolation code	Gene sequence	Microbe /strain code	GeneBank/ Accession number
U15	LU20	TTCCTTGAGCGACAGCGCCGCGATGATGGCTTTCAGGAGTTCATCCACGGAGGTT TCCTCTTGGGTGGGGTAAAGGCCAAAGGTGGCCGCCGCTCGGCGGGCGAGCGGC TCCATGCCATCGATGGCGGGTCGTTGGTGATGGCGCCCATCAGAATCGACAGGAC CGTGCCGTCCGGGGCGTAGCTGAAGACCGGCGAGAAGTAGAGGTACTCACCGTC CTCGATCATCCGTGCGGCGCGGGCGGTGTATTCGACACGGCCCCACAGGCCGGA GCCTTCGCGCCATTCGAAATCGAGGAAGCGGCCGGCAGCAGGCGCCGGCTGGCC GTTTTCCTCTTTCTGAGGGTCTGGTGCTCATAGTCCAGGACAGGCGGTGTCTTGCG TGCCCTGGCCCGGTC	*Pseudomonas aeruginosa* strain M37351, 16S rRNA gene	CP 008863.1

Table 9. Nucleotide sequence of the isolate code LU22 based on 16S ribosomal RNA

Specimen code	Isolation code	Gene sequence	Microbe /strain code	GeneBank/ Accession number
U17	LU22	TTGTTGATATTCTGTTTTTTCTTTTTTAGTTTTCCACATAAAAAATAGTTGAAAACAATAG CGGTGTCACCTTAAAATGACTTTTCCACAGGTTGTGGAGAACCCAAATTAACAGTGTT AATTTATTTTCCACAGATTGTGGAAAAACTAACTATTATCCATTGCTCTGTGGAAAACTA GAATAGTTTATGGTAGAATAGTTCTAGAATTATCCACAAGAAGGAACCTAGTATGACTG AAAATGAACAAATTTTTTGGAACAGGGTCTTGGAATTAGCTCAGAGTCAATTAAAACAG GCAACTTATGAATTTTTTGTTCATGATGCCCGTCTATTAAAGGTCGATAAGCATATTGC AACTATTTACTTAGATCAAATGAAAGAACTCTTTTGGGAAAAAAATCTTAAAGATGTTAT TCTTACTGCTGGTTTTGAAGTTTATAACGCTCAAATTTCT	*Streptococcus pyogenes* MGAS315, 16S rRNA gene	AE 014074.1

Table 10. Nucleotide sequence of the isolate code LU23 based on 16S ribosomal RNA

Specimen code	Isolation code	Gene sequence	Microbe /strain code	GeneBank/ Accession number
U18	LU23	ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAGCACAGAGAGCTTG CTCTCGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAG GGGGATAACTACTGGAAACGGTAGCTAATACCGCATAANGTCGCAAGACCAAAGAGGG GGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGG TAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTG GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG GGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGT ACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAAT	*Enterobacter cloacae* strain NBRC 13535, 16S ribosomal RNA gene	NR 113615.1

Table 11. Nucleotide sequence of the isolate code LU24 based on 16S ribosomal RNA

Specimen code	Isolation code	Gene sequence	Microbe /strain code	GeneBank/ Accession number
U19	LU24	GACTTCTCACAGTTCGTTGTTACTAGGTTTTCCCTCTGTGAGACCTCACGGGATAAG TCGTCAGTCTTTCCTCGTCTACCTGCCTAATTTACTTACATAAGTTACGTTTGCCTTTT GGACTTCGTGACTTTGGGCCCACTCATCCTTTATGTAAGCCTTTATATTAGGTTTCTG TACGTCAGGCTACGATTTTGCTATTGCTTCTTCTCGCCATCACCTCACGGTGATAAC CTTGCAAGTTGCTATGAGGTTCGTCGGCAACTACGCCCTACGTGGACTTTCACCACA GACTGACGGCATGCCCGTCATACCCAAAAAAAGAAGCCCACAGTATATAACTGAGG GCTTGTATCTTCTATTATTTTATTAAATAAACTCGGCTGCCTTTACCTTTGAAGGTATA AATTTACCTATAACACCACCTATGATAGCTGGTACTACCCAGTTGAATCCTAGGAATC CAAGTGGTAGGTTGTTTACAGGTGCTAGTGATGGTATACTTGCTGATAATAGTGTAA GAGCACTTGTTATCACAGTAAATAGTACAGACACCTTGTAGA	*Peptostreptococcus* strain *anaerobius* DSM 2949	KB 906605.1

Table 12. Nucleotide sequence of the isolate code LU25 based on 16S ribosomal RNA

Specimen code	Isolation code	Gene sequence	Microbe /strain code	GeneBank/ Accession number
U20	LU25	CAGAGCTTTGGAATAAGATAGGTGATAATAAAAATACATATAGTGACTTATTAGAGCTG TTTAATAAAGTAGGAGAAAAATATTCAGATACAATAAAAAAGACTATTTGGGTTTATAAT AGAGAATTTTTTATATACTAATATGACAGAAATAACTGAAAAATACAAAAAATGAATATTT AACATTGAAAAAAGAGGAATTTAAGGGTAAAATAAGTTTATAAATAAAAATAATTTGAAT TTATATAAACAAAAAAATAAAAACTCTTAAAGGCTGAAAGTTTCGCGGCTTTACCTTTAA GAGTTATAAGAGTACCCTATATAGAAAAATTCTACATAGGAGAACTTTATTTACTTAAAG ACATTTTAATATATTTATTGTATATAGTCAATAGTAAAAAGTTCTTCTATAACTTTGGGTA CAAGATAGGAGGACTTTATTTGTTAGCAAAACAATTAAGCTTATATGATTATATAGACAA TACATTAAAAAACTTAGTAACCTGTGAAGTGGAAATAAAGGCAGAGGAAGATAACATAC AAAAGGCAACACTT	*Clostridium tetani* strain E88	AF 528097.1

Table 13. Nucleotide sequence of the isolate code LU26 based on 16S ribosomal RNA

Specimen code	Isolation code	Gene sequence	Microbe /strain code	GeneBank/ Accession number
U21	LU26	TGTGGATAACTCTCAAGTTTATGTCGAAACTTTCCACATAGAATTGAATTTACGCAGGTT AAAAGCAAAATTCTTCACAGTGAGCGTTATCCACAAGGTTGTGAAATAACTGTGGATAA CTTTTCACACACCCCAGTGTAGGAAGTTATCCACAGTTGTGGAAAACTCTGTGGAATAC GCGGTCACAGCCCCAAAGCGTTGTGAACAACTCGGTGAAATCCCCGTGGACAGTGAA ATAACATTTTTCACCGCCGGATGGGGAAATCTCGTTGCGTTGGACATTATTTTTGGGGC TAGTGAGCGCTGCTGATAGGGCACTGACCCGCGAATGCACTGCCATATCAGCATGAAA ATTAGTTGTTGTTCATTCGCACGAGGTAAAGGAAATACGAAGTGTCGGAGACTCCATC GACATGGAACGAGCGGTGGCAGGAAGTTACTAACGAGCTGCTGTCACAGTCTCAGGA CCCAGATAGTGGTATTTCCATTACTCGCCAGCAAAGTGCATACCTGCGATTGGTAAAG CCAGTCGCGTTTGTAGAGGGTATTGCAGTTTTAAGCGTCCCTCACGCCCGAGCGAAAA AAGAGATTGAAACTACGCT	*Corynebacterium ulcerans* strain0102	AP 012284.1

Table 14. Prevalence of lower ulcer types in Ebonyi State

Category of patients	No examined	No. infected (%)	No. uninfected (%)
Venous leg ulcer	300	250 (20.7)	50 (17.2)
pressure ulcer	600	450 (37.2)	150 (51.7)
Diabetic foot ulcer	350	300 (24.8)	50 (17.2)
Non-healing surgical ulcer	250	210 (17.4)	40 (13.8)
Total	1500	1210 (80.6)	290 (19.3)

Table 15. Occurrence of microorganisms isolated from lower leg ulcer patients according to ulcer type

Ulcer type	*Staphylococcus aureus*	*Staphylococcus aureus*	*Staphylococcus saprophyticus*	*Staphylococcus epidermidis*	*Enterococcus faecalis*	*Proteus mirabilis*	*Klebsiella aerogenes*	*Proteus vulgaris*	*Escherichia coli*	*Serratia marcescens*	*Pseudomonas aeruginosa*	**Total**
Venous	30(12.0)	20(8.0)	10(4.0)	10(4.0)	5(2.0)	10(4.0)	15(6.0)	13(5.2)	12(4.8)	6(2.4)	20(8.0)	250(20.7)
Pressure	31(6.9)	29(6.4)	14(3.1)	16(3.6)	13(2.9)	25(5.6)	26(5.8)	22(4.9)	34(7.6)	14(3.1)	37(8.2)	450(37.2)
Diabetic	20(6.7)	17(5.7)	12(4.0)	11(3.7)	10(3.3)	16(5.3)	16(5.3)	14(4.7)	20(6.7)	10(3.3)	19(6.3)	300(24.8)
Non healing	35(16.7)	9(4.3)	2(1.0)	0(0.0)*	0(0.0)*	3(1.4)	10(4.8)	1(0.5)	2(1.0)	0(0.0)*	32(15.2)	210(17.4)
Surgical												
Total	116(9.6)	75(6.2)	38(3.1)	37(3.1)	28(2.3)	54(4.5)	67(5.5)	50(4.1)	68(5.6)	30(2.5)	108(8.9)	1210(100)

$p \geq 0.05$: Ulcer type differed significantly except for values with asterisks.

Table 15. Occurrence of microorganisms isolated from lower leg ulcer patients according to ulcer type (continued)

Ulcer type	*Streptococcus pyogenes*	*Streptococcus pyogenes*	*Enterobacter cloacae*	*Peptostreptococcus*	*Clostridium tetani*	*Corynebacterium ulcerans*	*Klebsiella pneumoniae*	**Total**
Venous	15(6.0)	15(6.0)	6(1.2)	6(1.2)	5(2.0)	13(5.2)	10(4.0)	250(20.7)
Pressure	24(5.3)	32(7.1)	11(2.4)	16(3.6)	14(3.1)	24(5.3)	13(2.9)	450(37.2)
Diabetic	15(5.0)	17(5.7)	12(4.0)	12(4.0)	6(2.0)	17(5.7)	11(3.7)	300(24.8)
Non healing	35(16.7)	32(15.2)	0(0.0)*	5(2.4)	0(0.0)*	32(15.2)	10(4.8)	210(17.4)
Surgical								
Total	89(7.4)	96(7.9)	29(2.4)	39(3.2)	25(2.1)	86(7.1)	44(3.6)	1210(100)

p ≥ 0.05: Ulcer type was significantly different except for values with asterisk.

As shown in Table 14, the ulcer type designated as pressure ulcers, which amounted to 450 (37.2%) were found to be the most commonly infected. All acute soft tissue wounds such as road traffic accidents, lacerations, domestic violence, burn sites and gunshot injuries were classified under pressure ulcer. This was closely followed by diabetic foot ulcers, 300 (24.8%). Non-healing surgical ulcers 210 (17.4%) were the least frequent.

The distribution of microorganisms isolated from lower leg ulcer patients according to the ulcer type showed that *Staphylococcus aureus* (12.0%) was the most predominant in venous leg ulcer, followed *Pseudomonas aeruginosa* which had 8.0%, whereas *Peptostreptococcus* (1.2%) was the least in occurrence. In pressure ulcer, the most predominant microbe was *Pseudomonas aeruginosa* (8.2%), followed by *Escherichia coli* (7.6%). Diabetic leg ulcer had *Staphylococcus aureus* (6.7%) and *Escherichia coli* strain 6.7%) as the highest in occurrence, followed by *Pseudomonas aeruginosa* (6.3%), as the least was *Clostridium tetani* strain (2.0%). The non-healing surgical ulcer had *Staphylococcus aureus* (16.7%) and *Streptococcus pyogenes* (16.7%) as the most occurring microbes, followed by 15.2% for *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Corynebacterium ulcerans* respectively (Table 15).

Out of 1500 specimens examined, 1210 (80.7%) showed positive microbial association, while 290 (19.3%) did not produce any growth due to the general purpose media employed.

The subjects in Ebonyi State are involved in active farming, 58% prevalent rate of leg ulcer was recorded in this occupation. The infection was less prevalent among civil servants.

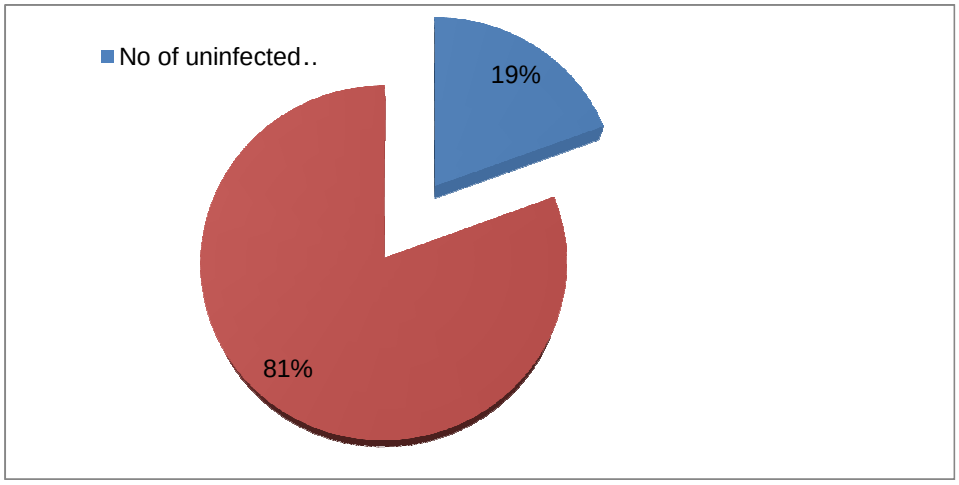

Fig. 2. Prevalence of lower leg ulcers in Ebonyi State

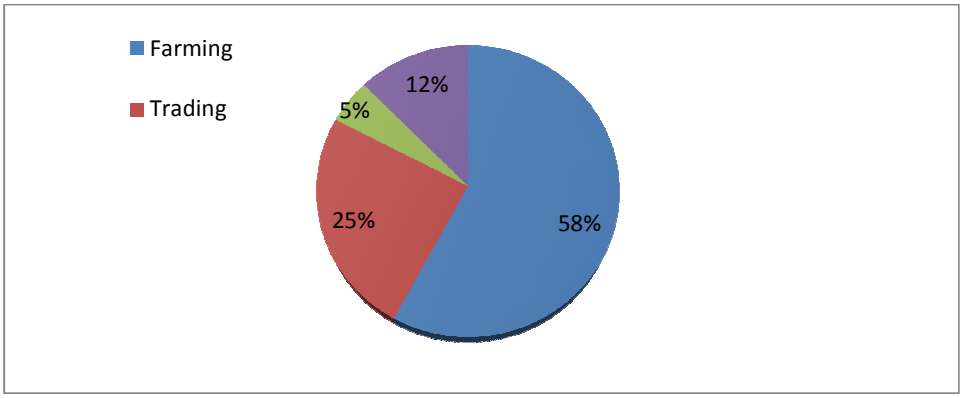

Fig. 3. Prevalence of lower leg ulcer in relation to occupation in Ebonyi State

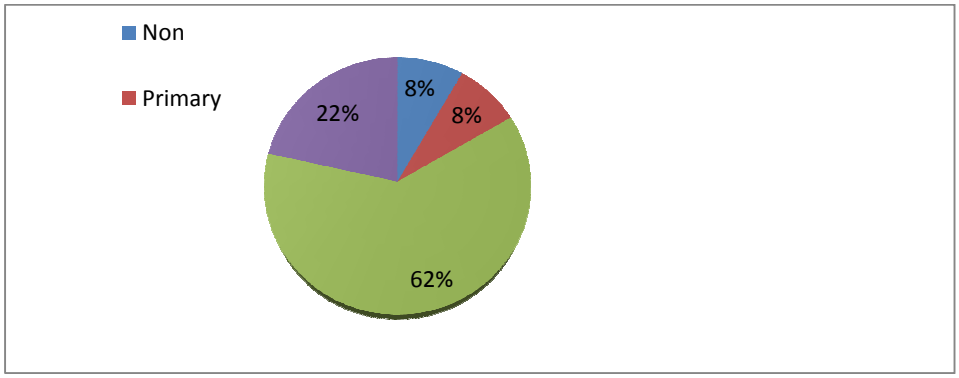

Fig. 4. Prevalence of lower leg ulcer in relation to educational level of patients in Ebonyi State

Table 16. Age and gender prevalence of lower leg ulcers in Ebonyi State

Age group (years)	No. of specimen examined	No infected (%)	No. uninfected (%)	Male (%)	Female (%)
0-10	5	1(0.1)*	4(1.4)	1(0.1)*	0(0.0)*
11-20	29	12(1.0)	17(5.9)	7(0.9)	5(1.2)
21-30	250	196(16.2)	54(18.6)	99(12.5)	97(23.1)
31-40	300	251(20.7)	49(16.9)	150(19.0)	101(24.1)
41-50	357	248(20.5)	109(37.6)	124(15.7)	124(29.5)
51-60	250	211(17.4)	39(13.5)	161(20.4)	50(11.9)
61-70	180	165(13.6)	15(5.2)	135(17.1)	30(7.1)
71-80	75	72(6.0)	3(1.0)	62(7.9)	10(2.4)
≥ 81	54	54(4.5)	0(0.0) *	51(6.5)	3(0.7)
Total	1500	1210(80.7)	290(19.3)	790(65.3)	420(34.7)

$p \geq 0.05$: Number of infected and uninfected among age groups and gender were significantly different except for values with asterisks

Prevalence of lower leg ulcer in relation to educational level of patients in Ebonyi State showed that the highest rate was seen among the secondary level (62%), followed by tertiary education (22%), while the least was 8% for primary and non-educated respectively.

The age and gender prevalence of patients of lower leg ulcer is shown in Table 16. The age group of 41-50 years (29.5%) was the most infected among females, while 51-60 years was mostly infected among males.

4. DISCUSSION

Modern molecular tools such as 16S rRNA gene-based sequencing provide powerful means to define chronic wound bacteria. We found that leg ulcers supported complex bacterial communities comprised of a wide-range of bacterial taxa including fastidious anaerobic bacteria that were not observed using culture-based methods. The bacterial communities characterized in this study were similar to those reported by other groups using 16S rRNA gene-based methods [14,15]. Of the numerous organisms that colonize chronic wounds, wound care experts believe *Staphylococcus aureus, Pseudomonas aeruginosa*, beta-hemolytic streptococcus, and anaerobes are the most likely bacterial causes of delayed healing and infection. Of these organisms, *S. aureus* is most commonly isolated from chronic wounds with the others occurring at relatively low rates. *S. aureus* is a known pathogen with an extensive array of virulence factors including proteases and toxins. As with most bacteria, these factors are primarily expressed at higher densities to enable the organism to further colonize, and subsequently invade surrounding tissues. Such factors are rarely expressed at lower densities where adherence and survival are paramount. Wound contaminants are likely to originate from the environment (exogenous microorganisms in the air or those introduced by traumatic injury), the surrounding skin (involving members of the normal skin microflora such as *Staphylococcus epidermidis*, micrococci, skin diphtheroids, and propionibacteria), and endogenous sources

involving mucous membranes, primarily the gastrointestinal, oropharyngeal and genitourinary mucosae [14].

The distribution of microorganisms isolated from lower leg ulcer patients in relation to the ulcer type showed that *Staphylococcus aureus* (12.0%) was the most predominant in venous leg ulcer, followed by *Staphylococcus aureus* subsp. anaerobius and *Pseudomonas aeruginosa* which had 8.0% respectively, whereas *Peptostreptococcus* (1.2%) was the least in occurrence. This is consistent with similar studies in Nigeria which reported that *Staphylococcus aureus* was the predominant pathogen in wound ulcers [16], but differed from the studies of [17] in Okolobiri, Bayelsa, Nigeria and [18] in Sagamu, Nigeria, who reported *P. aeruginosa* and *Klebsiella* respectively as the most common pathogens. Although the presence of *P. aeruginosa* among subjects may be attributed to contamination of wounds with contaminated medical devices, it also shows local variability in wound management procedures.

It was observed that pressure ulcers 450 (37.2%) was the most commonly infected, and these include acute soft tissue infections such as road traffic accidents, lacerations, domestic violence, burn sites and gunshot injuries. This was closely followed by diabetic foot ulcer 300 (24.8%). Infections of non-healing surgical ulcers 210 (17.4%) were the least frequent. This report deferred from the result of [19], who observed that surgical site infection ranked highest among wound infections. Taiwo, et al [19] further attributed the claim that patients are likely to undergo surgical operations and more likely to have breaks in their local defence system. The predominant microbe in pressure ulcers was *Pseudomonas aeruginosa* (8.2%), followed by *Escherichia coli* (7.6%). Similar findings had also been reported by Aizza, et al [20]. In wound sepsis, *Pseudomonas aeruginosa* was also the most prevalent infectious organism caused by incision or fluid collection under the skin surface. This finding differed from that obtained by [21]. The susceptibility of burn wound to opportunistic colonization by bacteria and fungi results from several factors, including the presence of coagulated proteins, the absence of blood-borne immune factors, and the avascularity of the burn wound [22]. Multiple studies had examined the microflora that affects these wounds; both superficial and deep tissue cultures had been used, and the results are fairly consistent. The organisms isolated by culture were *S. aureus, S. epidermidis, and Streptococcus* spp. [23,24]. Other bacteria that consistently appeared include *Proteus mirabilis, P. aeruginosa,* and *Propionibacterium* spp.

Diabetic leg ulcers had *Staphylococcus aureus* (6.7%) and *Escherichia coli* (6.7%) as the highest in occurrence, followed by *Pseudomonas aeruginosa* (6.3%), while the least was *Clostridium tetani* (2.0%). Bowler, et al [25] reported *S. aureus* as the most prevalent isolate in diabetic foot ulcers, together with other aerobes such as *S.epidermidis*, *Streptococcus* spp., *P. aeruginosa*, *Enterococcus* spp., and coliform bacteria. In addition, increased plasma glucose stimulates the growth of Gram-positive organisms. High glucose levels have been associated with an increased risk of wound infection in both humans and animal models, and hyperglycemia has been demonstrated to be associated with Gram-positive septicemia [26].

The non-healing surgical ulcers had the most occurring microbes as *Staphylococcus aureus* and *Streptococcus pyogenes* (16.7%), followed by 15.2% for *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Corynebacterium ulcerans* respectively. This finding is similar to that reported by Nwachukwu, et al [27] who found that 21.3%, 19.0% and 10.9% were *S. aureus*, *E. coli*, *P. mirabilis* and *P. aeruginosa*, respectively. This research finding indicates that the presence of enteric organisms in fresh wounds or at operation probably leads to their subsequent sepsis. These findings, therefore, imply that both enteric and non organisms are important determinants of healing traumatic and other in surgical wounds. The incidence of enteric bacteria also confirms the observation that most wound infections arising from abdominal procedures are mainly acquired from the patient's own faecal flora [28]. However, various traumatic and surgical wounds are potentially heavily contaminated with exogenous and endogenous aerobic and anaerobic bacteria derived from the disruption of mucosal surfaces [29].

The number of specimens collected from male patients with leg ulcer infections were much higher than those from female patients (900 males compared to 600 females) and the proportions with infection in each gender class were 790 (65.3%) for males and 420 (34.7%) for females. There was significant correlation ($r = 0.12$) between gender and contracting wound infection. A similar result was also

reported in India, the difference in the number of males to females with wound infection might have been due to the social behavior where males were given superiority to the females, and if contacted disease were immediately taken to hospitals in comparison to delays in bringing females for treatment [20]. Age prevalence in relation to number of microbial isolates among patients showed that 31-40 years had the highest rate, followed by 41-50 years while the least was 0-10 years. The proportion of adults with wound infection was much higher than children, and there was a moderate correlation (r = 0.43) between age and contracting wound infection. This might have been due to the fact that more adults are involved in farming, laboring jobs and more exposed to occupational hazards and therefore likely to have more wounds and injuries which then provide the sites for microbial infections.

5. CONCLUSION

This work discovered a high index of bacterial involvement in lower leg ulcers in Ebonyi State, Nigeria and the isolates had their highest occurrence in males except for *Escherichia coli* which occurred mostly in females. *Staphylococcus aureus* and *Pseudomonas aeruginosa* had the high prevalence rate. In addition, the findings of this study might also guide policy makers to implement specific intervention strategies to reduce the bacterial infections and their transmission.

CONSENT AND ETHICAL APPROVAL

Prior to the commencement of the study, ethical clearance was sought from the Federal Teaching Hospital Abakaliki (FETHA) ethical committees. In addition, letters requesting for collaboration was written to the management of all the private hospitals from which specimens were collected and consent was obtained from parents or close relatives.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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