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Detection of Multidrug resistant cariogenic bacteria among Diabetic Patients attending a Tertiary Hospital in Ado Ekiti, South-Western, Nigeria

[*1,2]Adewumi Funmilayo Ajoke; [3,4]Adegoke, Anthony Ayodeji; [5]Uzairue, Leonard Ighodalo; [5]Ojerinde, Amos Kunle; [2]Ibeh Isaiah and [6]Okiki Pius

[1]Department of Medical Microbiology and Parasitology, Faculty of Basic Clinical Sciences, College of Medicine, Ekiti State University, Ado Ekiti, PMB 5363, Ado-Ekiti, Ekiti State, Nigeria
[2]Department of Medical Laboratory Sciences, School of Basic Medical Sciences, College of Medicine, University of Benin, Benin City, Nigeria. P.M.B. 1154, Benin City, Edo State.
[3]Department of Microbiology, Faculty of Science, University of Uyo, PMB 1017 Uyo, Akwa, Ibom State, Nigeria.
[4]Applied and Environmental Microbiology Research Group (AEMREG), Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa.
[5]Department of Medical Laboratory Science, Federal University of Oye Ekiti, Ekiti State, Nigeria.
[6]Department of Biological Sciences, Afe Babalola University, Ado Ekiti, Nigeria.
*Corresponding author: adewumiolufunmi@gmail.com
Running Headline: New strains of oral streptococci among diabetics

Abstract

Microorganisms colonizing the oral cavity have a significant probability of spreading on epithelial surfaces to neighboring sites. Many reports have associated oral bacteria to oral infectious diseases as well as systemic diseases such as cardiovascular disease and diabetes. Oral care is usually neglected in the control of health problems linked with diabetes which may contribute to the increased morbidity and complications from oral health problems. The study aimed to identify the resident microbial flora and assess the possible carriage of cariogenic bacteria among people with diabetes in Ado- Ekiti, Nigeria. Oral swabs were collected from 306 consenting patients. Culture-based isolation was done using standard techniques on Mitis salivarius agar supplemented with 1% Potassium Tellurite, 10% chocolate agar and MacConkey agar at 37°C for 18-48 hours. The susceptibility patterns of the isolates were detected using standard techniques as recommended by CLSI. Selected bacteria isolates were genomically identified using the bacterial universal primer 16S rRNA and STR-1 and STR 2 primers (partial tuf genes). Amplified genes were sequenced and phylogenetic analysis was carried out using MEGA 7 software. A total of 447 microbial isolates were identified among which were Staphylococcus spp (14.5%), Klebsiella pneumoniae (3.4%), Escherichia coli (2.5%), Candida spp (8%) while the streptococcal organisms such as Streptococcus pyogenes (35.2%), Streptococcus mitis (14%), Streptococcus mutans (7.3%) and Streptococcus salivarius (15.1%). Streptococcus mutans was susceptible to meropenem (71.4%) but highly resistant to Penicillin (100%), cefuroxime (71.4%) cefotaxime (68.6%). Streptococcus mitis was highly resistant to Amoxicillin/Clavulanate (100%), Cloxacillin (100%) and Cefuroxime (70.1%) but was found susceptible to Meropenem (77.3%). 16S ribosomal RNA gene amplification and subsequent sequencing of the isolates revealed twenty-six (26) de-novo bacterial strains. In conclusion, the study showed that diabetic individuals carry multidrug-resistant cariogenic bacteria that may predispose them to other oral diseases. There is a need to incorporate routine oral microbiological screening into routine care for diabetic patients in Nigeria.
Keywords: *Antimicrobial resistance, Diabetics, Streptococcus mitis, Streptococcus mutans, Streptococcus pyogenes, Streptococcus salivarius.*

INTRODUCTION

Microorganisms resident in the human oral cavity are termed oral microbiota. They are also known as oral microflora or the oral microbiome (Kilian *et al*., 2016). The term microbiota was introduced to explain the ecological populace of commensals, pathogenic and symbiotic microorganisms that occupy the body space and have been implicated as determinants of health and disease(Dewhirst *et al*., 2010; WHO, 2022). The environmental conditions in the human mouth encourage the growth of micro-organisms resident there(Willey *et al*., 2013). The oral flora comprises a complex group of micro-organisms such as bacteria, fungi, protozoa and viruses(Marsh *et al*., 2016). Resident flora of the mouth includes cariogenic Streptococci such as S. mutans, S. salivarius and S. pneumoniae, Staphylococci species, Haemophilus species, Neisseria species, Branhamella species, Escherichia coli and other Enterobacteriaceae as well as anaerobes such as Bacteroides melaninogenicus, Peptostreptococcusspp, Lactobacillussppand Peptococcusspp. (Ochei & Kolhatkar, 2009; Sarkonen *et al*., 2005). Fungal organisms often isolated in the oral cavity include Candida, Penicillium,Aspergillus and Cryptococcus

(Cui *et al*., 2013). Microorganisms of the oral cavity origin, have been reported to cause oral infectious diseases, including caries (tooth decay), periodontitis (gum disease), alveolar osteitis (dry socket), endodontic (root canal) infections and tonsillitis.Also, Oral microbial dysbiosis has been linked to oral inflammation and thus may contribute to systemic conditions through bacteremia (Han & Wang, 2013). Many reports have associated oral bacteria to systemic diseases such as cardiovascular disease(Beck & Offenbacher, 2005; Offenbacher *et al*., 1998), diabetes(Genco *et al*., 2005), stroke(Joshipura *et al*., 1996), preterm birth (Offenbacher *et al*., 1998) and pneumonia (Awano et al., 2008; Gao *et al*., 2018; Seymour *et al*., 2007).

Diabetes mellitus is a clinical metabolic syndrome characterized by hyperglycemia caused by an absolute or relative deficiency of Insulin(American Diabetes Association, 2009).According to World Health Organization (WHO), Diabetes mellitus is a Silent epidemic that affects many people around the world and is directly associated with the oral health status of the patients (Wild *et al*., 2004). It has been estimated that about halfa billion people are living with diabetes globally and the number is projected to increase by 25% in 2030 especially in Asia and Africa (Saeedi *et al*., 2019). Oral care is usually neglected in the control of health problems linked with diabetes which may contribute to the increased morbidity and complications from oral health problems (Bharateesh *et al*. 2012). Periodontitis has been identified as a chronic inflammatory disorder affecting the gingivae and the periodontal tissue, usually caused by bacteria. Oftentimes, periodontal disease coexists with severe diabetes mellitus and thus, diabetes has been identified as a risk factor for severe periodontal disease (Douglass, 2006; Nazir, 2017; Taylor, 2001). Currently, periodontal disease has received tremendous attention and interest to understand whether periodontal disease predisposes or worsens the diabetic condition. Available reports have supported the existence of a bi-directional relationship between glycaemic control and periodontal disease with each disease having a potential impact on the other (Khader *et al*., 2008; Taylor, 2001; Ussar *et al*., 2016), oral fungal and bacterial infections have also been reported in patients with diabetes. The micro-flora in the oral cavity can accumulate to form dental plaque adjacent to the teeth, which can induce inflammatory processes due to the toxins released by the microorganisms in the dental plaque if not removed daily. There is a growing interest in knowledge and attempts to understand the human microbiome compositions of all ethnic nationalities. Thus, it is important to have local data on oral bacterium which can be collected into a database or used for further research and analysis. In Nigeria, there is a paucity of information on the resident oral bacteria and its implications on oral health and disease among diabetics. This study is aimed at identifying the resident microbial flora among diabetics attending a tertiary healthcare facility in Ekiti State, Nigeria as well as assessing the possible carriage of cariogenic bacteria among these patients

MATERIALS AND METHODS.

Study Setting: The study was conducted among patients with Type II diabetes mellitus attending the medical outpatients' clinic of the Ekiti State University Teaching Hospital (EKSUTH) Ado-Ekiti, Nigeria between June 2019 and December 2019.

Ethical Consideration

Ethical approval for this study was obtained from Ekiti State University Teaching Hospital, Ado-Ekiti (EKSUTH) Ethics and Research Committee with the reference number: EKSUTH/A67/2019B/09/002.Patients attending the clinic were approached and invited to participate in the study after the aims and objectives of the study were explained to them.

Informed consent was obtained from patients who agreed to participate. The demographic characteristics of patients were collected and entered into a data collection form.

Patient Enrolment and sample collection

A total of 306 oral swabs from 136 Male and 170 Female were collected from consenting diabetic patients at the study site. Patients with type II diabetics and attending clinic at the centre within the period of the study were recruited.

Isolation and Identification

Mouth swabs were put in sterile Brain heart infusion broth and transported immediately to the laboratory. Samples were inoculated on Mitis salivarus agar supplemented with 1 % potassium tellurite, Chocolate agar and MacConkey agar. Plates were incubated aerobically at 37^{o}C and all inoculated plates were incubated for 18-48 hours. On Mitis salivarius agar, plates were incubated anaerobically using a gas pak (Oxoid), on chocolate agar, they were incubated in reduced CO_2 condition in a candle jar and MacConkey agar plates were incubated at 37^{o}C. Presumptive isolates were identified phenotypically using biochemical tests and confirmed using API 20E

MOLECULAR IDENTIFICATION OF BACTERIAL ISOLATES

DNA extraction from pure bacteria culture.

Genomic DNA was extracted from all bacterial isolates from culture using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions after initial treatment with lysozyme (20 mg/ml, 37°C for 1 h).

DNA Quantification

This was done using the Nano-Drop spectrophotometer (microvolume sample retention system) (Thermo Scientific Nanodrop 2000). The software robotically calculated the nucleic acid concentration and purity ratios

displayed and saved on the computer. The final quality of DNA was evaluated by Nanodrop2000 (Thermo, USA). A standard concentration of10 ng/µl was prepared for each sample for all Polymerase Chain Reaction (PCR) assays.

Oligonucleotides.
The partial *tuf* gene (STR-I and STR-2) and the 16S rRNA bacteria universal primers used in this study (shown in Table 1) were obtained from Inqababiotec (IB OLO -001).

Polymerase Chain Reaction using 16S ribosomal RNA gene
PCR amplification was done using bacterial universal primer. Routine reactions were carried out using a PCR master mix composed of 3µldeoxynucleoside triphosphate (dNTPs), 4 µl X10 Tris EDTA buffer, 0.2 µl thermos aquaticus (Taq) polymerase, 0.5 µl MgCl2, 5 µlDNA lysates,15.3µl of PCR water and 1µl of primers (reverse and forward) as shown in table1. A total of thirty (30µl) volume reaction was used containing 25µl of the aliquot of master mix with 5µl of DNA lysate. Controls were set along with the samples. The denaturation phase was performed at 95^{o}Cfor 30sec, the annealing phase was done for 30 seconds at 72^{o}C and a final elongation step of 10 min at 72^{o}C

Polymerase Chain Reaction Detection of streptococcal organisms using primers targeting tuf genes (elongation factor Tu)
PCR amplifications using the *Streptococcus*-specific primers were performed using 1 µl of a genomic DNA preparation at 1 ng/µl anda 19-µl Polymerase chain reaction mix containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.4 µM concentrations of each of the *Streptococcus*-specific primers, 200 µM (each) deoxynucleoside triphosphate. 3.3 µg of bovine serum albumin (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) per µl, and 0.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.) As previously described, thermal cycling conditions for PCR amplification and agarose gel analysis of the amplified products were performed (Martineau *et al*., 1998)

Agarose Gel Electrophoresis
Agarose gel electrophoresis was done using 1% agarose gels suspended in 0.5x Tris borate EDTA buffer (44.5 mM Tris-borate and 1Mm EDTA, $_{p}$H 8.3). Five microliter of PCR product was mixed with equal volume of loading buffer (containing 0.25% bromophenol blue, 0.25% xylene cyanole and 40% sucrose) and was loaded into wells on the gel. DNA ladder was also loaded to one end of the gel. Following electrophoresis at 300V for 15 min, the products were visualized under ultra violet (UV) light (Syngene, Cambridge, U.K).

Sample preparation for Gene Sequencer (ABI 3130xl machine)
A 10µl volume Cocktail mix composed of 9µl of Hi di Formamide with 1µl of Purified PCR product was used. The samples were loaded on the machine (3130xl genetic analyzer, Applied Biosystems) and the data were generated in form of A, C, T, and G.

Alignments and Phylogenetic analyses.
This was determined based on 16S rRNA sequences generated. All generated sequences were blasted on a public Genbank. National Center for Biotechnology Information (NCBI) (Nucleotide Blast) (http://www.ncbi.nlm.nih.gov) and the results were used for identification of the isolated organisms and phylogenetic analysis was done using MEGA 7 software (version 1.0).

Susceptibility test
Antimicrobial susceptibility of pure colonies of each of the identified Streptococci to Amoxicillin/Clavulanate (AUG) (30µg), Cefotaxime (CTX) (30µg), Ceftazidime (CAZ) (30µg), Cefuroxime (CXM), Ceftriaxone (CRO) (30µg), Cefixime (CRX) (5µg), Ofloxacin(OFL) (5µg), Penicillin (GN) (10µg), Erythromycin (ERY) (10µg), Cloxacillin (CXC) (5µg), Meropenem (MEM) (10µg), Bacitracin (B)(10U), Optochin (OP) (5µg)was determined by the disc diffusion technique following standard procedures as recommended by Clinical and Laboratory Standard Institute (CLSI, 2018). The zone diameter of inhibition (mm) of the organism to the antibiotic tested was measured with a calibrated ruler and the diameter (mm)was interpreted as significantly susceptible, intermediate or resistant to the antibiotic tested following standard techniques as recommended by Clinical and Laboratory Standard Institute (2018).

RESULTS
Among the diabetic patients, a total 447 isolates were identified. These organisms include *Staphylococcus spp (14.5%), Klebsiella. pneumoniae (3.4%), Escherichia coli (2.5%), Candida spp*(8%)while the streptococcal organisms such as *Streptococcus pyogenes (35.2%), Streptococcus mitis*(14%), *Streptococcus mutans*(7.3%) and *Streptococcus salivarius*(15.1%) were isolated as presented in Table 1.

Antibiotics Susceptibility Testing of Oral Streptococci
The susceptibility patterns of the streptococcal organisms are presented in Tables 2a, 2b, 2c and 2d. *Streptococcus salivarius* isolated among the diabetic patients showed high resistance to Cloxacillin (80.6%), Amoxicillin/Clavulanate (58.3%) and Penicillin (88.9%) but was susceptible to Meropenem (72.2%) (Table 2a). *Streptococcus mutans* was susceptible to meropenem (71.4%) but highly resistant to Penicillin (100%), cefuroxime (71.4%) and cefotamime (68.6%) (Table 2b). *Streptococcus pyogenes* showed high

resistance to Erythromycin (75.6%), Cloxacillin (98.2%), Amoxicillin/Clavulanate (95.2%), Ceftazidime (99.4%) and Cefuroxime (98.2%) but highly susceptible to Meropenem (78.6%) (Table 2c). *Streptococcus mitis* was highly resistant to Amoxicillin/Clavulanate (100%), Cloxacillin (100%) and Cefuroxime (70.1%) but was found susceptible to Meropenem (77.3%) (Table 2d).

Polymerase Chain Reaction of the streptococcal tuf-gene and 16S universal primers.
Only eighty (80) of the total one hundred (100) multidrug resistant streptococcal isolates that were randomly chosen for molecular analysis had pure and amplified bands and were chosen for sequencing. Seventy-six (76) of the eighty (80) amplicons showed retrievable pure sequences upon sequencing the amplified region of the tuf gene for all streptococcal species using the 16S universal primer.

Sequencing
The chosen tuf sequence (253 bp) was found to be evolutionary similar among streptococci species. Following nucleotide sequence analysis, it was observed that streptococcal tuf genes encoding the elongation factor-Tu were just as variable as genes encoding 16S rRNA, despite the fact that streptococcal tuf sequences had been presumed to have a higher PCR detection rate than 16S sequences.

Alignments and Phylogeny
The amplicons produced by the chosen Streptococcus-specific primers were purified, and sequences of the resulting amplicons provided sequence data for the phylogenetic relationships among all the isolated organisms, particularly the streptococcal species (Table 3). Phylogenetic analyses are shown on Figures 1. Twenty-six (26) denovo organism strains from patients with diabetes were found among these sequences. These are shown in Table 4 along with their assigned accession numbers. They were submitted to the gene bank (http://www.ncbi.nlm.nih.gov) and received their approval.

DISCUSION
There is a growing interest in knowledge and attempts to understand the human microbiome compositions of all ethnic nationalities. This study employed culture-based molecular techniques to identify the oral Streptococci compositions in sampled patients with diabetes mellitus in Ado Ekiti, South-Western Nigeria.

The organisms that were recovered in this study were *Staphylococcus* spp*., Klebsiella* spp*., Candida* spp while the streptococcal organisms were *Streptococcus pyogenes, Streptococcus mitis, Streptococcus mutans and Streptococcus salivarius*. Our study's results are in keeping with findings from a study by Lydia Rajakumari & Saravana Kumari (2016) who identified *Streptococcus salivarius, Actinobacillus actinomycetemcomitans, Bacteroides oralis, Staphylococcus aureus and Streptococcus mutans* as the most common organisms among the studied diabetic group. Previous studies have also reported that these organisms were recovered from diabetic patients (Murray *et al*., 2003; Mustard & Packham, 1977). In another study, Amadi *et al*. (2007) identified *S. mutans* (78%), *S. sobrinus* (6%), alongside *S. mitis* (4%) and *S. salivarius* (12%), from forty carious dental lesions in patients attending Ebonyi State University Teaching Hospital Dental Clinic. They concluded that *Streptococcus mutans* is the prominent causative organism in dental caries. Although *Streptococcus mutans* is considered important to dental caries due to its virulence factors, other organisms such as *Streptococcus salivarius* and *Streptococcus oralis* also participate in the initial formation of dental plaque and also contribute to make the local environment adequate for *S. mutans* colonization (Meng *et al*., 2017; Santiago *et al*., 2018) its organoleptic properties, microbial contamination and its antibacterial action in vitro. This mouthwash was assessed in vivo to control dental plaque in humans. The presence of microorganisms was analyzed and the minimum inhibitory concentration against Streptococcus mutans was determined. A comparative study was done in vivo using propolis, chlorhexidine, and propolis plus chlorhexidine in lower concentrations for 14 days. Dental plaque was analyzed by the Patient Hygiene Performance (PHP).

The streptococcal isolates in this showed multi resistance to more than two groups of the tested antibiotics. This was in agreement with a study by Lemos *et al*. (2019) Streptococcus mutans resides primarily in biofilms that form on the tooth surfaces, also known as dental plaque. In addition to caries, S. mutans is responsible for cases of infective endocarditis with a subset of strains being indirectly implicated with the onset of additional extraoral pathologies. During the past 4 decades, functional studies of S. mutans have focused on understanding the molecular mechanisms the organism employs to form robust biofilms on tooth surfaces, to rapidly metabolize a wide variety of carbohydrates obtained from the host diet, and to survive numerous (and frequent who have reported multi-resistance in the oral streptococci isolated from healthy children in Greek, also Davidovich *et al*.(2020) S. salivarius, S. mitis, S. sanguinis, S. anginosus and S. mutans. All streptococci were sensitive to linezolid and meropenem. The proportion of penicillin-resistant streptococci in the subgroup S. oralis / mitis / mutans was 47,8% versus 23,5% in the subgroup S. salivarius / sanguinis / anginosus ($p = 0.020$ reported an extensive resistance pattern for oral streptococci in study of clinical isolates in Russia.

Streptococcus-specific assay used in the PCR detection and identification of streptococcal species was very sensitive and specific for the detection of the streptococcal organisms. The *tuf* gene targeting the elongation factor -Tu provided a high discrimination PCR power for the amplification and identification at the streptococcal

species level for the identification of very closely related and de-novo species. Our results further support other studies that have reported that the use of tuf-gene encoding the elongation factor Tu (EF-Tu) are highly evolutionarily conserved in the core genome and said to be equally discriminative as the 16S rRNA gene for identifying strains belonging to the genera *Enterococcus*, *Staphylococcus*, and *Streptococcus* (Ke *et al*., 1999; Martineau *et al*., 1998; Picard *et al*., 2004). The data in this study was in agreement with a study by Picard et al. (2004)including the 28 streptococcal species. Genomic DNA purified from all streptococcal species was efficiently detected, whereas there was no amplification with DNA from 72 of the 74 nonstreptococcal bacterial species tested. There was cross-amplification with DNAs from Enterococcus durans and Lactococcus lactis. However, the 15 to 31% nucleotide sequence divergence in the 761-bp tuf portion of these two species compared to any streptococcal tuf sequence provides ample sequence divergence to allow the development of internal probes specific to streptococci. The Streptococcus-specific assay was highly sensitive for all 28 streptococcal species tested (i.e., detection limit of 1 to 10 genome copies per PCR who concluded their study that tuf-genes are suitable for the development of diagnostic assays for the detection and identification of streptococcal species due to its higher level of specie specific genetic diverge.

Oral bacteria have been suggested to play a crucial role in establishing a number of systemic problems such as cardiovascular disease, adverse pregnancy outcomes, rheumatoid arthritis, inflammatory bowel disease, respiratory tract infection, stroke, meningitis, pneumonia, and diabetes (Dewhirst *et al*., 2010; Han & Wang, 2013; Zarco *et al*., 2012) including the teeth, gingival sulcus, tongue, cheeks, hard and soft palates, and tonsils, which are colonized by bacteria. The oral microbiome is comprised of over 600 prevalent taxa at the species level, with distinct subsets predominating at different habitats. The oral microbiome has been extensively characterized by cultivation and culture-independent molecular methods such as 16S rRNA cloning. Unfortunately, the vast majority of unnamed oral taxa are referenced by clone numbers or 16S rRNA GenBank accession numbers, often without taxonomic anchors. The first aim of this research was to collect 16S rRNA gene sequences into a curated phylogeny-based database, the Human Oral Microbiome Database (HOMD. *Streptococcus mutans* is the main cause of dental decay, while various lactobacilli have been reported to be associated with the progression of periodontal lesions. Also, severe periodontitis has been identified to adversely affectglycaemic control and management in diabetics and glycaemia in hyperglycemic non diabetic individuals (Chapple & Genco, 2013)given the ubiquity of periodontal diseases and the emerging global diabetes epidemic, the complications of which contribute to significant morbidity and premature mortality, it is timely to review the role of periodontitis in diabetes. AIMS To report the epidemiological evidence from cross-sectional, prospective and intervention studies for the impact of periodontal disease on diabetes incidence, control and complications and to identify potential underpinning mechanisms. EPIDEMIOLOGY Over the last 20 years, consistent and robust evidence has emerged that severe periodontitis adversely affects glycaemic control in diabetes and glycaemia in non-diabetes subjects. In diabetes patients, there is a direct and dose-dependent relationship between periodontitis severity and diabetes complications. Emerging evidence supports an increased risk for diabetes onset in patients with severe periodontitis. Biological mechanisms: Type 2 diabetes is preceded by systemic inflammation, leading to reduced pancreatic b-cell function, apoptosis and insulin resistance.Increasing evidence supports elevated systemic inflammation (acute-phase and oxidative stress biomarkers.

Furthermore, among the studied diabetic group, 16S ribosomal RNA gene amplification and subsequent sequencing of the isolates revealed twenty-six (26) de-novo strains of bacteria which were identified and approved by NCBI. Further studies are therefore recommended to enhance understanding on the role of diabetes mellitus in the modulation and alteration of oral microbiome in clinical samples.

In conclusion, the study showed that patients with diabetes mellitus carry cariogenic bacteria that may predispose them to other diseases. In a high number of subjects who participated in this study. *Streptococcus* species, which are considered as normal flora in the oral cavity, were shown to be resistant to multiple drugs. Furthermore, to the best of our knowledge, this study, is the first report of oral bacterium among Diabetics in Ado Ekiti, South Western Nigeria. Also, the genera and species-level oral Streptococci from this Nigerian population isolated in this study could be used as a reference in comparison with oral diseased conditions and future investigations.

Conflict of Interest Statement
We declared no conflict of interest.

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We would like to thank the patients that consented to participate in this study

Author Contribution Statement
Adewumi Funmilayo Ajoke - Conceptualization, investigation, methodology, project administration, first draft and editing; Uzairue Leonard Ighodalo and Ojerinde Amos Kunle (OAO) - Conceptualization, formal analysis, visualization and editing; Ojerinde Amos Kunle, Adegoke Anthony Ayodeji, Ibeh Isaiah and Okiki Pius - Supervision and editing.

Table 1: Microbial isolates from mouth swabs from diabetic patients

ISOLATES	NUMBER	%
Staphylococcus spp.	69	14.5
K. pneumoniae	16	3.4
E. coli	12	2.51
Candida spp.	38	8.0
S. pyogenes	168	35.2
S. mitis	67	14.0
S. mutans	35	7.3
S. salivanus	72	15.1

Table 2a: Antibiotic susceptibility pattern of *Streptococcus salivarius* (n = 72) isolated from Diabetic subjects against selected antibiotics

ANTIBIOTICS	Sensitive n (%)	Intermediate n (%)	Resistant n (%)
Erythromycin	25 (34.7)	7 (9.7)	40 (55.6)
Cloxacillin	5 (6.9)	9 (12.5)	58 (80.6)
Ofloxacin	15 (20.8)	25 (34.7)	32 (44.4)
Amoxicillin/Clavulanate	18 (25.0)	12 (16.7)	42 (58.3)
Ceftazidime	25 (34.2)	14 (19.4)	33 (45.8)
Meropenem	52 (72.2)	5 (6.9)	15 (20.8)
Cefuroxime	9 (12.9)	24 (33.3)	39 (54.2)
Cefixime	5 (6.9)	10 (13.9)	47 (65.3)
Penicillin	0 (0.0)	8 (11.1)	64 (88.9)
Ceftriaxone	25 (34.7)	5 (6.9)	42 (58.3)
Cefotaxime	18 (25.0)	8 (11.1)	46 (63.8)

Table 2b: Antibiotic susceptibility pattern of *Streptococcus mutans* (n = 35) isolated from diabetic subjects against selected antibiotics

ANTIBIOTICS	Sensitive n (%)	Intermediate n (%)	Resistant n (%)
Erythromycin	9 (25.7)	15 (42.9)	11 (31.4)
Cloxacillin	7 (20.0)	5 (14.3)	23 (65.7)
Ofloxacin	8 (22.9)	12 (34.3)	15 (42.9)
Amoxicillin/Clavulanate	12 (34.3)	5 (14.3)	18 (51.4)
Ceftazidime	15 (42.8)	3 (8.6)	17 (48.6))
Meropenem	25 (71.4)	6 (17.1)	4 (11.4)
Cefuroxime	9 (25.7)	1 (2.9)	25 (71.4)
Cefixime	15 (42.9)	7 (20.0)	13 (65.3)
Penicillin	0 (0.0)	0 (0.0)	35 (100)
Ceftriaxone	5 (14.3)	11 (31.4)	19 (54.3)
Cefotaxime	5 (14.3)	6 (17.1)	24 (68.6)

Table 2c: Antibiotic susceptibility pattern of *Streptococcus pyogenes* (n = 168) isolated from diabetic subjects against selected antibiotics

ANTIBIOTICS	Sensitive N (%)	Intermediate N (%)	Resistant N (%)
Erythromycin	20 (11.9)	21 (12.5)	127 (75.6)
Cloxacillin	0 (0.0)	3 (1.8)	165 (98.2)
Ofloxacin	33 (19.6)	35 (20.8)	100 (59.5)
Amoxicillin/Clavulanate	8 (4.8)	0 (0.0)	160 (95.2)
Ceftazidime	1 (0.6)	0 (0.0)	167 (99.4)
Meropenem	132 (78.6)	5 (3.0)	52 (30.9)
Cefuroxime	3 (1.8)	0 (0.0)	165 (98.2)
Cefixime	47 (27.9)	28 (16.6)	93 (55.3)
Penicillin	34 (20.2)	27 (16.1)	107 (63.7)
Ceftriaxone	34 (20.2)	40 (23.8)	94 (55.9)
Cefotaxime	34 (20.2)	0 (0.0)	134 (79.7)

Table 2d: Antibiotic susceptibility pattern of *Streptococcus mitis* (n = 66) isolated from diabetic subjects against selected antibiotics

ANTIBIOTICS	Sensitive N (%)	Intermediate N (%)	Resistant N (%)
Erythromycin	46 (68.7)	0 (0.0)	20 (29.8)
Cloxacillin	0 (0.0)	0 (0.0)	67 (100)
Ofloxacin	30 (44.7)	1 (1.4)	36 (53.7)
Amoxicillin/Clavulanate	0 (0.0)	0 (0.0)	67 (100)
Ceftazidime	5 (7.4)	0 (0.0)	62 (92.5)
Meropenem	51 (77.3)	0 (0.0)	15 (22.7)
Cefuroxime	10 (14.9)	15 (22.3)	47 (70.1)
Cefixime	46 (68.6)	10 (14.9)	11 (16.4)
Penicillin	5 (7.4)	0 (0.0)	62 (92.5)
Ceftriaxone	21 (31.3)	0 (0.0)	46 (68.6)
Cefotaxime	31 (46.2)	0 (0.0)	36 (53.7)

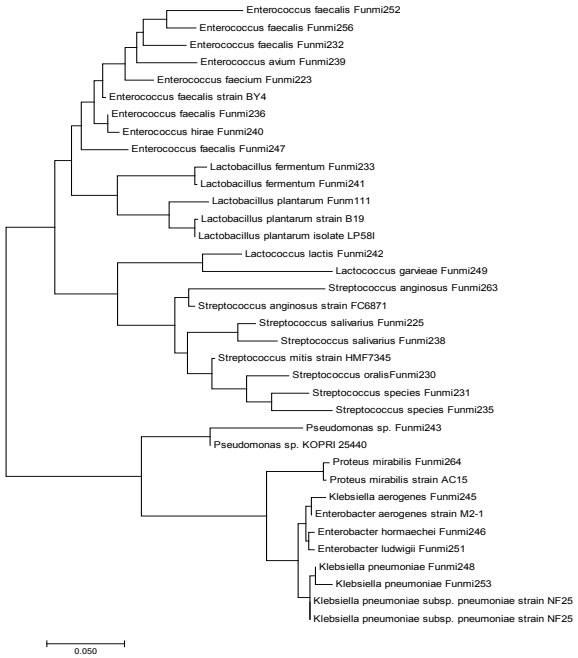
Table 3: Bacterial strains from the study and their accession numbers as identified by NCBI, USA

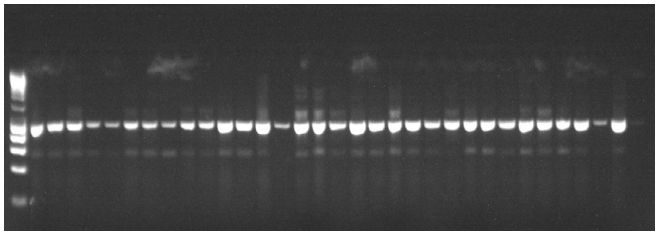
S/n	Sequence No	Organism	Acession No
1	SEQ1	*Lactobacillus plantarum*	MH665833.1
2	SEQ4	*Lactobacillus plantarum*	MG646692.1
3	SEQ6	*Enterococcus faecium*	MH817747.1
4	SEQ7	*Leuconostoc lactis*	MH307960.1
5	SEQ8	*Streptococcus salivarius*	MH813970.1
6	SEQ9	*Staphyloccus epidermidis*	KX170747.1
7	SEQ14	*Entrococcus faecalis*	MH105763.1
8	SEQ15	*Streptococcus oralis*	CP019562.1
9	SEQ18	*Staphylococcus epidermidis*	CP018842.1
10	SEQ31	*Enterococcus faecium*	KR363176.1
11	SEQ35	*Enterococcus hirae*	JF923641.1
12	SEQ36	*Enterococcus faecalis*	MF370045.1
13	SEQ38	*Lactobaccilus fermentum*	MH393160.1
14	SEQ39	*Streptococcus mitis*	MF578794.1
15	SEQ40	*Streptococcus mitis*	MF578796.1
16	SEQ41	*Bacillus cereus*	HQ219836.1
17	SEQ42	*Enterococcus faecalis*	MH105763.1
18	SEQ43	*Bacillus licheniformis*	AY871103.1
19	SEQ 46	*Streptococcus salivarius*	MH813970.1
20	SEQ47	*Entrococcu savium*	MH473244.1
21	SEQ48	*Entrococcus hirae*	JF923641.1
22	SEQ49	*Lactobacillus fermentum*	IQ293047.2
23	SEQ50	*Lactobacillas* subspp. *Cremoris*	CP031538.1
24	SEQ51	*Pseudomonas extremaustralis*	MH482280.1
25	SEQ52	*Pseudomonas extremaustralis*	MH482293.1
26	SEQ53	*Klebsiella aerogenes*	MH542333.1
27	SEQ54	*Enterobacter hormaechei*	CP031574.1
28	SEQ55	*Enterococcus faecalis*	JX536091.1
29	SEQ56	*Klebsiella pneumoniae*	MG201995.1

S/n	Sequence No	Organism	Acession No
30	SEQ57	*Lactococcus garvieae*	MH701935.1
31	SEQ58	*Klebsiella pneumoniae*	MF417488.1
32	SEQ59	*Enterobacter ludwigli*	KP772100.1
33	SEQ60	*Enterococcus faecalis*	MH813960.1
34	SEQ61	*Klebsiella pneumoniae*	KP772069.1
35	SEQ62	*Bacillus cereus*	HQ219925.1
36	SEQ63	*Bacillus cereus*	HQ219835.1
37	SEQ64	*Enterococcus faecalis*	KX752889.1
38	SEQ65	*Lactobacillus plantarum*	MH392883.1
39	SEQ66	*Lactobacillus plantarum*	MH665798.1
40	SEQ67	*Lactobacillus plantarum*	KM462843.1
41	SEQ68	*Streptococcus mutans*	GU561376.1
42	SEQ69	*Lactobacillus casei* strain	CP026097.1
43	SEQ70	*Klebsiella aerogenes*	MF682950.1
44	SEQ71	*Streptococcus anginosus*	KX661067.1
45	SEQ72	*Proteus mirabilis* strain	FJ906732.1
46	SEQ73	*Lactobacillus fermentum*	HQ111079.1
47	SEQ74	*Streptococcus salivarius*	MH813969.1
48	SEQ75	*Streptococus mutans*	MF578808.1
49	SEQ76	*Klebsiella pneumoniae*	KP723532.1
50	SEQ77	*Streptococcus salivarius*	MH813962.1

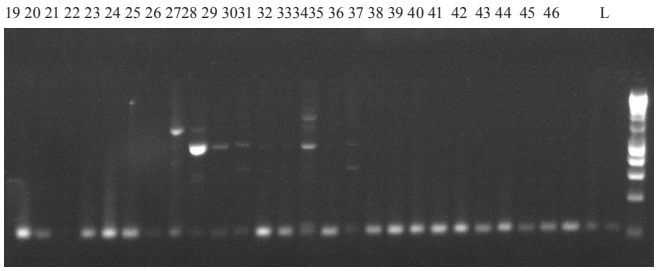
Table 4: Unique Identity of *denovo* bacterial strains from the study and their accession number as deposited and approved by NCBI, USA

Seq #	Identity	Accession #
Seq1	*Lactobacillus plantarum* Funm111	MN121715
Seq3	*Enterococcus faecium* Funmi223	MN121716
Seq5	*Streptococcus salivarius* Funmi225	MN121717
Seq10	*Streptococcus oralis* Funmi230	MN121718
Seq11	*Streptococcus* species Funmi231	MN121719
Seq12	*Enterococcus faecalis* Funmi232	MN121720
Seq13	*Lactobacillus fermentum* Funmi233	MN121721
Seq16	*Streptococcus species* Funmi235	MN121722
Seq17	*Enterococcus faecalis* Funmi236	MN121723
Seq19	*Streptococcus salivarius* Funmi238	MN121724
Seq20	*Enterococcus avium* Funmi239	MN121725
Seq21	*Enterococcus hirae* Funmi240	MN121726
Seq22	*Lactobacillus fermentum* Funmi241	MN121727
Seq23	*Lactococcus lactis* Funmi242	MN121728
Seq24	*Pseudomonas* species Funmi243	MN121729
Seq26	*Klebsiella aerogenes* Funmi245	MN121730
Seq27	*Enterobacter hormaechei* Funmi246	MN121731
Seq28	*Enterococcus faecalis* Funmi247	MN121732
Seq29	*Klebsiella pneumoniae* Funmi248	MN121733
Seq30	*Lactococcus garvieae* Funmi249	MN121734
Seq32	*Enterobacter ludwigii* Funmi251	MN121735
Seq33	*Enterococcus faecalis* Funmi252	MN121736
Seq34	*Klebsiella pneumoniae* Funmi253	MN121737
Seq37	*Enterococcus faecalis* Funmi256	MN121738
Seq44	*Streptococcus anginosu* Funmi263	MN121739
Seq45	*Proteus mirabilis* Funmi264	MN121740

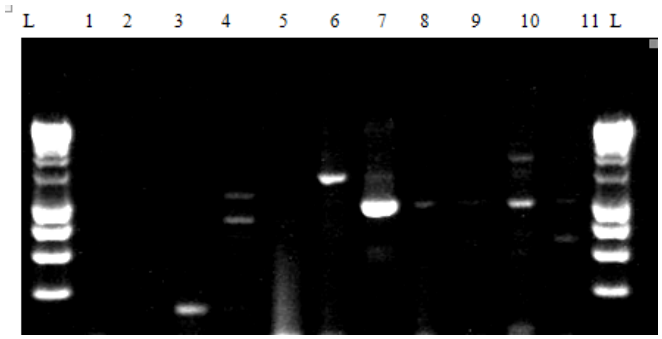




Gel electrophoresis for 16S Universal primer showing expected amplification at 1000bp
L- hyperladder lane (200bp-10kb)
Lanes 1-32: Sample DNA



Gel Electrophoresis using STR primer for *Streptococcus* targeting the *Tuf*genes showing expected amplification at 253bp, L- hyper ladder lane (200bp-10kb) Lanes 1-46: Sample DNA



Electrophoresis gel for the bacteria samples showing variations using the STR primer targeting the *Tuf*genes.L- hyperladder lane (200bp-10kb) Lanes 1-11: Sample DNA

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