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CLINICAL RESEARCH

In Vitro Susceptibility Assays Identify Fluoroquinolones, Gentamicin and Nitroimidazole as Effective Agents against *S. intermedius* and *C. albicans* Isolated from Diabetic Foot Ulcers

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ABSTRACT

BACKGROUND

Diabetic foot ulcer (DFU) is a common acute complication of diabetes mellitus, characterized by peripheral vascular disease and neurological disorders. Severe deterioration of DFU is often due to extensive gangrene and chronic infections that are responsible for 85% of limb amputations among diabetics.

AIM

The aim of this study was to isolate and identify microbial organisms responsible for DFU infections and determine their susceptibility to some common antibiotics.

METHODOLOGY

Deep wound swabs were collected, and organisms cultured in nutrient agar, using standard techniques. A modified cetyltrimethylammonium bromide (CTAB)-based protocol was used for microbial DNA extraction and purity of extracted DNA assessed by agarose gel electrophoresis. This was followed by amplification and sequencing of target 16S ribosomal and subsequent identification of isolated organisms based on results obtained from the NCBI Basic Local Alignment Search Tool (BLAST) software. Antimicrobial susceptibility test was carried out on identified organisms, using standard antibiotic disk diffusion methods.

RESULTS

Findings revealed presence of *Candida albicans* and *Streptococcus intermedius* in infected DFU, with both organisms showing susceptibility to fluoroquinolones (ciprofloxacin and ofloxacin), gentamicin and nitroimidazole. They were, however, resistant to ceftazidime, ceftriaxone, cefuroxime, erythromycin, cloxacillin and Augmentin.

CONCLUSION

Ciprofloxacin, ofloxacin, gentamicin and nitroimidazole, appear to be some antibiotics with retained efficacy against some common organisms responsible for DFU infections, including *Candida albicans* and *Streptococcus intermedius* despite the current wave of antimicrobial resistance. These antibiotics should be considered for both topical and systemic management of DFU infections.

KEYWORDS

Diabetic Foot; Fluoroquinolones; *C. albicans*; *S. intermedius*; Ulcer

INTRODUCTION

The global incidence of diabetes was estimated to be 8.5% in 2014, with several studies predicting an increase from 422 million to 642 million (52% increase) over the next 26 years [1]. An estimate of the global burden of diseases between 1990 and 2010 also revealed that diabetes moved from 15th to 9th position during the period under review [2]. The rapid increase in the incidence of diabetes across the globe is attributable to increases in unhealthy dietary habits, sedentary lifestyles, obesity, hereditary factors, and age of the population [3]. Poor management of diabetes mellitus could lead to some serious acute complications [4], among which diabetic foot ulcer (DFU) is the most common and is characterized by peripheral vascular disease, neurological disorders and inadequate or complete lack of foot care [5]. The complications of DFU are believed to be responsible for 85% of amputations among diabetics making it an important public health issue [6].

For proper evaluation and treatment, DFU has been classified into different stages. For example, the Wagner classification recognizes five stages of DFU, graded according to the depth of the ulcers. Wagner grade 0 indicates presence of foot pain, without any obvious signs of ulceration. In grade 1, there is superficial ulceration, while grade 2 is characterized by presence of deep ulcers, involving a tendon, bone or joint. Wagner grade 3 DFU has abscess or osteomyelitis, while gangrene is the major sign of grade 4 (gangrene of the forefoot) and 5 (gangrene of the entire foot). The Wagner classification is based on the depth of an ulcer alone without taking into consideration, presence of other pathologies such as vascular and neurological disorders or other systemic manifestations of a DFU. A more comprehensive and widely accepted classification of DFU is the one developed by the University of Texas which takes into consideration, the presence of ischemia and infections in diabetic feet. Inclusion of ischemia and infection in the grading of DFU could be used to evaluate the prognosis of a DFU during management and improve treatment outcome [7].

Majority of the diabetes-associated amputations are due to severe deterioration of DFU, with widespread gangrene and bacterial infections [8]. Consequently, early identification and treatment of ongoing infections in DFU is important for quick recovery and prevention of amputations. The early signs of infection and inflammation, including pain, warmth, redness and swelling of ulceration sites must be treated as soon as possible, even when there is minimum evidence of systemic involvement [9]. There has been a wide debate on the bacteria species commonly isolated from DFU, with some studies reporting presence of *Staphylococcus aureus*, *Streptococcus species* and *Pseudomonas aeruginosa* as the prevalent organisms, while *E. coli* is rarely observed. The aim of this study is to identify *in vitro*, the most active antibiotic class against a host of microorganisms isolated from DFU.

METHODOLOGY

Study location was the University of Medical Sciences Teaching Hospital (UNIMEDTH), Department of Biochemistry, University of Medical Sciences, Ondo City and the Bioscience Center of International Institute of Agriculture (IITA), Ibadan, Oyo State, Nigeria. Ethical approval for the study was obtained from the Research Ethics Committee of the UNIMEDTH (UNIMEDTH/REC'OFFICE), dated January 11, 2021. Informed consent was also obtained from participating patients.

Sample Collection, Culturing and Isolation of Microorganisms

Wound swabs were collected from three diabetic patients with DFU under strict adherence to established protocols for infection control including the proper use of nose masks, surgical hand gloves, laboratory coats, eye shields and hair scarf. Culturing and isolation of microorganisms from collected samples were done according to the methods of [10].

Bacterial DNA Extraction

The DNA extraction process involves a disruption of bacterial cell wall and release of intact DNA molecule into solution, followed by precipitation of extracted DNA from solution and its purification from contaminants such as proteins, lipids, polysaccharides and other metabolites.

Bacterial/fungal DNA extraction was achieved, using a modified cetyltrimethylammonium bromide (CTAB)-based method of [11]. The major modification involved a prior culturing and sub-culturing of bacteria from wound swabs in order to obtain pure strains, use of Proteinase K for protein degradation and final suspension of extracted DNA in Tris EDTA (TE) buffer.

Briefly, single colonies of isolated bacteria from samples 1, 2 and 3 were suspended in nutrient broth and centrifuged at 46000 grams for 5 minutes. Following centrifugation, supernatant was discarded, and pellets re-suspended in 520 µl of TE buffer (10 mMTris-HCl, 1 Mm EDTA, Ph 8.0). This was followed by addition of 15 µl of 20% SDS and 5 µl of 20 mg/ml of Proteinase K. Mixture was incubated at 37°C for 1 hour, after which 100 µl of 5 M NaCl and 80 µl of 10% CTAB detergent in 0.7 M NaCl was added and vortexed. Mixture was again incubated at 65°C for 10 minutes and kept in ice for 15 minutes. After 15 minutes, an equal volume (720 µl) of a 24:1 chloroform:isoamyl alcohol (CIA) was added and mixture left in ice for another 5 minutes, followed by centrifugation at 7200 grams for 20 minutes. A total of 500 µl of supernatant was transferred to a new tube and equal volume (500 µl) of CIA (1:0.6) added. Extracted DNA was purified from CIA by centrifugation at 46000 grams for 10 minutes, after which supernatant was discarded and pellets washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours and finally suspended in 50 µl of TE buffer. The integrity of extracted DNA was analyzed by Agarose Gel Electrophoresis.

Amplification of the 16S rRNA Gene by PCR

Thermo cycling conditions involved an initial denaturation at 94°C for 5 minutes, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and elongation at 72°C for 45 seconds. There was a final elongation step at 72°C for 7 minutes and hold temperature of 10°C. Amplified fragments were visualized on Safe view-stained 1.5% agarose electrophoresis gels. The size of the amplicon was about 1500 bp and the DNA ladder used was 1kb ladder from NEB. The PCR protocol used was optimized for Taq polymerase and primer pair 27F - 1525R. The cocktail mix consisted of 2.5 µl of 10X PCR buffer, 1 µl of 25 Mm Magnesium

chloride, 1 µl each of forward primer and reverse primer, 1 µl of DMSO, 2 µl of 2.5 mM DNTPs, 0.1 µl of 5 µ/µl Taq DNA polymerase and 3 µl of 10 ng/µl DNA. The primer sequence for bacterial identification was 27F: AGAGTTTGATCMTGGCTCAG; 1525R: AAGGAGGTGWTCCARCCGCA.

Antibiotics Susceptibility Test

The susceptibility of isolated organisms to selected antibiotics was studied using methods previously described by [12].

RESULTS AND DISCUSSION

Physical examination of cultures was carried out to determine the morphological characteristics of isolated bacteria, including their shape, size, colour, cell arrangement, surface texture, and surface elevation (Table 1).

Sample	Size (mm)	Shape	Colour	Cell Arrangement	Surface Texture	Surface Elevation
1	11	Rod-like	Cream	Clustering	Moist	Elevated
2	10	Round and irregular	Cream	Singling	Moist	Flat
3	9	Oval	Cream	Clustering	Moist	Elevated

Table 1: Morphological features of isolated bacteria.

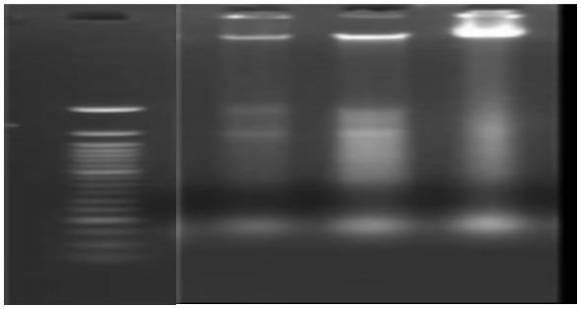

Figure 1: Agarose gel electrophoresis results for DNA extracted from samples 1, 2 and 3.

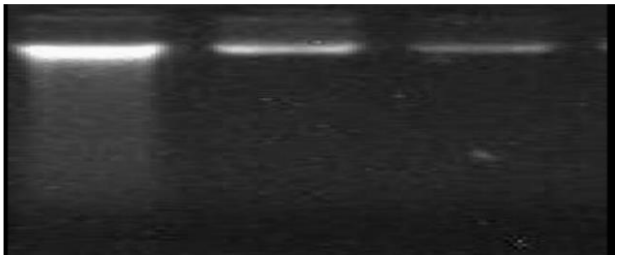

Figure 2: PCR products of DNA from samples 1, 2 and 3.

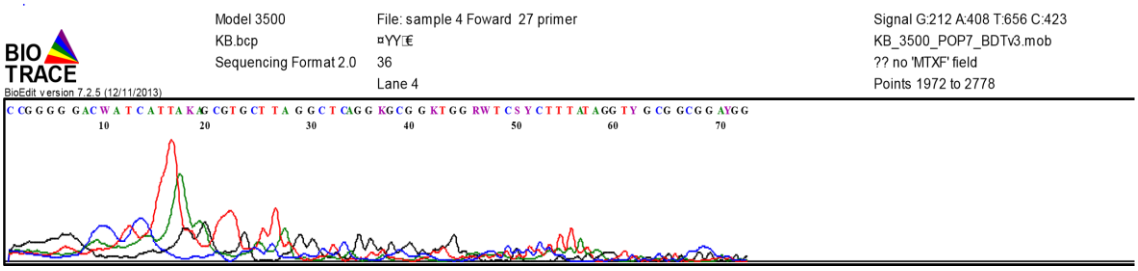

Figure 3: Sequence of DNA obtained for sample 1 using forward primer.

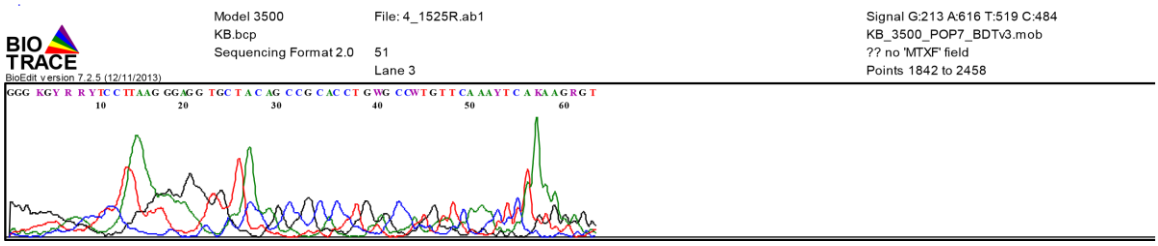

Figure 4: Sequence of DNA obtained for sample 1 using reverse primer.

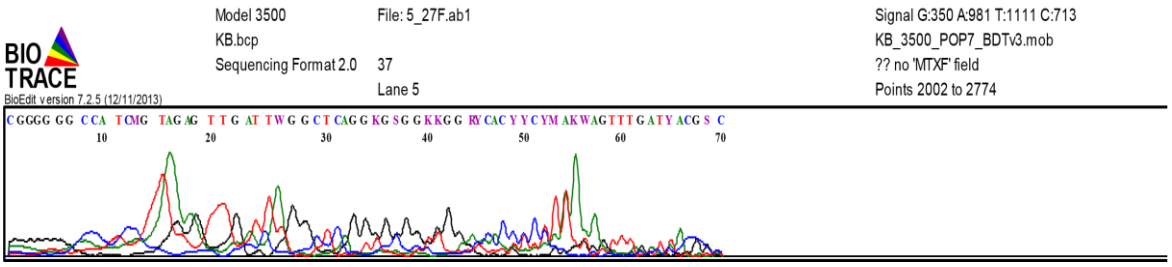

Figure 5: Sequence of DNA obtained for sample 2 using forward primer.

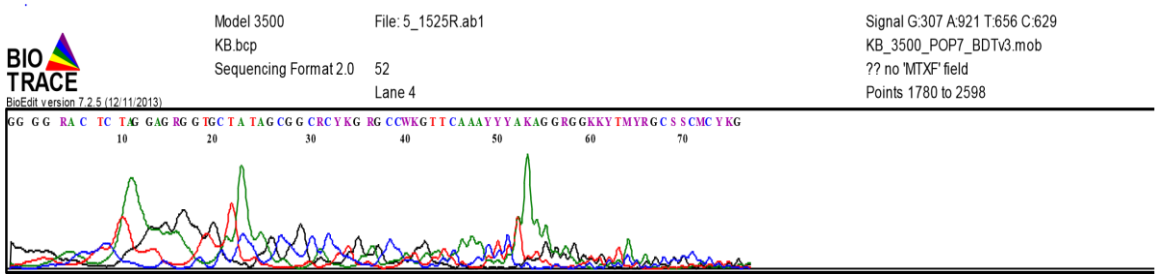

Figure 6: Sequence of DNA obtained for sample 2 using reverse primer.

Sample ID	Sequence alignment	NCBI BLAST report
Sample 1	GGGKGYRRYTCCTTAAGGGAGGTGCTA CAGCCGCACCTGWGCCWTGTTCAAAYT CAKAAGRGTGGGGGG AACTCCTTAAGGGAGGTGCTACAGCCG CACCTGTGCCA TGTTCAAACTCAGAAGAGT	*Streptococcus intermedius* strain FDAARGOS_769 chromosome, complete genome
Sample 2	CGGGGGGCCATCMGTAGAGTTGATTW GGCTCAGGKGSGGKKGGRYCACYYCY MAKWAGTTTGATYACGSCCGGGGGGC CATCCGTAGAGTTGATTTGGCTCAGGG GCGGGGGGACCACCCCTAATAAGTTTG ATAACGGCGGGCGGGGGGACCACCCCT AATAAGTTTGA	*Candida albicans* strain TIMM 1768 chromosome6, complete sequence. GenBank: CP032016.1

Table 2: Sequence alignments and NCBI BLAST report of DNA Samples 1 and 2.

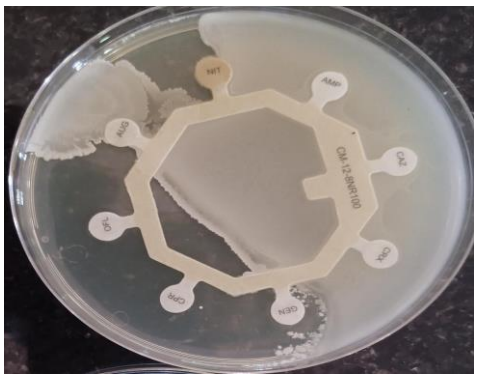

Figure 7: Antibiotic susceptibility of *C. albicans*, showing susceptibility to ciprofloxacin, ofloxacin, gentamicin and nitroimidazole (limited sensitivity) in antibiotic disk 1.

Figure 8: Antibiotic susceptibility of *S. intermedius*, showing susceptibility to ciprofloxacin, ofloxacin, gentamicin and nitroimidazole in antibiotic disk 1.

Figure 9: Antibiotic susceptibility of *C. albicans*, showing susceptibility to ofloxacin.in antibiotics disk 2.

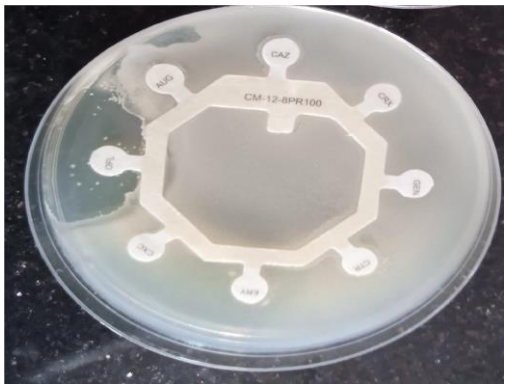

Figure 10: Antibiotic susceptibility of *S. intermedius*, showing susceptibility to ofloxacin in antibiotic disk 2.

This study was aimed at identifying the common bacterial infections present in DFU and specific antibiotics to which these bacteria are susceptible, in order to enhance treatment outcomes and minimize the problem of antibiotics resistance. There is widespread emergence of multi-drug resistant bacteria across the globe, a situation mostly caused by the inappropriate use of antibiotics [13]. For un-infected DFU, proper wound care practices and use of topical antiseptic agents are sufficient. However, when infected, DFU could develop complications and lead to partial or complete limb amputations. A diagnosed DFU infection is therefore an urgent clinical indication for the prescription of an appropriate systemic antibiotic, based on antibiotics susceptibility test results.

Several antibiotics have been recommended for the treatment of DFU infections, including ceftazidime, ceftriaxone, carbapenems and metronidazole. Others are ciprofloxacin, levofloxacin and combination therapies such as piperacillin-tazobactam and ampicillin-sulbactam combinations [14]. However, recent studies have revealed that most of the bacteria found in DFU have become resistant to some of these common antibiotics used

for the treatment of DFU infections, including carbapenems, ceftazidime, ceftriaxone, cefuroxime and aztreonam [15]. Findings from the present study also observed resistance of *Streptococcus intermedius* and *Candida albicans* isolated from DFU to ceftazidime, ceftriaxone, cefuroxime, erythromycin, cloxacillin and Augmentin. Both organisms showed susceptibility to the fluoroquinolones (ciprofloxacin and ofloxacin), gentamicin and nitroimidazole. Similar results were also observed for an unknown organism (results not shown) which therefore could either be *C. albicans, S. intermedius*, or a related bacterial species. These observations suggest that there is the need for continuous evaluation and review of the antibiotic regimen for the treatment of localized or systemic DFU infections.

Although this study was targeted at isolating bacteria species present in DFU, the observation of fungal features in culture plates was not surprising. Non-fastidious fungi such as *C. albicans* when present in biological samples, can grow easily on common laboratory media such as nutrient agar [16]. Also, *C. albicans* are common infections of DFU, where they often impair wound healing if not identified and treated urgently [17]. Fungal organisms are therefore widely implicated in DFU infections just like bacteria, with Candida species being the most commonly involved fungus [18,19].

Bacterial populations are believed to inhibit fungal growth through the synthesis and release of antifungal compounds [20]. Consequently, antibiotic therapy could theoretically promote the proliferation of fungi through loss of competitive inhibition by bacteria. However, the exact influence of antibiotics-mediated reduction in bacterial load on fungal populations remain largely undefined [21]. In contrast, there is massive scientific evidence in support of the direct antifungal effects of broad-spectrum antibiotics, especially fluoroquinolones, including moxifloxacin, ciprofloxacin, gatifloxacin and levofloxacin.

Cell cycle arrest and inhibition of fungal Topoisomerase II have been identified to be responsible for moxifloxacin-mediated killing of *Candida albicans* [22]. Also, 0.5% concentration of moxifloxacin and gatifloxacin have been reported to inhibit the growth of over 95% of *Candida albicans*, when topically applied for the treatment of ophthalmic infections [23]. Findings from similar studies have also revealed that ciprofloxacin, moxifloxacin, levofloxacin, travofloxacin and sitafloxacin could enhance the anti-Candida and anti-Aspergillus effects of several antifungal agents [24].

Patients suffering from diabetes and malignancies are highly susceptible to *Streptococcus anginosus* Group (SAG) of infections comprising *S. anginosus, S. constellatus* and *S. intermedius* infections. Also, the greater percentage (61.1%) of Diabetics with SAG infections have been observed to have DFU [25]. Diabetes mellitus is therefore a predisposing factor to *S. intermedius* infections, especially in the presence of DFU. Although not currently classified as opportunistic pathogens, presence of SAG has been reported in the upper respiratory, digestive and reproductive tracts, where they could induce non-invasive infections and cause systemic manifestations at sterile body sites, including blood and serous cavities [26]. Consequently, detection of *S. intermedius* infections should be considered as significant in the diagnosis and management of systemic diseases, including diabetes and cancers, especially in the presence of localized ulcers such as DFU.

CONCLUSION

Findings from the present study indicate that *S. intermedius* and *C. albicans* are common infections encountered in DFU and should be adequately treated, in order to prevent further complications that could lead to limb

amputations. Apart from these two organisms, other bacteria and fungi that have been widely associated with DFU include *Pseudomonas species*, *S. aureus*, *Enterococcus spp*, *Klebsiella*, *Enterobacter*, *Proteus*, *E. coli*, *Aspergillus spp*, *Fusarium*, *Trichophytons*, *Trichosporon*, *Penicillium* and *Acremonium spp*. *In vitro* antibiotics susceptibility test results suggest that ciprofloxacin and ofloxacin could effectively inhibit the proliferation of *S. intermedius* and *C. albicans* in DFU, with gentamicin and nitroimidazole also showing evidence of activity against both organisms.

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