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ORIGINAL RESEARCH

Prevalence of Extended-Spectrum β-Lactamase and Carbapenemase Producers of Gram-Negative Bacteria, and Methicillin-Resistant *Staphylococcus aureus* in Isolates from Diabetic Foot Ulcer Patients in Ethiopia

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Background: Infectious diabetic foot ulcers (IDFU) are a widespread health issue that affects people all over the world. IDFU, like other medical disorders, can have negative implications if drug resistance develops. Clinicians will be able to choose the optimal antibiotics to treat impacted patients based on the antibiotic susceptibility pattern of bacterial strains. In this project, we attempted to evaluate the levels of extended-spectrum beta-lactamase (ESBL), carbapenemase, and MRSA in patients with diabetic foot ulcers.

Methods: A sterile swab was used to collect a sample from the leg ulcer, while a sterile needle was used to collect the aspirated pus. Bacteria identification and antibiotic susceptibility tests are carried out based on conventional bacterial culture. The double-disc inactivation method and modified carbapenem (meropenem) were used to screen the production of ESBL and carbapenemase.

Results: Of the 76 isolates tested, 53.9% (41/76) were phenotypically ESBL producers. *K. pneumoniae* 75% (6/8), *Acinetobacter* species 75% (9/12), Serratia species 75% (3/4), *Pseudomonas* species 64.3% (14/9), *E. coli* 57.8% (11/19), *Citrobacter* species 50% (2/4) and *Proteus mirabilis* 25% (1/4) had the highest frequency of ESBL production. Of the 68 GNB isolates tested, 27.9% (19/68) were carbapenemase-producing organisms. *K. pneumoniae* 62.5% (5/8), *Serratia* 50% (3/6), *Acinetobacter* species 40% (4/10), *Pseudomonas* species 23.5% (4/17) and *E. coli* 15.8% (3/19) are the top five major carbapenemase producers. In this study, 32 isolates of *Staphylococcus aureus* were tested for methicillin resistance. Among them, 81.3% (26/32) were methicillin-resistant *Staphylococcus aureus* (MRSA), and 18.7% (6/32) of the remaining isolates were methicillin-sensitive *Staphylococcus aureus* (MSSA).

Conclusion: The result draws attention to the management of diabetic foot ulcer infections based on the results of microbiological analysis and drug susceptibility testing.

Keywords: prevalence, DM, DFU, ESBL, carbapenemase, MRSA

Introduction

Diabetes mellitus is a chronic disease that can be seen all over the world and over time causes serious damage to many organs and systems in the body. Retinopathy, cardiovascular disease, high blood pressure, kidney failure, neuropathy, diabetic foot ulcers, and many other complications are considered signs of long-term specific effects.[1]

Diabetic foot ulcer (DFU) is one of the most serious and devastating complications of diabetes mellitus (DM). It is defined as the foot below the ankle affected by ulceration/thickening wound/involving neuropathy and/or peripheral arterial disease of the lower limbs in diabetics.[2,3] As various studies report, the main risk factors for developing DFU are diabetic neuropathy (90%), peripheral artery disease (210%), and subsequent table trauma to the legs.[2]

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Other risk factors include deformities and structural abnormalities of the foot, a history of ulcers or amputations of the foot, poor blood sugar control, and smoking. In addition, the risk of foot ulcers and amputation increases with age and duration of diabetes.[4,5] According to various parameters (such as extent, size and depth, location, presence of infection, and ischemia), the severity of DFU is divided into different categories by different groups, such as Meggitt–Wagner Classification System, The Infectious Diseases Society of America (IDSA) Classification Scheme and the International Working Group Diabetic Foot (IWGDF).[6,7] Meggitt–Wagner classification is one of the most common DFU classification systems and it has six grades based on the depth of ulcer, presence of gangrene, and extent of tissue necrosis.[7,8]

Although peripheral neuropathy and peripheral arterial disease (PAD) are considered the most significant risk factors for DFU occurrence, microorganisms facilitate the severity of diabetic foot ulcers. As different articles currently show different pathogenic microbes,[9] infect greater than 50% of the ulcerated foot of diabetes patients. Signs like inflammation (erythema, oedema, heat, pain) and purulent discharge classically characterize infection, but in diabetic foot wounds, this is not typical due to ischemia and neuropathy. The Infectious Diseases Society of America (IDSA) and International Working Group on the Diabetic Foot (IWGDF) have put criteria (definition) to measure the severity of DFIs such as uninfected, mild, moderate, and severe infection.[10,11] Due to DFU, intact skin loses many of its barriers or defense mechanisms, and invading microorganisms can easily enter through the wound portal. Pathogenic microorganisms that colonize diabetic foot wounds cause local tissue damage.

Bacterial growth is promoted by the presence of tissue ischemia (leading to hypoxia) or necrosis, as well as by hyperglycemia affecting the host defenses.[12]

Infections start as a minor problem, but progress to conditions that cannot be controlled (involving deep tissue, joints, or bones). DFU occurs over a long period and severe infection is often associated with bacterial colonization.[7,12]

Several authors have reported that both aerobic and anaerobic microorganisms including multidrug resistance microorganisms (MDRO) with the ability to form biofilm are isolated from foot ulcers patients.[13] Among gram-positive bacteria; *Enterococcus* spp.,*Bacteroides* spp., *Peptostreptococcus* spp., *Veillonella* spp., *and Clostridium perfringens* have been identified.[14,15] The most commonly isolated gram-negative bacteria include Enterobacteriaceae (*E. coli, Proteus* spp., *K. pneumonia*, etc) and none fermenters include (*P. aeruginosa*, and *Acinetobacter* spp.) and have also been identified.[16,17] Additionally, fungi (such as candida) are also common and have been implicated in the delayed healing of DFUs.[18]

The source of these bacteria is in the environment, adjacent skin, or other endogenous sources, including the gastrointestinal tract.[19] Risk factors associated with MRSA, such as invasive devices, previous hospitalization, and comorbid illness, were found to be significantly associated with MRSA.[20] Diabetic foot ulcer infection is also a characteristic or comorbid related to immune compromised diseases.

Nowadays, the incidence and prevalence of ESBL have been increasing. ESBL production is an important resistance mechanism hampering the antimicrobial treatment of infections caused by Enterobacteriaceae and poses a serious threat to the arsenal of currently available antibiotics.

Currently, there are little data on ESBL-producing organisms and carbapenemase producers for diabetic foot infections in Ethiopia. Therefore, this study aimed to determine the burden of resistant microorganisms, ESBL and carbapenemase production, and methicillin-resistant *Staphylococcus aureus* that infect diabetic foot ulcers.

Materials and Methods

A multicenter-institutional-based cross-sectional study was conducted in Addis Ababa, Ethiopia, from November 2020 to May 2021.

Ethical Consideration

The ethical review was first obtained from the ethics and review committee of the Department of Microbiology, Immunology and Parasitology, College of Health Sciences, Addis Ababa University, and was approved by the Department Ethical Review Committee (meeting no. DERC/005/2020). The Addis Ababa Public Health Research and Emergency Management Agency had also approved it. The formal written letter had been distributed to each hospital included in this study. Before collecting data, study participants were informed of the study and obtained their consent,

and confidentiality was maintained by omitting their names and personal identifiers throughout the study (Supp1). This study was also conducted per the Declaration of Helsinki.

Sample Collection, Transport, Process, Culture and Identification

A sterile swab was used to collect a sample from the leg ulcers, while a sterile needle was used to collect the aspirated pus. Staurt's transport medium was used for transporting swabbed and tissue samples within 2 hours. After the sample reaches in the laboratory, Gram stain, culture on (Blood Agar, MacConkey, Manito Salt Agar), subculture, and a panel of biochemical testing were done to isolate the bacteria.

Antibiotics Susceptibility Testing

Per CLSI guidelines (2021), the Kirby–Bauer disk diffusion method was used for antibiotic susceptibility testing.[22] The antibacterial discs used for the test are ampicillin (20 µg), aztreonam (30 µg), gentamicin (10 µg), amikacin (30 µg), cefazolin (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), (30 µg), cefepime (30 µg), cefoperazone/sulbactam (75/10 µg), piperacillin/tazobactam (100/10 µg), Imipenem (10 µg), meropenem (10 µg), and polymyxin B (300 units) against gram-negative bacilli.

Penicillin, ampicillin, azithromycin (15µg), cefoxitin (30µg), cefotaxime (30µg), chloramphenicol (30µg), clindamycin (2µg), erythromycin (15µg), oxacillin (1µg), vancomycin (30µg), and teicoplanin (30µg)), ciprofloxacin, ofloxacin (5µg), linezolid (30µg) and tetracycline (30µg) were used to study the susceptibility patterns of the gram-positive cocci. The production of MRSA, ESBL, and carbapenemase had been detected according to the CLSI 2021 guidelines.[22]

Methicillin-Resistant *Staphylococcus aureus* (MRSA) Detection

Cefoxitin discs (30 µg) were used for phenotypic testing for MRSA. A zone of inhibition equal to or greater than 22 mm was considered sensitive to cefoxitin and the organism was reported as methicillin-sensitive *Staphylococcus aureus* (MSSA). Isolates with a zone of inhibition less than or equal to 21 mm were considered methicillin-resistant *Staphylococcus aureus* (MRSA).

Extended-Spectrum β-Lactamase (ESBL) Production

The production of ESBL was confirmed using ceftazidime tablets (30 µg) and ceftazidime clavulanate (30/10 µg). The test organisms were inoculated on Mueller–Hinton agar plates, and the above discs were placed on the plates.

Plates were incubated overnight at 37°C and checked the next day. Compared with the antimicrobial agent tested alone, the area diameter of the antimicrobial agent tested in combination with clavulanic acid increased by 5 mm or more, indicating that the strain is a producer of ESBL.

Carbapenemase Production Screening

The production of carbapenemase was detected by a modified Hodge test. The 0.5 Mac Farland suspension of ATCC *E. coli* 25922 was diluted 1 to 10 in sterile saline. As a conventional disk diffusion test, it was inoculated on the MHA media. The plate was dries was for 5 minutes; then a 10 µg meropenem plate was placed in the center of the agar plate. A few colonies were picked from the test organism and directly inoculated on the edge of the disc at least 20 mm away. The plate was incubated overnight at 37°C and checked the next day. Around the test organism, an improvement in growth was observed at the intersection of the straight line and the zone of inhibition. The presence of improved growth indicates that carbapenemase is produced, while no improvement in growth means that the test isolate does not produce carbapenemase.

Data Analysis

The data were entered into EpiData v.4.6.0.4 and cleaned and analyzed with statistical software SPSS 25 version (IBM Corporation, Comp.soft-sys.stat.spss). Descriptive statistics such as frequency and percentage were used to report a numerical summary of the survey results. The quantitative value model is presented through graphical representation and statistical tables.

Result

Sociodemographic Data of the Study Participants

One hundred and thirty participants were included and out of the total 88 (67.89%) were males and 42(32.3%) were females. The majority of the study participants were in the age group of 50–75 years. Among the study participants, type-I DM was in 51.9%, and 48.4% participants had type-II DM. In this study, the Meggitt–Wagner classification system was used to classify DFU. The majority of study participants came with grade three in 48.4%, followed by grade two 33.59%, 14.8% with grade four, 2.3% with grade one, and grade five in 0.8%.

Magnitude of ESBL-Productions

In this study, 76 gram-negative isolates were tested for the production of ESBL. Of the tested 76 GNB isolates, 53.9% (41/76) were phenotypically ESBL-producer while 46.05% (35/76) were non-ESBL-producers. High ESBL production rates was observed among *K. pneumoniae* 75% (6/8), *Acinetobacter* species 75% (9/12), *Serratia* 75% (3/4), *Pseudomonas* species 64.3% (9/14), and followed by *E. coli* 57.8% (11/19), *Citrobacter* species 50% (2/4), *Proteus mirabilis* 25% (1/4) (Figure 1).

The Magnitude of Carbapenemase Production

Carbapenemase-producers of gram-negative bacteria isolate phenotypical determination of carbapenemase-producer pathogenic gram-negative bacterial isolate. Based on the modified disk inactivation (meropenem) method,[22] the carbapenemase test was done for 68 ESBL positive and Imipenem or Meropenem resistant gram-negative isolates.

Out of 68 pathogenic gram-negative bacteria isolates, 27.9% (19/68) were carbapenemase-producer, whereas 73.53% (50/68) were non-carbapenemase-producer. High carbapenemase-producers rate was observed among *K. pneumoniae* 5/8 (62.5%), followed by *Serratia* 3/6(50%), *Acinetobacter* species 4/10(40%), *Pseudomonas* species 4/17(23.5%), E. coli 3/19(15.8%), and the rest of the isolates were non-producers (Figure 2).

The Burden of Methicillin-Resistant *S. aureus* (MRSA)

In this study, 32 isolates of *Staphylococcus aureus* were screened for methicillin resistance. Of these, the majority of the isolates 81.3 (26/32) were methicillin-resistant *Staphylococcus aureus* (MRSA) and the rest of the isolates were methicillin-sensitive *Staphylococcus aureus* (MSSA) 18.7% (6/32). Almost 50% of the isolates screened for MRSA were susceptible to amikacin, clindamycin, chloramphenicol, vancomycin, and ciprofloxacin.

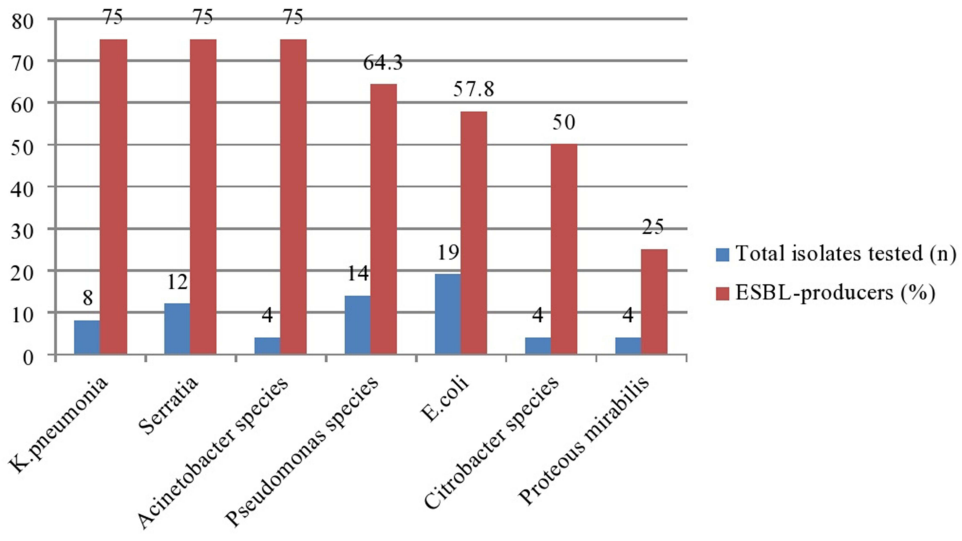

Figure 1 Magnitude of ESBL-producers among pathogenic gram-negative isolates.

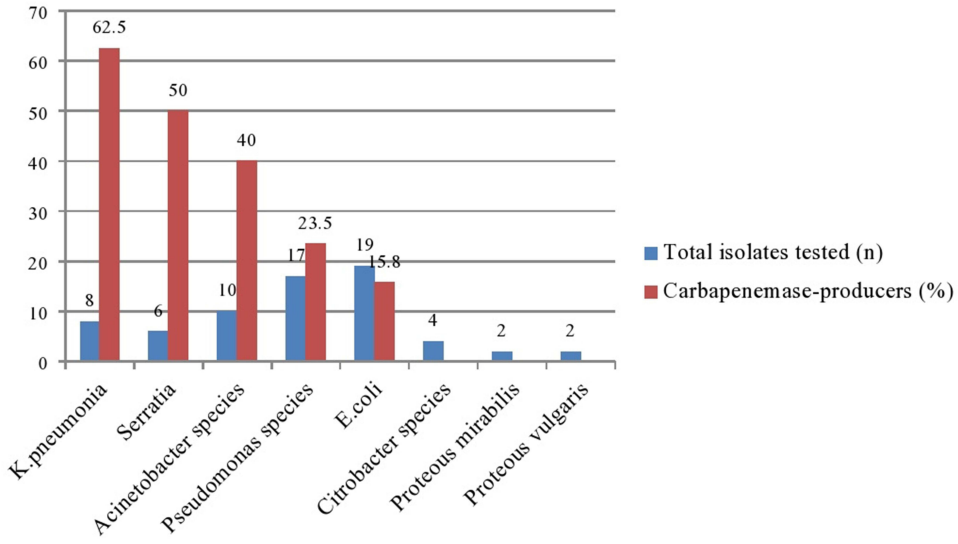

Figure 2 Magnitude of carbapenemase-producers among gram-negative isolates.

Discussion

Antimicrobial resistance is a deep-rooted scientific problem in hospitals and community settings. Rapid tests in clinical laboratories are crucial for the correct identification of antibiotic-resistant microorganisms. The production of extended-spectrum β-lactamases (ESBL) is an important mechanism of drug resistance, which makes antibacterial treatment of Enterobacteriaceae infections difficult and represents a serious threat to the currently available group of antibiotics.[23]

Of the 76 gram-negative bacteria, more than half of the isolates (53.95%) were ESBL-producers, which are consistent with the study documented in India (53%),[24] in Egypt 49%.[36]

In poor resource settings, the burdens of ESBL producing gram-negative bacteria are more prevalent among DFU patients. Some reports, from the Middle East and North Africa, showed that the ESBL-producing bacteria range from 11% to 53%.[25] Other studies also supported this finding 46% in eastern India,[26] 42% in Odisha, India,[27] 38% in Istanbul Turkey,[28] 33% in Nigeria,[29] and 31% in Iran.[30]

The present study indicated that gram-negative ESBL-producers in patients with DFU was high among *K. pneumoniae* 75% (6/8), *Acinetobacter* species 75% (9/12), *Serratia* 75% (3/4), *Pseudomonas* species 64.3% (9/14), and followed by *E. coli* 57.8% (11/19), *Citrobacter* species 50% (2/4), and *Proteus mirabilis* 25% (1/4). This finding is somehow different from the study conducted in Iran that reported *Acinetobacter* species (50%) followed by *E. coli* (36%), *P. aeruginosa* (33%), and *Enterobacter species* (25%).[30]

Of the 68 gram-negative bacteria analyzed in this study, 19 (27.9%) were positive for the carbapenemase phenotype. A high rating was recorded among *K. pneumoniae* followed by *Acinetobacter* species,*E. coli, Pseudomonas* species, and *Serratia* species. The results of the current research are superior to those carried out in Egypt, of which 3.6% are carbapenemase producers,[36] including a recent study carried out in the same country that shows 11.7% from carbapenemase producers[32] and 3.1% carbapenemase producers in Nigeria.[27]

Methicillin-resistant *Staphylococcus aureus* (MRSA) has long been considered a significant human pathogen and the most common cause of hospital-acquired infections. Developing resistance to treatment options for treating infections caused by Methicillin-resistant *Staphylococcus aureus* is an emerging issue.[33]

In this study, 32 isolates of *Staphylococcus aureus* were screened for methicillin resistance. Of these, the majority of the isolates 81.3% (26/32) were methicillin-resistant *Staphylococcus aureus* (MRSA) and the rest of the isolates were methicillin-sensitive *Staphylococcus aureus* (MSSA) 18.7% (6/32). This finding is in line with the study documented in Arbaminch, Ethiopia (82.3%) and Eretria 72%.[31,32] However, the present prevalence rate is higher than the study documented previously in Egypt 10.1%, 15.8%.[25,34]

In this study, nearly 50% of the isolates screened for MRSA were sensitive to amikacin and chloramphenicol. These results far exceed previous reports, which showed that methicillin-susceptible isolates of *Staphylococcus aureus* were susceptible to clindamycin, vancomycin, and ciprofloxacin. However, these results are consistent with studies previously reported in Ethiopia,[31] Egypt,[25,34] and Sudan.[35]

Limitations of the Study

Because of the lack of molecular methods and primes, molecular testing of ESBL, carbapenemase producers, and genes encoding MRSA in the analyzed isolates was not performed as a confirmatory test.

Conclusion

The result shows that greater than 50% of the isolated gram-negative bacteria were phenotypically ESBL producers, significant number of isolated gram-negative bacteria were phenotypically carbapenemase producers, and high number of the *S. aureus* isolates were methicillin-resistant. This draws attention to the management of diabetic foot ulcer infections based on the results of microbiological analysis and drug susceptibility testing.

Abbreviations

CLSI, Clinical and Laboratory Standards Institute; DFU, diabetic foot ulcer; ESBL, extended-spectrum beta-lactamase; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; MDR, multidrug-resistant organism.

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Disclosure

The authors have no conflicts of interest to be declared.

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