Original Article

The Antimicrobial Resistance Patterns & Distribution of Antibiotic Resistance Genes among Carbapenem Resistant Escherichia Coli Isolated from Diabetic Foot Infection

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Abstract

Background: Diabetic foot infection (DFI) is an infection in soft tissue or bone in patient with diabetes mellitus, treatment of DFIs requires appropriate antibiotic selection, continuous updates of the microorganisms that are responsible of infection and their resistance pattern, the aim of this study to determine the frequency of antimicrobial resistance patterns, ESBLs, and MBLs produced carbapenem resistant *Escherichia coli* isolated from patients with DFI.

Methods: about 111 swabs specimen were collection from patient suspected with DFI during the period of study, determinethe antibiotic susceptibility of *E coli* isolatesby using Kirby-Bauer disk diffusion method to 23 antimicrobial agents. ESBL production was screened by two tests initial method by the disk diffusion method of ceftazidime, ceftriaxone, cefotaxime, and aztreonam (30µg/disk each), accordingto the CLSI (2021) and confirmatory method by Double disk diffusion test, MBL production by the diskdiffusion method of imipenem and meropenem. Molecular detection for ESBL genes by monoplex PCR and MBL genes by multiplex PCR.

Results: from 111 swabs specimen *E.coli* isolates had represented in 19 (27.5%) among Gram-negative bacteria. Antibiotic susceptibility test revealed that10 carbapenem resistant *E.coli* isolates, and the highest resistant to β- lactam class and cephalosporin, results indicated also 100% of isolates were MDR and 63.1 XDR. PCR assay to ESBL genes revealed that *bla*CTX-M was found in 3 (30%) and *bla*OXA was noticed in 4 (40%). The PCR data of MBL genes revealed that, the frequency of MBL genes among isolates as following *bla*VIM1 (50%), *bla*NDM2 (100%), and *bla*SIM 2 (100%).

Conclusion: the study show high frequency of antibiotic resistant pattern and the presence of ESBL and MBL genes among carbapenem resistant *E.coli* isolated from DFI.

Keywords: Diabetic Foot Infection, TEM, SHV.

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INTRODUCTION

Diabetic foot infection (DFI) is an infection in soft tissue or bone for peoples with diabetes, and has grown to be a major reason for non-traumatic amputation [1]. contamination of bacteria among diabetic foot can cause foot ulceration and this can lead to development gangrene and then amputation, according to some studies about 15-34% of diabetic patients are at risk for developing foot ulcers during their lives [2].The characterization of *Escherichia coli* isolates from skin and soft tissue infections have been previously published [3 4]. However, this description still poor from DFIs, while *E. coli* is a major pathogenic Gram-negative bacteria isolated from these ulcers [5 6 7].Treatment of DFIs requires appropriate antibiotic selection, continuous updates of the microorganisms that are responsible of infection and their resistance pattern[8]. Antibiotic resistance rates vary by country and geographical region [9]. Rising levels of resistance to main antibiotics were initial caused by Extended-Spectrum-Beta- Lactamase (ESBL) producing Gram-negative bacteria in the mid-2000's, and more lately by carbapenemase-producers[10].The emergence of carbapenem as a therapy for bacteria that are resistant to β -lactamase is a significant advance in clinical practice [11]. Carbapenems have been utilized as a last choice of antibiotics to treat severe infections caused by

Gram-negative bacteria (GNB). However, the therapeutic use of carbapenem is in threat with the development of carbapenemases, mainly metallo-β-lactamase (MBL) [12].Extended spectrum β-lactamases is an enzyme produced by Gram negative bacteria *Enterobacteriaceae*, which can hydrolyze penicillin but also third generation cephalosporin and monobactam and other antibiotics by inactivation of penicillin and cephalosporin by using plasmid-mediated ESBLs such as the TEM, SHV, or CTX-M groups [13].This study purposed to determine the frequency of antimicrobial resistance patterns for Carbapenem resistant *Escherichia coli*isolated from patients with diabetic foot infection, and investigation the presence of ESBLs and MBL using conventional and multiplex PCR technique.

MATERIAL AND METHODS

Sample collection and Identification of Bacteria Isolates

This cross section study was conducted in Al-Sader Medical City in the Najaf province during the period from December 2021to March 2022. The study population consisted of 111 swabs specimen from patients clinically suspected by the physician to have DFI, and the specimens immediately transported to the microbiology laboratory. All Gram-negative isolates from swab in the current study had been identified by depending on Morphological characteristic, Microscopically examination (gram-stain), and Biochemical testsaccording to standard method described by MacFaddin and Hart [14] [15]. *E.coli* isolates were also identified by the VITEK-2 compact system.

Antimicrobial Sensitivity Testing

Antimicrobial sensitivity testing of *E. coli* isolateswas performed by using disk diffusion methods (Kirby-Baur method). The selection of antibiotic disks (table 1) were performed according to the guidelines recommended by the CLSI. All susceptibility results were interpreted according to the standardvalues performed by CLSI (2021).

Phenotypic detection for the Production of (ESBLs)

Initial test

All the *E.coli* isolates screened for susceptibility to cefotaxime, ceftazidime and ceftriaxone (30mg/disk each) using disc diffusion sensitivity testing.The isolates were classified as potential ESBL producers if the zone diameter for cefotaximewas ≤26 mm, ≤21 mm forceftazidime and ≤23 mm for ceftriaxone.

Confirmatory test using the double disc diffusion test (DDT)

The DDT was carried out by using a standard diffusion assay on Mueller Hinton agar. Disks containing ceftriaxone, cefotaxime, ceftazidime, andaztreonam (30 mg) were placed at different distances (20—30 mm from center to center, depending on the species) around a disk containing amoxicillin- clavulanic acid (30 mg). Extension ofthe inhibition zone to the amoxicillin-clavulanic acid disk was indicated suggestive of ESBL production.

Phenotypic detection for carbapenem resistance

In the present study, carbapenem resistancewas defined as resistant to at least one of the two-carbapenem antibiotics tested: imipenem, and meropenem based on the CLSI (2021) guidelines. Isolate that showed as zone of inhibition ≤19 mm in diameter for imipenem and\ or meropenem was considered as carbapenem resistant.

Molecular Detection of Antimicrobials Resistance Genes

ESBLs genes

All carbapenem resistant *E.coli* were screening for detection the presence ESBLs genes by monoplex PCR, the primers sequence and PCR condition illustrated in previous references [16] [17] [18]. Amplicons were separated by agarose gel electrophoresis in 1.5 % (w/v) agarose gel, stained with ethidium bromide. The positive results were detection when the DNA band base pairs ofsample equal to the target product size. The PCR were prepared in total volume25µl PCR mixture including 12.5µl Promega Master mix, 2µl forward primer(10µM), 2µl reverse primer (10µM), 5µl DNA template (10-250 ng), and 3.5µl nuclease free water, prepared according protocol kit of the manufacturing company (Favorgen). PCR conditions had performed in T3000 thermocycler (Biometra).

MBLs genes

All carbapenem resistant *E.coli* were screening for detection the presence MBLs genes by multiplex PCR, the primers sequence and PCR conditions were published elsewhere [19]. Amplicons wereseparated by agarose gel electrophoresis in 1.5 % (w/v) agarose gel, stained with ethidium bromide. The positive results were detection when the DNA band base pairs of sample equal to the target product size. The PCR were prepared in total volume 50µl PCR mixture including 25µl Promega Master mix, 1.5µl forward primer(10µM), 1.5µl reverse primer (10µM), 2µl DNA template (10-250 ng), and 8µl nuclease free water, preparedaccording protocol kit of the manufacturing company (Favorgen). PCR conditions had performed in T3000 thermocycler (Biometra).

RESULTS

The results indicated that out of 111 swabs were cultured, there 93 (83.7%) of specimens were positive for the presence of growth bacterial, while 18 (16.2%) had non growth bacterial. Out of 93 of bacteria isolates were

bacterial growth and the rate of Gram-negative bacteria was 69(74.1%) and 34 (36.5%) was Gram positive bacteria, 19 (27.5%) were identified as *E. coli* based upon colonial characteristics and conventional biochemical tests and VITEK 2 compact system test.

Antibiotic susceptibility test of E. coli isolates

The resistance profile to 23 antibiotics disks belonged to 12 classes of antibiotics as showed in table (1). The highest resistance rate to ampicillin (100%), piperacillin (95%), ceftriaxone (95%),and followed bycefotaxime, cefepime, cefixime, ciprofloxacin, tetracycline (89.4%),respectively, and followed by tobramycin (84.2%), norfloxacin (84.2%), ceftazidime (79%), Aztreonam (79%), levofloxacin (79%), and nitrofurantoin (79%), respectively. While highest sensitivity of the *E. coli* has been to the colistin (95%), imipenem (63,1%) and amoxicillin-clavulanic acid (63.1%). According to the resistanceprofile (100%) were MDR resistant to at least one antimicrobial for three different categories, and (63.1%) were XDR the isolates were resistant to at least one antibiotic in allbut two or fewer antimicrobial categories.

Table 1: Antibiotics sensitivity test of E.coli isolated from patients of DFI (n=19)

Antibiotic class	Antimicrobial agent	No.(%) of isolates exhibited		
		Resistant	Intermediate	Sensitive
Penicillin	Ampicillin	19(100%)	0(0%)	0(0%)
	Piperacillin	18(95%)	1(5.2%)	0(0%)
Beta-lactam	Amoxicillin clavulanate acid	7(37%)	0(0%)	12(63.1%)
	Piperacillin tazobactam	9(47.3%)	5(26.3%)	5(26.3%)
cephems	Ceftazidime	15(79%)	2(10.5%)	2(10.5%)
	Ceftriaxone	18(95%)	0(0%)	1(5.2%)
	Cefotaxime	17(89.4%)	0(0%)	2(10.5%)
	Cefepime	17(89.4%)	0(0%)	2(10.5%)
	Cefixime	17(89.4%)	0(0%)	2(10.5%)
monobactams	Aztreonam	15(79%)	1(5.2%)	3(16%)
carbapenem	Imipenem	6(32%)	1(5.2%)	12(63.1%)
	Meropenem	10(53%)	0(0%)	9(47.3%)
aminoglycosides	Amikacin	10(53%)	0(0%)	9(47.3%)
	Gentamicin	12(63.1%)	0(0%)	7(37%)
	Tobramycin	16(84.2%)	0(0%)	3(16%)
quinolones	Ciprofloxacin	17(89.4%)	1(5.2%)	1(5.2%)
	Levofloxacin	15(79%)	0(0%)	4(21%)
	Norfloxacin	16(84.2%)	0(0%)	3(16%)
Folate pathway	Trimethoprime-Sulphamethazole	13(68.4%)	0(0%)	6(32%)
nitrofurans	Nitrofurantoin	15(79%)	0(0%)	4(21%)
phenicol	Chloramphenicol	10(53%)	1(5.2%)	8(42.1%)
tetracyclines	Tetracycline	17(89.4%)	0(0%)	2(10.5%)
lipopeptides	Colistin	1(5.2%)	0(0%)	18(95%)

Detection of ESBLs production

The presence of ESBLs was investigated in all *E.coli* isolates using phenotypic tests as follows:

Phenotypic screening for the Production of (ESBLs)

The *E.coli*isolates tested by initial screening test, 100% showedresistance to at least one of the third generation cephalosporins (potential ESBLproducers). A positive result was noted with 89.41% for cefotaxime, 95% forceftriaxone, 79% for ceftazidime and aztreonam respectively. All isolates thatexpressed antibiotic resistance to any of the third generation cephalosporinsand aztreonam tested were subjected to confirmatory methods by DDT. However, based on the double disc diffusion test, no isolatewas found to be ESBL producer.

Molecular Detection and Distribution of ESBLs genes

All 10 carbapenem resistant *E.coli* isolates were screening by conventional PCR for the occurrence of ESBL genes, the result revealed that *bla*OXA was found to be themost prevalent gene among isolates, which are noticed in 4 (40%) isolates Figure (1), followed by *bla*CTX-M that was found in 3 (30%) isolates Figure(2), while *bla*TEM and *bla*SHV were not found in any isolate.

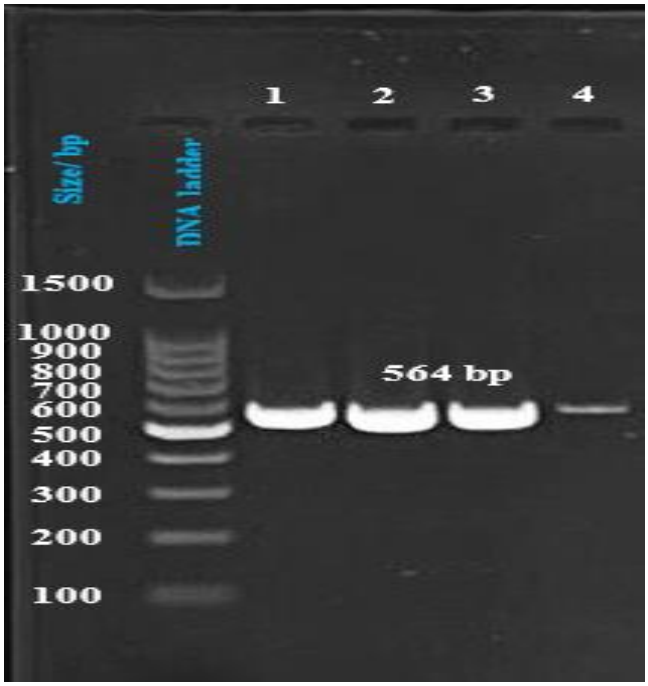

Figure 1: Agarose gel electrophoresis of image that show monoplex PCR amplified product from extract DNA of *E.coli* isolates with *bla*OXA genes primers, Lane (1,2,3,4) exhibit positive results with *bla*OXA gene (564 bp). DNA ladder with size\bp (100-1500bp).

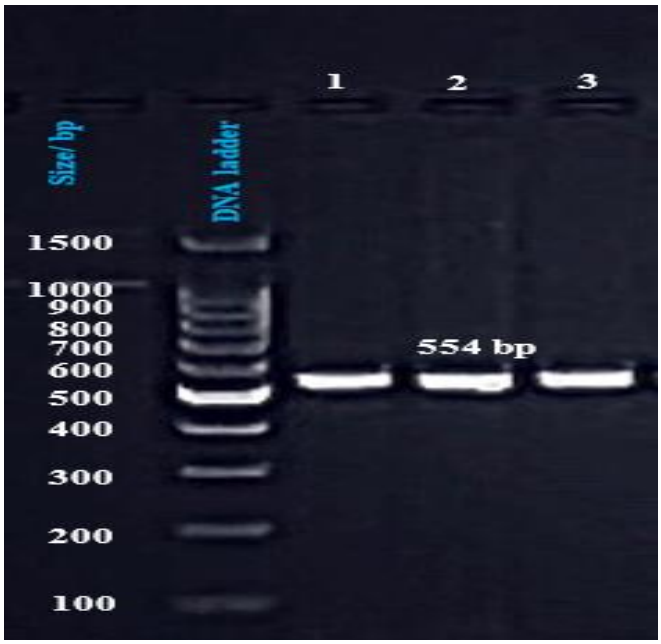

Figure 2: Agarose gel electrophoresis of image that show monoplex PCR amplified product from extract DNA of *E.coli* isolates with *bla*CTX-M genes primers, Lane (1,2,3) exhibit positive results with *bla*CTX-M gene (554 bp). DNA ladder with size\bp (100-1500bp).

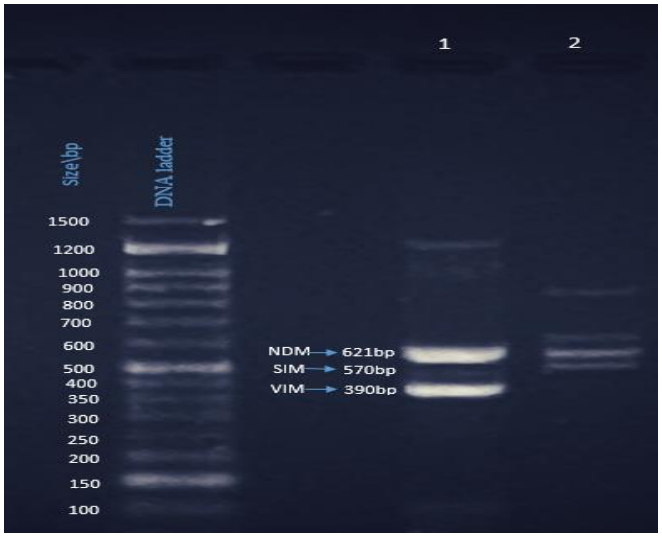

Figure 3: Agarose gel electrophoresis of image that show multiplex PCR amplified product from extract DNA of *E.coli* isolates with *bla*NDM, *bla*VIM, *bla*IMP, *bla*SIM, *bla*SPM genes primers, Lane (1) show positive results with *bla*NDM gene (621 bp),*bla*SIM gene (570 bp),and *bla*VIM gene (390 bp). Lane (2) show positive results with *bla*NDM gene (621 bp) and *bla*SIM gene (570 bp). DNA ladder with size\bp (100-1500bp).

Molecular Detection and Distribution of MBLs genes

All 10 carbapenem resistant *E.coli* isolates were examined by multiplex PCR for the occurrence gene determinants encoding MBL genes (*bla*IMP, *bla*VIM, *bla*NDM, *bla*SPM, and *bla*SIM). The results indicated that presence only two *E.coli* isolate carry the MBL genes (*bla*VIM, *bla*NDM,, and *bla*SIM). The frequency of MBL genes among isolates as following *bla*VIM1 (50%), *bla*NDM2 (100%), and *bla*SIM 2 (100%) as shown in figure (3). While *bla*SPM and *bla*IMP were not found in any isolate.

DISCUSSION

Diabetic foot (DF) is refersto the alterations and abnormalities that occur either separately or together in the feet and legs of diabetic patients[20].These reasons are less likely to penetrate antimicrobial therapies in DFI, making it more challenging to deliver effective antimicrobial activity to the target location and accelerating the emergence of bacterial resistance[21]. In the current study the prevalence of *E.coli* was 19 (27.5%) among isolates from DFI, similar results have been found previous studies done in Iraq and India with frequency 30% and 25.5% respectively[22],[23]. Additionally, other studies conducted in different regions around world with the prevalence of *E.coli* were 19.9%, 14.4%, and 13% done in France, Romanian, and UK respectively [24],[25],[26].The other findings the prevalence of *E.coli* was low compared with present study among specimens diabetic foot patients such as study done in Malaysia, Pakistan, and China, which reported 3.8%, 7.2%, and 2.4% respectively [27],[28],[21].

The main purpose of this study is to determine the frequency of antimicrobial resistance patterns of *E.coli* isolated from patients with DFI. The results of current study indicated that the highest resistance rate to ampicillin (100%), piperacillin (95%),ceftriaxone (95%), and followed by cefotaxime, cefepime, cefixime, ciprofloxacin, tetracycline (89.4%), respectively, this results are in agreement with previous studies done in Iraq,India, and Ethiopia [29],[30],[31],[32]. While other findings revealed low resistance to this antbiotics such studies conducted in different regions around the world [30], [32], [33], [34]. In contrast the highest sensitivity of the *E. coli* has been to the colistin (95%), imipenem (63,1%) and amoxicillin-clavulanic acid (63.1%),This results are consistent with previous studies performed in South Korea and India that indicated high sensitive to colistin (100%) [35] [31] and another study performed in Iraq found the sensitive to Amoxicillin-clavulanic acid was 82% [33]. The current study demonstrated a high sensitive to imipenem (63.1%), the results is consistent with a study done in Kurdistan Region of Iraq indicatedthat high senstive to imipenem 82% [36].In contrast, a study performed in India revealed that sensitivity rate to imipenem was 33% [37]. In current study, multi resistant isolates to *E. coli* were divided into MDR, XDR and possible PDR according to criterion proposed by Magiorakos *et al.* (2012)[38].The results indicate that prevalence MDR (100%), XDR (63.1%).Moreover, studies worldwide, including those in Najaf and Egypt which indicated that MDR is the most common among*E. coli* was 83.1% and 93% [39],[40]. The MDR isolates are frequent correlated with hospital acquired infections and their extended isassociated with raised mortality, morbidity, and healthcare costs. Among other microorganisms, *Enterobacteriales* family indicated the biggest danger because of the presence of ESBLs [41].

In the present study the prevalence to ESBL genes among carbapenem-resistance *Escherichia coli* isolates, which reported that *bla*OXA was 40% and *bla*CTX-M was 30%, this results similar to study in Nepal carried out on *E.coli* indicated that *bla*CTX-M and *bla*OXA genes were detected in 15% and 37.4% of ESBL isolates, respectively [42]. In contrast another studies performed in Iraq that found ESBL genes among isolates of *Escherichia coli* and indicated high prevalence compared with the present study where reported the prevalence of *bla*CTX-M was 95% and *bla*OXA was 83.6% among isolates of *Escherichia coli* [39]. Besides, many studies in different sites around world indicated that *bla*CTX-M genes more incidence among *E.coli* in clinical isolates such as study done in USA, India, Korea, and Nigeria who reported the prevalence of *bla*CTX-M genes were16%, 72%,25%, and 50% respectively [43] [44] [45] [46].

In this study the occurrence of only two carbapenem-resistance *E.coli* isolates and the percentage of this genes were *bla*VIM 50% and 100% to both *bla*NDM and *bla*SIM respectively, this result similar to result of study conducted in India by Tewari *et al*., (2022)[47] revealed that occurrence of MBL genes as following *bla*VIM (n=4; 22%), *bla*SIM (n=4; 22%), and *bla*NDM (n=2; 11%) among *E.coli* isolates. In contrast, the other finding in Pakistan who reported the high occurrence of *bla*VIM (n= 30; 25%) and *bla*NDM (n=43; 36%) [34].

CONCLUSIONS

In this study revealed the high frequency to antimicrobial resistant pattern among *E.coli*isolates from patients with DFI, the results showed that 100% of isolates were MDR, andthe results also indicated that the highest resistance rate toward beta-lactam class and the generation of cephalosporin, while highest sensitivity of the *E. coli* has been to the colistinand imipenem, and also there is the presence of MBL genes (*bla*VIM, *bla*NDM, and *bla*SIM) and ESBL genes (*bla*OXA and *bla*CTX) among carbapenem resistant *E.coli* isolates.

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