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MOLECULAR AND PHENOTYPIC CHARACTERIZATION OF CARBAPENEM RESISTANCE AND EXTENDED SPECTRUM BETA-LACTAMASES AMONG URINARY ESCHERICHIA COLI ISOLATES

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ABSTRACT

Uropathogenic Escherichia coli isolates are responsible for around 90% of urinary tract infections (UTIs) and are becoming more-commonly associated with extended-spectrum beta-lactamases (ESBL) and carbapenemase production, leading to failure of antimicrobial treatment, with resultant increases in the rates of morbidity and mortality. The aims of this study were to determine the prevalence of ESBL and carbapenemase producing E. coli from UTI patients, to determine their antibiotic susceptibility, characterize genes responsible for ESBL and carbapenem resistance and to identify the co-associations between these two types of genes. One hundred clinical isolates of E. coli were collected from patients suffering from UTIs from King Abdallah University Teaching Hospital, Jordan. Antimicrobial susceptibility was performed using the Kirby Bauer disk diffusion method and phenotypic identification of ESBLs and carbapenemase production was confirmed by the double disk synergy test and the modified Hodge test, respectively. Conventional and multiplex PCR were conducted for detection of ESBL and carbapenem resistance genes, respectively. The highest rates of resistance were observed against ampicillin (85%), followed by sulfamethoxazole/trimethoprim (73%), ceftriaxone (58%), cefotaxime (57%), ciprofloxacin (53%), and ceftizoxime (50%). All isolates were sensitive to imipenem and meropenem. Furthermore, 96% of the isolates were sensitive to ertapenem, 84% to tigecycline, 79% to nitrofurantoin, 77% to doripenem, 51% to ceftazidime, 48% to aztreonam and levofloxacin each, and 41% to amoxicillin/clavulanic acid. The prevalence of ESBL and carbapenemase producers was 54% and 23%, respectively. Aztreonam, alone identified the most ESBL isolates, while ertapenem, alone identified the most carbapenemase isolates. Having a carbapenemase phenotype was associated with resistance to most antimicrobial agents used, but not imipenem and meropenem. The co-expression of ESBLs and carbapenemases was seen among20% of the isolates. The blaTEM gene was the most prevalent (69%), followed by blaCTX (55%), blaNDM (53%), blaVIM (41%), and blaSHV (1%). None of the isolates harbored blaKPC. A statistically significant association of resistance genes was observed between blaTEM and blaVIM, as 41% of E. coli isolates had both blaTEM and blaVIM. The ESBL phenotype was correlated with the presence of blaTEM and blaCTX genes. BlaCTX gene was significantly associated with resistance to almost all cephalosporins, levofloxacin, ciprofloxacin, and doripenem, and the presence of carbapenemase phenotype. Overall, a high prevalence of blaTEM type ESBL and co-presence of ESBLs and carbapenemase genes, was observed. The prompt detection of these multi-resistant organisms ultimately enhances patient prognosis and limits the further spread of these organisms in the hospitals and community settings.

Keywords: *carbapenem, extended spectrum beta-lactamases, antimicrobial resistance, KPC, NDM, VIM, SHV, TEM, CTX, PCR urinary escherichia coli, urinary tract infection*

1. INTRODUCTION

Urinary tract infection (UTI) is a general term which encompasses microbial colonization of the urine and infection of the urinary tract structures involving kidney, renal pelvis, ureters, bladder, and urethra, in addition to nearby structures such as the perinephric fascia, prostate, and epididymis [1]. UTIs are the most common of all infections caused by bacteria that are experienced in both nosocomial and community settings. The infections can affect any part of the urinary tract and are sometimes described by site of infection or identified as complicated or uncomplicated infections [1, 2]. Although UTIs occur in all age groups irrespective of gender, clinical studies reveal that the overall occurrence of UTI is more in females than in males and *Escherichia coli* was shown to be the most frequent recovered bacterial pathogen [3, 4]. *E. coli* is a leading cause of urinary tract infections and intra abdominal infections in which the extent of the disease can range from cystitis to life threatening sepsis [5]. It is one of the most commonly studied Gram-negative bacteria in microbiology. Although it is well known that *E. coli* inhabits the human bowel as part of normal microbiota, some strains are capable of causing a significant intestinal/diarrheal and extra intestinal infections [6, 7]. Three main strains of extraintestinal pathogenic *E. coli* (ExPEC) are of clinical concern, namely; neonatal meningitis-associated *E. coli* (NMEC), sepsis-associated *E. coli* (SEPEC), and uropathogenic *E. coli* (UPEC). These types have been associated with meningitis in infants, systemic infections, and urinary tract infections, respectively [6, 7].

Uropathogenic *Escherichia coli* (UPEC) represents the most common causative agent of UTIs in humans, resulting in more than 80% of urinary tract related infections. Strains of UPEC infect the urinary tract via a range of urovirulence mechanisms that contribute to colonization and invasion of the bacterium. These include specialized adhesins, fimbriae, biofilm formation, iron uptake systems, specific O, K, and H serotypes, synthesis of cytotoxins, and aversion of host responses[8-10]. UPEC cause urinary tract infection by binding firmly to the cells of urinary tract epithelium. This attachment prevents the bacteria from being washed away by the flow of urine [9]. UPEC can also adhere to the epithelial cells of the bladder, where they multiply, and initiate formation of biofilm-like

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microbial communities that may harbor other pathogenic organisms [9].

The introduction of antimicrobial therapy contributes immensely to the management of UTIs. However the major problem with current antibiotic therapies is the rapid development of antimicrobial resistance, including multidrug resistance in hospitals as well as in the community by pathogenic organisms [3, 11, 12]. According to the world health organization (WHO), these resistant microorganisms are able to resist antimicrobial drugs, leading to ineffective treatment and persistent infections [13]. Although the emergence of multi-drug resistance (MDR) is a natural phenomenon, the extensive rise in the number of immunocompromised individuals such as those with HIV or diabetes, organ transplant recipients, and severe burn patients, makes these individuals an easy target for hospital acquired infections, thereby contributing to further spread of MDR [13].

Since the discovery of penicillin, beta-lactam antibiotics were the most successful treatment regimen for human infectious bacterial diseases. Unfortunately, many beta-lactam-resistant bacteria produce a beta-lactamase enzyme that degrades and thus inactivates these life saving antibiotics leading to modern extended spectrum cephalosporin and even carbapenem resistance [14, 15]. A number of these enzymes has been described but the most clinically significant are the extended spectrum beta-lactamases (ESBLs), AmpC beta-lactamases, *Klebsiella pneumoniae* carbapenemases (KPC), and the Metallo beta-lactamases (MBLs) [16].

Currently, beta-lactamases are the leading cause of resistance to beta-lactam drugs. The ability of these enzymes to cause antimicrobial resistance is primarily due to its activity, site of production, volume produced, and the permeability of the producer strain [17]. Genes encoding for beta-lactamases are mostly present on either bacterial chromosomes or plasmids. A large number of Gram-negative bacteria acquired naturally-occurring chromosomally-mediated beta-lactamases, considered to have originated from penicillin binding proteins, due to their several common sequence characteristics [17, 18].

Carbapenemases represent the most diverse class of beta-lactamases. They are capable of efficiently hydrolyzing a wide range of beta-lactam antibiotics such as penicillins, cephalosporins, monobactams, and carbapenems. Carbapenemases are mostly found within the molecular class B (e.g., IMP and VIM), D (e.g., OXA-23 to -27) or A (e.g., IMI, KPC, NMC, and SME) [19, 20]. For many years these enzymes were regarded as species specific, chromosomally-encoded beta-lactamases, until many of the genes encoding these enzymes were detected on plasmids of some pathogenic bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*, thus allowing further spread of carbapenem resistance genes between bacterial species [19].

Several Gram-negative bacteria are able to produce other beta-lactam hydrolyzing enzymes known as ESBLs which contribute to resistance to many beta-lactam antimicrobial agents [21, 22]. The ESBLs are predominantly plasmid-mediated enzymes. They can hydrolyze and inactivate a broad spectrum of beta-lactam drugs, including third-generation cephalosporins, penicillins and aztreonam, but clavulanic acid and other beta-lactamase inhibitors can block their activities [21]. The ESBLs can be classified into three main types, designated as TEM, SHV, and CTX-M [5]. Several ESBLs of clinical relevance belong to the TEM and SHV families. The TEM ESBL was given this designation because it was initially described in *Escherichia coli* recovered from a patient called Temoniera while the SHV designation was due to the enzyme having a sulf-hydryl variable active site [5, 23]. All these classes are widespread among various members of *Enterobacteriaceae* [24, 25]. The coexistence of different ESBLs and carbapenemase resistance genes on mobile genetic elements (such as plasmids) in the same bacterial isolate are of major concern because this can lead to the widespread transfer of these elements between the same, as well as different bacterial species, and will result in failed antimicrobial therapy [26, 27].

This study aims to determine the prevalence and the potential co-association of carbapenem and ESBL resistance and their corresponding genes, among *E coli* isolated from urinary tract infections. This knowledge will greatly help reduce the rates of morbidity and mortality associated with UTIs caused by this organism.

2. MATERIALS AND METHODS

Bacterial Isolates

One hundred isolates of *E. coli* were collected from pure cultures from King Abdullah University Hospital (KAUH) microbiology laboratory. All pure cultures were from urine samples of patients suffering from UTIs. The isolates were subcultured on Muellar Hinton (MH) agar. The overnight colonies were suspended in LB broth supplemented with 16% (final concentration) glycerol and stored frozen at -80ºC.

Preparation of Bacterial Suspensions

Three to five well separated colonies having the same morphological characteristics were transferred using a sterile loop, from an MH agar pure culture plate, into a tube containing 5 mL of sterile normal saline. The colonies were resuspended by vortexing to create a 0.5 McFarland bacterial suspension for antimicrobial susceptibility testing.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed for the isolates using the Kirby-Bauer disk diffusion method according to the Clinical Laboratory Standard Institute (CLSI) guidelines. Briefly, a sterile cotton swab dipped into the bacterial suspension was used to inoculate the surface of each

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MH plate in three directions. The plates were covered and left for about 5 minutes to dry. The antimicrobial disks were placed using a sterile lancet and the plates were incubated inverted overnight at 37 °C. The zones of inhibition, if any, around each disk was measured using a transparent ruler and recorded.

The antimicrobial disks used were amoxicillin-clavulanic acid (30µg), ceftizoxime (30µg), ceftazidime (30µg), ceftriaxone (30µg), cefotaxime (30µg), aztreonam (30µg), ampicillin (10µg), sulfamethoxazole-trimethoprim (25µg), nitrofurantoin (300µg), levofloxacin (5µg), ciprofloxacin (5µg), tigecycline (15µg), imipenem (10µg), meropenem (10µg), ertapenem (10µg), doripenem (10µg).

Detection of ESBL Production by Double Disk Synergy Test (DDST)

The production of ESBL was detected by the double disk synergy test according to CLSI guidelines using a disk of amoxicillin/clavulanic acid along with ceftizoxime, ceftazidime, ceftriaxone, cefotaxime, aztreonam. An MH agar plate was inoculated with each isolate as described above. Next, an amoxicillin/clavulanic acid disk was placed in the centre of the plate, and ceftizoxime, ceftazidime, ceftriaxone, cefotaxime, and aztreonam disks were placed 25 mm (center to center) from the amoxicillin/clavulanic acid disk. After overnight incubation at 37 °C, any distortion or increase in the zone of inhibition (i.e., augmentation of inhibition) towards the amoxicillin/clavulanic acid disk was considered a positive result for the ESBL production [28].

Detection of Carbapenemase Production by Modified Hodge Test

Carbapenemase production was tested using the MHT according to CLSI guidelines. A 0.5 McFarland suspension of *E. coli* ATCC 25922 in 5 mL of sterile saline was prepared. A 1:10 dilution was prepared by adding 0.5 mL of the 0.5 McFarland suspension to 4.5 mL sterile saline, this was then used as inoculum for an MH agar plate. The plate was dried for 5 min and a disk of either imipenem (10 µg), ertapenem (10 µg), meropenem (10 µg), or doripenem (10 µg), was placed in the center of the agar plate. Two-to-four colonies of the test organism were selected and streaked in a straight line, from the edge of the disk, up to the edge of the plate. After overnight incubation at 36 °C, carbapenemase production was identified by observing a clover leaf-like indentation of *Escherichia coli* 25922 growing along the test organism growth streak within the disk diffusion zone, while negative results showed no growth [29,30].

Table 1. Antimicrobial agents used in this study

Antimicrobial Agent	Antimicrobial Class	Abbreviation	Potency	Interpretation criteria for inhibition zone diameter in mm (CLSI 2013)		
				R	I	S
Ceftizoxime	3rd generations cephalosporin	ZOX	30 µg	≤21	22-24	≥25
Ceftazidime		CAZ	30 µg	≤17	18-20	≥21
Ceftriaxone		CRO	30 µg	≤22	23-25	≥26
Cefotaxime		CTX	30 µg	≤22	23-25	≥26
Aztreonam	Monobactam	ATM	30 µg	≤17	18-20	≥21
Amoxicillin/Clavulanic acid	Penicillin/beta-lactamase inhibitor	AMC	30 µg	≤13	14-17	≥18
Ampicillin	Penicillin	AM	10 µg	≤13	14-16	≥17
Doripenem	carbapenem	DOR	10 µg	≤19	20-22	≥23
Imipenem		IPM	10 µg	≤19	20-22	≥23
Ertapenem		ETP	10 µg	≤18	19-21	≥22
Meropenem		MEM	10 µg	≤19	20-22	≥23
Levofloxacin	Fluoroquinolones	LEV	5 µg	≤13	14-16	≥17
Ciprofloxacin		CIP	5 µg	≤15	16-20	≥21
Nitrofurantoin	Nitrofuran	F	300 µg	≤14	15-16	≥17
Tigecycline	Glycylcycline	TGC	15 µg	≤14	15-18	≥19
Sulfamethoxazole/trimethoprim	Sulfonamides	SXT	25 µg	≤10	11-15	≥16

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DNA Extraction

Crude DNA was extracted from the pure cultures of *E. coli*. Briefly, a number of pure bacterial colonies were inoculated into 5 mL of LB broth and the tubes were incubated overnight at 37 °C for 16 hours. 1.5 mL of overnight culture was transferred to an Eppendorf tube and centrifuged at 13.000 x g for 10 minutes to pellet the cells. The bacterial pellet was then suspended in 300 µL sterile water and heated at 100°C for 10 min to ensure cell lysis. The leftover cells were removed by centrifugation at 13.000 x g for 10 minutes and the supernatant was transferred into new Eppendorf tube and used as template for PCR reactions. The extracted DNA was stored at -20 °C until used.

Molecular Characterization of Carbapenem Resistance Genes

Multiplex PCR assay was performed to detect carbapenem resistance genes (bla_{KPC}, bla_{NDM}, and bla_{VIM}). Amplification of these genes was done using three pairs of primers [31], as shown in Table 2. The 25µL PCR was contained 12.5 µL 2×PCR master mix Solution, 1 µL template bacteria DNA solution, 0.75 µL of both forward and reverse primers (10 pmoles/µL) for each of the targeted genes, and 7 µL nuclease free water. The following PCR conditions were used; 10 min at 94 °C; 36 cycles of amplification consisting of 30 s at 94 °C, 40 s at 52 °C , and 50 s at 72 °C; final extension at 72 °C for 5 min [31].

Table 2. Primers used for amplification of carbapenemase genes

Primer	Gene amplified	Primer sequence (5'-3')	PCR product size (bp)
KPC-Fm	bla_{KPC}	CGTCTAGTTCTGCTGTCTTG	798
KPC-Rm		CTTGTCATCCTTGTTAGGCG	
NDM-F	bla_{NDM}	GGTTTGGCGATCTGGTTTTC	621
NDM-R		CGGAATGGCTCATCACGATC	
VIM-F	bla_{VIM}	GATGGTGTTTGGTCGCATA	390
VIM-R		CGAATGCGCAGCACCAG	

Molecular Characterization of ESBL Genes

Conventional PCR was performed for detection of ESBL genes (bla_{CTX}, bla_{TEM}, and bla_{SHV}). Three pairs of primers (Table 3) [24], were used for amplification of these genes. The 25µL PCR contained 12.5 µL 2×PCR master mix solution, 3 µL template bacteria DNA solution, 0.83 µL of both forward and reverse primers (10 pmoles/µL) of each targeted gene, and 7.84 µL nuclease free water. The following PCR conditions were used; 5 min at 94 °C, followed by 35 cycles of amplification at 30 sec at 94 °C denaturation, 30 sec at (50 °C for bla_{CTX}, 52 °C for bla_{TEM}, and 56 °C for bla_{SHV}) for annealing, 30 sec (60 sec for bla_{SHV}) at 72 °C for extension, and a final elongation step of 5 min at 72 °C [24].

Table 3. Primers used for amplification of ESBL genes.

Primer	Gene amplified	Primer sequence (5'-3')	PCR product size (bp)
TEM-F	bla_{TEM}	ACATGGGGGATCATGTAACT	421
TEM-R		GACAGTTACAATGCTTACT	
SHV-F	bla_{SHV}	ATGCGTTATATTCGCCTGTG	859

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SHV-R		AGCGTTGCCAGTGCTCGATG	
CTX-MU1	*bla*$_{CTX}$	ATGTGCAGYACCAGTAARGT	593
CTX-MU2		TGGGTRAARTARGTSACCAGT	

Controls used were *Klebsiella pneumoniae* ATCC BAA-1706 (*bla*$_{KPC}$ and *bla*$_{NDM}$ negative control), *K. pneumoniae* ATCC BAA-1705 (*bla*$_{KPC}$ positive control), *K. pneumoniae* ATCC BAA-2146 (*bla*$_{NDM}$ positive control), *K. pneumoniae* ATCC 700603 (*bla*$_{SHV}$ positive control),and *E. coli* ATCC 35218 (*bla*$_{TEM}$ positive control) were used as control stains for PCR and antimicrobial susceptibility testing.

Gel Electrophoresis

Products of each PCR were separated on 2% agarose. A total of 5.0µLof PCR product was loaded per well of the gel. Electrophoresis was done at 145V for 45 min. DNA was visualized under a UV transilluminator provided with a gel documentation system using the Quantity One software (Biorad, USA). Fragment sizes of each PCR were determined by comparison with a 100 bp DNA ladder and with the positive controls.

Statistical Analysis

The IBM SPSS software version 21 was used to generate descriptive analysis of raw data, including generation of all frequency tables and cross tabulations. Pearson's Chi-square test was used to compare frequency data. A *P* value equal to or less than 0.05 was considered statistically significant.

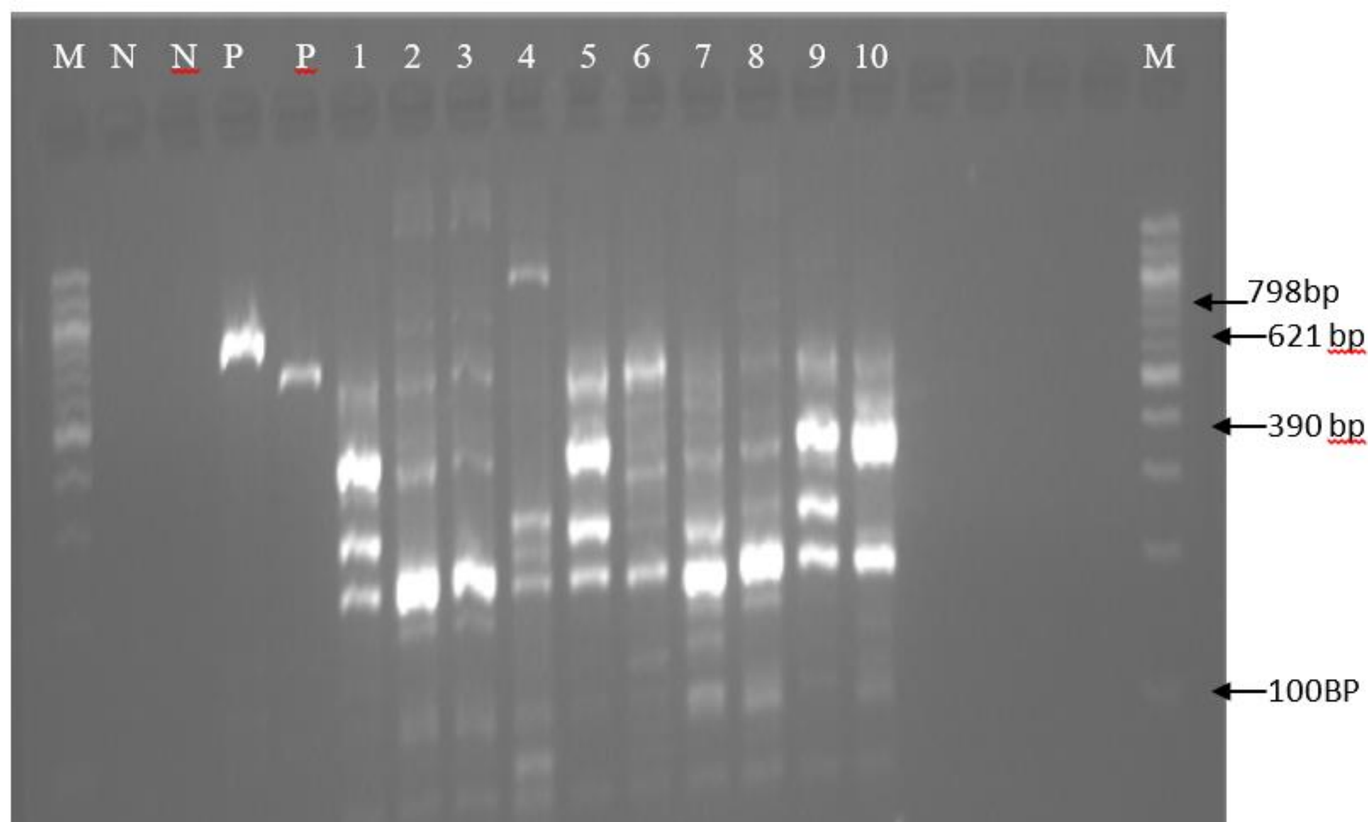

Figure 1. Representative gel for detection of *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{VIM}$ genes: Lane M: 100 bp ladder. First lane N: negative control without sample. Second lane N: negative control for *bla*$_{KPC}$ and *bla*$_{NDM}$. First lane P: positive control for *bla*$_{KPC}$. Second lane P: positive control for *bla*$_{NDM}$. Lanes 1, 5, 9, and 10: samples demonstrating *bla*$_{VIM}$. Lanes 5 and 6: samples demonstrating *bla*$_{NDM}$.

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3. RESULTS

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility profile of 100 *E. coli* isolates to various antimicrobial agents is shown in table 4**.** The highest rates of resistance were observed against ampicillin (85%), followed by sulfamethoxazole/trimethoprim (73%), ceftriaxone (58%), cefotaxime (57%), ciprofloxacin (53%), and ceftizoxime (50%). All isolates were sensitive to imipenem and meropenem. Furthermore, 96% of the isolates were sensitive to ertapenem, 84% to tigecycline, 79% to nitrofurantoin, 77% to doripenem, 51% to ceftazidime, 48% to aztreonam and levofloxacin each, and 41% to amoxicillin/clavulanic acid.

Using the double disk synergy test (DDST) and the modified Hodge test, to detect ESBL and carbapenemase producers, 54% and 23% of the isolates, respectively, demonstrated a positive result.

Multiple antimicrobial agents were used in the DDST to detect ESBL producers. Using the results of all agents, 54 total ESBL producers were identified. Aztreonam detected the highest number of ESBL producers (90.7%; 49/54), followed by ceftizoxime and ceftazidime (88.9% each; 48/54), cefotaxime (68%; 37/54), and ceftriaxone (64.8%; 35/54). There was a statistically significant association between presence of an ESBL producer and obtaining an augmentation pattern using the DDST for all antimicrobials used. Any isolate showing a resistant or intermediately susceptible result using the Kirby-Bauer DDT was considered nonsusceptible to the respective antimicrobial agent. Furthermore, there was a statistically significant association between the ESBL phenotype and obtaining a nonsusceptible phenotype against ampicillin (100%; 54/54), ciprofloxacin (75.9%; 43/54), levofloxacin (72.2%; 39/54), and amoxicillin/clavulanic acid (68.5%; 37/54).

By considering the modified Hodge test results for all four carbapenem drugs, 23 carbapenemase producing isolates were identified. Ertapenem identified the highest number of carbapenemase positive isolates (78.3%; 18/23), followed by doripenem (34.8%; 8/23), and imipenem (21.7%; 5/23). Meropenem only identified one isolate (4.3%). There was a statistically significant association between the carbapenemase phenotype and obtaining a nonsusceptible phenotype against ampicillin (100%; 23/23), ceftizoxime (95.7%; 22/23), ceftriaxone (95.7%; 22/23), cefotaxime (95.7%; 22/23), aztreonam (95.7%; 22/23), ceftazidime (87%; 20/23), (78.3%; 18/23), doripenem (47.8%%; 11/23), ertapenem (13%; 3/23).

Isolates having a carbapenemase producing phenotype were also more likely to have an ESBL phenotype. Notably, 87% (20/23) of the carbapenemase producing isolates were also ESBL producers.

Molecular Characterization of Beta-lactamase Resistance Genes

The most frequently detected gene was bla_{TEM} (69%; 69/100), followed by bla_{CTX} (55%; 55/100), bla_{NDM} (53%; 53/100), bla_{VIM} (41%; 41/100), and bla_{SHV} (1%; 1/100). The bla_{KPC} gene was not detected in any of the isolates.

Having bla_{TEM} and the bla_{CTX} genes was significantly associated with having an ESBL phenotype. Only the bla_{CTX} gene was significantly associated with nonsusceptibility to each of ceftizoxime, ceftazidime, ceftriaxone, cefotaxime, and aztreonam. Furthermore, having the bla_{CTX} gene was significantly associated with having a carbapenemase phenotype and nonsusceptibility to doripenem. Having the bla_{CTX} gene was significantly associated with nonsusceptibility to levofloxacin and ciprofloxacin. Having the bla_{VIM} gene was significantly associated with nonsusceptibility to nitrofurantoin and ciprofloxacin.

Statistically significant co-association of resistance genes was only observed between bla_{TEM} and $bla_{VIM.}$

4. DISCUSSION

Escherichia coli causes many infections and is frequently associated with urinary tract infections in both nosocomial and community settings [2,32]. Urinary tract infections are commonly caused by UPEC strains which accounts for around 90% of all UTIs [33]. Antimicrobial therapy remains the standard treatment for UTIs, and the most active of these agents being third-generation cephalosporins and carbapenem drugs [26]. However, the worldwide emergence of ESBL and carbapenemase producing *E. coli* poses a great challenge for the clinical therapy of infections caused by these organisms leading to increased morbidity and mortality [26,34]. The prompt detection of these multi-resistant organisms ultimately enhances patient prognosis and limits potential misuse of antimicrobial agents [26].

The present study was conducted on *Escherichia coli* isolated from UTI patients, for the phenotypic detection of carbapenem and ESBL resistance and the molecular characterization of the genes responsible for these resistance phenotypes. Furthermore, susceptibility of isolates to various antimicrobials was investigated.

In the present study ESBLs were identified in 54% of the isolatesby double disk synergy test (DDST). A strong statistically significant association was found between presence of an ESBL producer and obtaining an augmentation pattern using the DDST for all antimicrobials used. Various types of antimicrobials were used for the phenotypic detection of ESBL producers but aztreonam identified the highest number of these enzymes (90.7%; 49/54), while ceftriaxone the least (64.8%; 35/54). Therefore, we recommend that the screening for ESBL isolates should be done with multiple

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agents to enhance detection sensitivity, and that aztreonam, ceftazidime, and ceftizoxime, be included with these agents, as they detected the highest rates of ESBL isolates. Incidentally, the CLSI (2013) recommends using multiple agents, including aztreonam, ceftazidime, and ceftizoxime for ESBL screening, which is consistent with our findings and recommendation. The ESBL positive isolates had high resistance rates to ampicillin (100%), ciprofloxacin (75.9%), levofloxacin (72.2%), and amoxicillin/clavulanic acid (68.5%). Similar results have been reported for urinary *E. coli* isolates in Switzerland, London and India[35 - 37]. This resistance could be attributed to the fact that ESBL phenotype is mediated by genes on mobile genetic elements capable of carrying other resistance determinants and relative ease for *E. coli* to obtain multiple resistance genes, especially in light of the increased selective pressure due to exposure to a wide range of antimicrobials in the human body as well as in the environment.

Our study indicated the prevalence of a slightly higher rate of ESBL isolates (i.e., 54%) compared to that of a recent study from southern and central Jordan in 2014 which identified ESBLs at a rate of 35.8% (34/95) [71]. The differences in the rates of ESBL isolates are most likely due to differences in screening methodology and study populations. Consistent with the findings of our study, 55% of *E. coli* isolates from China were ESBL positive [101]. However, within the Middle East, low rates of ESBL producing *E coli* have been reported; 12% in Turkey, 11.7% in Kuwait, 16.8 % in Lebanon, and 33.3% in Iran [24, 72, 102, 103]. The higher prevalence of ESBL isolates in Jordan compared to the aforementioned countries is most likely due to the trend of self-medication, unregulated prescription of antimicrobial agents, and the extensive prophylactic misuse of antimicrobials by Jordanian patients and physicians.

While the CLSI (2013) only recommends using ertapenem and meropenem for carbapenemase screening, this study utilized four carbapenem drugs for the screening of carbapenemases using the modified Hodge test. Based on this, 23% of the isolates were identified as carbapenemase producers. Ertapenem identified the highest number of carbapenemase positive isolates (78.3%; 18/23), while meropenem detected only one isolate (4.3%)). There was a statistically significant association between having a carbapenemase positive isolate and resistance to most antimicrobial agents used including doripenem (47.8%; 11/23) and ertapenem (13%; 3/23), but not imipenem and meropenem. This is also likely explained by *E. coli*'s potential carriage of multiple resistance genes on mobile genetic elements.

The prevalence of carbapenemases has been well-described particularly in countries where the organisms harboring these enzymes are becoming endemic, such as in USA, Israel, among others [104]. In this study, the frequency of carbapenemase producers (23%) among the isolates was very high compared to several studies in India that reported incidence rate of 8-10% among the *Enterobacteriaceae* [105, 106].

Surprisingly, when correlating the carbapenemase producing phenotype with carbapenem susceptibility results, all isolates were 100% susceptible to imipenem and meropenem. This could be attributed to low carbapenemases production levels, or a false positive modified Hodge test likely due to low level carbapenem hydrolysis by ESBLs. In support of this phenomenon, false positive results by the modified Hodge test were previously reported by studies from Brazil, China, and Argentina [107-109].

In the present study, ESBL isolates were significantly associated with carbapenemase producers. Notably, 20% of *E. coli* isolates co-expressed ESBLs and carbapenemases. The co-existence of different ESBL enzymes in *E. coli* isolates has similarly been reported elsewhere, such as in Taiwan (40.6%) [68]. Infections with such highly resistant organisms may result in repeated hospitalization or even death. Thus, it is imperative to apply better screening protocols to detect these isolates and to devise an empirical therapy policy for patients at high risk of infection by such isolates.

Six gene-specific primers pairs were used for the molecular detection of ESBL and carbapenem resistance genes by PCR in this study. Different beta-lactamase genes were detected among the isolates; namely, bla_{TEM} (69%; 69/100), bla_{CTX} (55%; 55/100), bla_{NDM} (53%; 53/100), bla_{VIM} (41%; 41/100), and bla_{SHV} (1%; 1/100).None of the isolates harbored bla_{KPC}. The bla_{TEM} was the most prevalent beta-lactamase gene in this study. This may be due to its presence on highly mobile genetic elements that facilitates its spread among bacteria and that it is one of first genes to have developed to mediate resistance to ESBL drugs.

Many studies in different parts of the world have reported increasing incidence of bla_{CTX} genes [110-114]. In the present study, bla_{TEM} was the most prevalent (69%), followed by bla_{CTX} (55%). These prevalence rates probably demonstrate inherent differences in the distribution of resistance genes among Jordanian isolates compared to those from elsewhere. For example, in Canada, SHV-type beta-lactamases were the most prevalent among the ESBL producing *E. coli*[115].While, in China and Portugal, TEM-type beta-lactamases were the major type of beta-lactamases [116, 117]. Compared to bla_{CTX} of this study, the frequency of bla_{CTX-M} in *E. coli* was higher in Lebanon (96.5%), Taiwan (57.9%), India (88.6%), and Bangladesh (100%) [110, 118-120].

Various patterns of beta-lactamase resistance genes and distributions were identified among the isolates. Significant statistical associations between beta-lactamase resistance genes may reflect co-existence of the genes and, hence the possible co-carriage on mobile genetic elements. Statistically significant association of resistance genes was observed between bla_{TEM} and bla_{VIM}. Overall, 41 *E. coli* isolates (41%) had both bla_{TEM} and bla_{VIM}. Comparably, co-existence of bla_{NDM-1} and bla_{OXA-48}, and of bla_{NDM-1} and $bla_{OXA-232}$ was

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previously described in *E. coli* isolates from India and the USA, respectively [121, 122]. Furthermore, a study from Norway revealed the co-presence of bla_{NDM} with bla_{TEM-1}, bla_{SHV-11}, and $bla_{CTX-M-15}$[123]. The concurrence of ESBLs and carbapenemases is worrisome, considering that carbapenems are usually the last line of defense against many pathogens.

The ESBL phenotype was significantly correlated with the presence of bla_{TEM} and bla_{CTX} genes. According to previous studies, in most *E coli* isolates, these genes were plasmid-borne, and for that reason, they could quickly spread to various bacterial species [22]. Data analysis also showed a significant correlation between the presence of the bla_{CTX} gene and the resistance to almost all cephalosporins, levofloxacin, and ciprofloxacin. Furthermore, significant correlations were found between the presence of the bla_{CTX} gene and carbapenemase phenotype, as well as, resistance to doripenem. This indicates that resistance to beta-lactam antimicrobials was due to the presence of carbapenemase and ESBLs genes.

The significant finding of this study was the identification of urinary *E coli* isolates having co-presence of ESBLs and carbapenemase genotypes and phenotypes.

5. CONCLUSIONS

Using the DDST, 54% of the UPEC isolates were identified as ESBL producers. Aztreonam alone identified the most ESBL isolates. However, we recommend that screening for ESBL isolates should be done with multiple agents to enhance detection sensitivity; namely, aztreonam, ceftazidime, and ceftizoxime.

Using the modified Hodge test, 23% of the isolates were identified as carbapenemase producers. Ertapenem identified the most carbapenemase positive isolates, while meropenem detected the least. Having a carbapenemase phenotype was associated with resistance to most antimicrobial agents used, but not imipenem and meropenem.

ESBL isolates were significantly associated with carbapenemase producers. Notably, 20% of *E. coli* isolates co-expressed ESBLs and carbapenemases.

Using PCR, the bla_{TEM} was the most prevalent beta-lactamase gene (69%), followed by bla_{CTX} (55%), bla_{NDM} (53%), bla_{VIM} (41%), and bla_{SHV} (1%). None of the isolates harbored bla_{KPC}. A Statistically significant association of resistance genes was observed between bla_{TEM} and bla_{VIM}, as 41% of *E. coli* isolates had both bla_{TEM} and bla_{VIM}.

The ESBL phenotype was correlated with the presence of bla_{TEM} and bla_{CTX} genes. Bla_{CTX} gene was significantly associated with resistance to almost all cephalosporins, levofloxacin, ciprofloxacin, and doripenem, and the presence of carbapenemase phenotype.

Recommendations

Based on the findings of this study, we recommend the following:

i) The need for continuous monitoring of ESBL and carbapenemase producers, as high prevalence of antimicrobial resistance was observed especially the ESBL producing *E. coli.*

ii) Due to the issue of potential false positive results associated with modified Hodge test when screening for carbapenemase producers, we recommend the use of alternate methods that are more reliable such as the Carba NP which was reported to have 100% sensitivity and specificity.

iii) Future studies may be required to determine the exact subtypes of beta-lactamase genes that are prevalent in the Jordanian population.

iv) The implementation of health awareness programs for physicians and the general public to prevent the misuse of antimicrobial agents.

v) The application of tighter restrictions when antimicrobial agents are prescribed to patients.

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