Detection of ESBL and AmpC producing *Klebsiella pneumoniae* ST11 and ST147 from urinary tract infections in Iran

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RESEARCH ARTICLE

ABSTRACT

In the present study a total of 200 *Klebsiella pneumoniae* isolates were collected from patients with urinary tract infections (UTIs) in Tehran, Iran. Antibiotic resistance was determined by disk diffusion and broth dilution methods. Detection of extended-spectrum β-lactamases (ESBLs) and AmpCs was performed using phenotypic tests. Polymerase chain reaction (PCR) was applied to detect the ESBL, AmpC, and integron genes. Analysis of AmpC and cassette arrays of integron genes was performed using DNA sequencing. Plasmids were analyzed by PCR-based replicon typing and conjugation. Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were applied to explore the genomic relatedness among the isolates. The highest levels of resistance were observed against ampicillin (100%), followed by piperacillin (57.5%), ceftazidime (46%), trimethoprim/sulfamethoxazole (44%), ciprofloxacin (32.5%), and imipenem (19%). Approximately, 66.5% of isolates harbored at least one of the beta-lactamase genes (bla_{TEM}, bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$, and $bla_{\mathrm{OXA\text{-}1}}$). In addition, 22.5% of isolates carried at least one of the AmpC genes including bla_{DHA} and bla_{CIT}. Integron class I was the most prevalent integron among resistant isolates. According to the results of replicon typing, IncFII, IncL/M, and IncA/C were the most frequent replicons, respectively. All selected isolates were able to transfer $bla_{\mathrm{CTX\text{-}M}}$, also two isolates transferred the $bla_{\mathrm{DHA\text{-}1}}$ gene to *Escherichia coli* K12 through conjugation. Finally, 21 isolates were categorized into 4 pulsotypes and 11 unique clusters in PFGE. MLST identified ST147 and ST11 sequence types but ST147 was the most prevalent in the current study.

KEYWORDS

Klebsiella pneumoniae, integron, ESBLs, MLST, PFGE

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INTRODUCTION

Klebsiella pneumoniae is an opportunistic bacterium that is responsible for a variety of nosocomial infections including urinary tract infections (UTIs), as one of the most common bacterial infections among hospitalized patients [1, 2]. β-lactam and fluoroquinolone antibiotics play an important role in the treatment of *K. pneumoniae* infections [1].

However, the spread of various plasmid-mediated drug resistance determinants among *K. pneumoniae* leads to the development of UTIs resistant to these antibacterial therapies [3]. Among the most common causes of bacterial resistance to broad-spectrum beta-lactam antibiotics we find the production of extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamase [4–6]. Recently, the prevalence of ESBLs and AmpC enzymes in *K. pneumoniae* has been increasingly reported around the world [7–10]. AmpC enzymes are weakly inhibited by

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clavulanic acid and they confer resistance to beta-lactam antibiotics other than cefepime, cefpirome, or carbapenems [3]. AmpC-type cephalosporinases are classified as Ambler class C β-lactamases. Most of them are intrinsic and chromosomally encoded however, plasmid-encoded AmpCs are also transferred through mobile genetic elements, such as plasmids, transposons, and integrons [3]. Various types of plasmid-encoded AmpC enzymes such as CMY-1 (the first plasmid-mediated AmpC discovered in South Korea), CIT (CMY-2, being considered the most common variant), MOX, DHA, FOX, ACC, and EBC have been described [3, 11]. AmpC genes are surrounded by several genetic elements such as insertion sequences IS26 (CMY-13), ISEcp1 (CMY-2-type, ACC-1-type), and ISCR1, associated with complex integrons (DHA-1 type), and they are involved in the mobilization of acquired AmpC (ac-*Amp*C) β-lactamase genes [3].

AmpC β-lactamases are highly expressed and can be transferred between bacterial species through mechanisms such as transformation and conjugation, and these resistance determinants consequently lead to resistance to beta-lactams [12, 13]. These AmpC genes can be located on large plasmids around more than 30 kb in size, as main vehicles, containing additional antimicrobial resistance genes, such as the ESBLs and plasmid-mediated quinolone resistance (PMQR) genes [14, 15] and resulting in multidrug-resistant (MDR) bacteria which makes antibacterial therapy more challenging. MDR *K. pneumoniae* is associated with significant morbidity and mortality in the world [16]. Treatment options for infections caused by multi-resistant *K. pneumoniae* are strongly limited. Hence, evaluating the antimicrobial resistance patterns of *K. pneumoniae* isolates may have great outcomes to select appropriate antimicrobial treatments and decrease the rate of antibiotic resistance. Therefore, in this study we aimed to define the drug susceptibility patterns of *K. pneumoniae* isolated from patients with UTIs referred to two hospitals in Tehran, Iran, and to assess the prevalence of ESBL and AmpC as well as class I integron genes. Then, the plasmid replicon typing and conjugation were performed, and finally, the genetic diversity of the isolates was determined using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) techniques.

METHODS

Bacterial strains

From February 2018 through September 2018, a total of 200 *K. pneumoniae* isolates were collected from the urine samples of symptomatic UTIs with significant counts ($\geq 10^5$ CFU mL^{-1}) in Tehran, Iran. Exclusion criteria included antibiotic treatment during sampling or one month after hospitalization and mixed infection. Conventional phenotypic and biochemical tests were used for bacterial identification.

Antimicrobial susceptibility patterns

Antibiotic susceptibility of the *K. pneumoniae* isolates to 16 antibiotics was determined by the disc diffusion agar method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [17]. Commercially available antibiotic discs (Mast, Co., Merseyside, UK) were included: Ampicillin (10 μg), Piperacillin (100 μg), Ceftazidime (30 μg), Cefotaxime (30 μg), Aztreonam (30 μg), Cefepime (30 μg), Cefoxitin (30 μg), Piperacillin/tazobactam (100/10 μg), Gentamicin (10 μg), Amikacin (30 μg), Ciprofloxacin (30 μg), Levofloxacin (5 μg), Ertapenem (10 μg), Imipenem (10 μg), Trimethoprim/sulfamethoxazole (23.75/1.25 μg), and Nitrofurantoin (300 μg). *Escherichia coli* ATCC 25922 was used as a control strain. Isolates resistant to three or more different classes of antibiotics were defined as MDR. The minimum inhibition concentrations (MICs) of ceftazidime, cefotaxime, aztreonam and cefoxitin for ESBL-producing isolates were determined using the broth dilution method [17].

Phenotypic detection of ESBLs and AmpC β-lactamases

K. pneumoniae isolates that showed inhibition zones of ≤27 mm for cefotaxime and ≤22 mm for ceftazidime by disc diffusion method were screened as potential ESBL producers. Phenotypic detection of AmpC was applied for isolates with inhibition zone ≤20 mm regarding cefoxitin. β-lactamase production was further confirmed by the total ESBL/AmpC confirm kit (Rosco diagnostica, Denmark). Since the detection of ESBLs can be obscured by chromosomal AmpC producers, ESBL confirmational kit (Rosco Diagnostica, Denmark) was used to detect ESBLs in such isolates. The results were interpreted according to the CLSI guidelines [17]. *K. pneumoniae* ATCC 700603 and *Enterobacter* spp. KEJ-3 were used as positive controls in ESBL and AmpC phenotype tests, respectively. *E. coli* ATCC 25922 was used as a negative control for both tests.

Molecular detection of β-lactamase and integron genes

DNA templates were prepared by DNA extaction Mini Kit (Qiagen, Germany). All *K. pneumoniae* isolates were screened for the presence of β-lactamase genes (bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, and $bla_{\text{OXA-1}}$) and class I integron (*intI1* gene) genes using the Polymerase chain reaction (PCR). For the isolates carrying integron genes, PCR amplification was also performed on variable regions. Primers used for PCR amplification are listed in Table 1. Also, multiplex PCR assays were performed on all isolates for the detection of AmpC family (FOX, CIT, DHA, EBC, MOX, and ACC) as described previously [18]. All PCR products from AmpC genes and variable regions of class I integron were confirmed and analyzed by DNA sequencing.

PCR-based replicon typing

Plasmid replicon typing was performed for isolates harboring $bla_{\text{CTX-M}}$ and at least one of the AmpC genes, simultaneously [19]. DNA was extracted using a DNeasy extraction kit (Qiagen) and template DNA was used in five multiplexes and three simplex PCR assays to amplify the FIA, FIB, FIC, HI1, HI2, I1, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA replicons. *E. coli* strain ATCC 25922 was used as a negative control.

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Table 1. Primers used for PCR amplification

Target	Primer Sequences	Size, bp	Ref.
bla_{CTX-M}	F: TTTGCGATGTGCAGTACCAGTAA R: CGATATCGTTGGTGGCATA	593bp	[69]
bla_{SHV}	F: GGTTATGCGTTATATTCGCC R: TTAGCGTTGCCAGTGCTC	867bp	[70]
bla_{TEM}	F: ATGAGTATTCAACATTTCCG R: CCAATGCTTAATCAGTGAGC	931bp	[70]
bla_{OXA-1}	F: TTTTCTGTTGTTTGGGTTTT R: TTTCTTGGCTTTTATGCTTG	427bp	[71]
intI1	F: ACGAGCGCAAGGTTTCGGT R: GAAAGGTCTGGTCATACATG	565bp	[72]
Conserved segment (CS)	F: GGCATCCAAGCAGCAAG R:AAGCAGACTTGACCTGA	Variable	[73]

Resistance transfer determination

At the subsequent experimental stage, our study was focused on 10 randomly selected *K. pneumoniae* isolates that harbored both bla_{CTX-M} and AmpC genes. To determine the transferability of plasmids coding for multiple antibiotic resistance enzymes, conjugation experiments were carried out using the filter mating method [20]. Nalidixic acid-resistant *E. coli* K-12 was used as the recipient.

Transconjugants were selected on nutrient agar plates containing nalidixic acid (30 $\mu g\ ml^{-1}$) and cefotaxime (2 $\mu g\ ml^{-1}$). Antimicrobial susceptibility testing, PCR amplification, and plasmid replicon typing were performed for all transconjugants to determine antibiotic phenotypes, resistance determinants, and incompatibility groups, respectively.

Pulsed-field gel electrophoresis (PFGE) analysis

PFGE analysis was performed to determine the genetic relatedness of 21 selected *K. pneumoniae* isolates, based on simultaneous presence of bla_{CTX-M} and at least one of the AmpC genes. Briefly, genomic DNA of *K. pneumoniae* isolates and *Salmonella braenderup* H9812 (DNA marker) was prepared in agarose plugs. Digestion was performed using 20U *Xba*I restriction enzyme (Fermentas, Lithuania) and DNA fragments were separated in a CHEF-DR III system (Bio-Rad, Richmond, CA, USA) as described previously [21]. The system setup was as follows: 6V cm^{-1} for 19 h with pulse time ramped from 5 to 35 s. The generated PFGE patterns were analyzed using the Gel Compare II V5.10 software (Applied Maths, St Martens-Latem, Belgium). Cluster analysis of Dice similarity indices based on the unweighted pair group method with arithmetic means (UPGMA) was used to generate a dendrogram describing the relationships among the PFGE profiles. According to Tenover's criteria, isolates were considered to be in the same PFGE cluster if their Dice similarity index was more than 85% [22].

Multilocus sequence typing (MLST)

Isolates with ≥85% similarity in PFGE analysis were selected for MLST. Seven housekeeping genes (*rpoB, gap A, mdh, pgi, phoE, infB,* and *tonB*) were used for the typing by MLST. Alleles and sequence types (STs) were determined using the MLST database (http://www.pasteur.fr/mlst/Kpneumoniae.html).

Statistical analysis

The SPSS software version 19 for Windows (IBM, Chicago, USA) was used for statistical data analysis. The chi-square or Fisher's exact tests were used to compare variables of different groups and compare the relationships between the antibiotic resistance properties. P-value ≤ 0.05 was considered as statistically significant.

RESULTS

Bacterial strains

Of the total samples, 80% ($n = 160$) were isolated from females and the remaining 20% ($n = 40$) were isolated from males between 21 and 98 years of age, with a mean age of 44.5 years. In addition, 65% ($n = 130$) and 35% ($n = 70$) were isolated from inpatients and outpatients, respectively. The incidence of UTI in female patients with the age of 20–40 years (47.5%) was higher than in the elder group (2%). Among male patients, the highest and lowest prevalence of UTI was observed in the age of 61–80 years (40%) and 21–40 years (8%), respectively. All isolates were confirmed as *K. pneumoniae* by the standard biochemical tests.

Antimicrobial susceptibility patterns

Based on disk diffusion method, all isolates were resistant to ampicillin (100%), followed by piperacillin (57.5%), ceftazidime (46%), cefotaxime (44.2%), trimethoprim/sulfamethoxazole (44%), aztreonam (40.5%), cefepime (41%), ciprofloxacin (32.5%), levofloxacin (30%), cefoxitin (30%), gentamicin (28.5%), piperacillin/tazobactam (26%), nitrofurantoin (25%), imipenem (19%), amikacin (16%), and ertapenem (14%). There were 36 different antibiotic resistance patterns among the isolates and 8% ($n = 16$) were

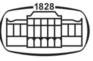

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Table 2. Distribution of antibiotic resistance according to inpatients and outpatients

Antibiotics	Inpatient, $n = 130$ No (%)	Outpatient, $n = 70$ No (%)	Total, $n = 200$ No (%)	P value
PRL	74 (56.9)	41 (58.6)	115 (57.5)	NS
CAZ	62 (47.6)	30 (42.6)	92 (46)	NS
CTX	62 (47.7)	26 (37.1)	88 (44)	NS
CPM	58 (44.6)	24 (34.3)	82 (41)	NS
ATM	58 (44.6)	23 (32.9)	81 (40.5)	NS
FOX	43 (33)	17 (24.3)	60 (30)	NS
PTZ	42 (32.3)	10 (14.3)	52 (26)	0.02
GEN	44 (33.8)	13 (18.6)	57 (28.5)	0.043
AN	23 (17.7)	9 (12.9)	32 (16)	NS
CIP	47 (36.2)	18 (25.7)	65 (32.5)	NS
LEV	45 (34.6)	15 (21.4)	60 (30)	NS
IMI	31 (23.8)	7 (10)	38 (19)	0.03
ETP	28 (21.5)	0 (0)	28 (14)	0.001
SXT	56 (43)	32 (45.7)	88 (44)	NS
NIT	40 (30.8)	10 (14.3)	50 (25)	0.02

Abbreviations: PRL, Piperacillin; CAZ, Ceftazidime; CTX, Cefotaxime; CPM, Cefepime; ATM, Aztreonam; FOX, Cefoxitin; PTZ, Piperacillin-tazobactam; GEN, Gentamicin; AN, Amikacin; CIP, Ciprofloxacin; LEV, Levofloxacin; IMI, Imipenem; ETP, Ertapenem; SXT, Trimethoprim/sulfamethoxazole; NIT, Nitrofurantoin.
Notes: NS, Non-significant.

resistant to all of the antibiotics (pan drug-resistant). The distribution of antimicrobial resistance in both genders was not statistically significant ($P > 0.05$). The antibiotic resistance rates among the elderly were higher than the other groups. Resistance to piperacillin-tazobactam, gentamicin, imipenem, and nitrofurantoin was significantly more prevalent in inpatients than outpatients ($P < 0.05$) (Table 2).

Out of 200 isolates, 49% ($n = 98$) were MDR strains with resistance to more than three classes of antibiotics. In addition, 67.3% and 32.7% MDR isolates were associated with inpatients and outpatients, respectively ($P > 0.05$).

The results of MIC indicated 44.5% ($n = 89$) of isolates were resistant to ceftazidime that 30 isolates had MIC higher 256 μg ml^{-1}. MIC for cefotaxime, aztreonam, and cefoxitin were 16- ≥64, 16- ≥128, and 16–64 μg ml^{-1}, respectively within the resistant isolates.

Phenotypic detection of ESBL and AmpC β-lactamases

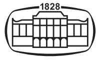
The results of the Combined Disc Diffusion Test (CDDT) showed that 40% ($n = 80$) of isolates were positive for ESBL production. In addition, all of the ESBLs producers were MDR isolates. The antibiotic resistance profiles among positive and negative ESBL isolates are detailed in Table 3. Resistance rates to piperacillin, ceftazidime, cefotaxime, cefepime and aztreonam were 100%, whereas resistance to gentamicin, amikacin, and ertapenem was only observed in ESBLs producing isolates. Most of the ESBLs producing isolates were collected from inpatients (57 isolates) ($P > 0.05$). The positive ESBL isolates were collected from different hospital wards include Intensive care unit (ICU): 22, Women: 8, Internal: 7, Infectious: 7, Urology: 5, Surgery: 3, Emergency: 3, and Endocrinology: 2 isolates.

The results indicated that 66.6% ($n = 40$) from 60 cefoxitin-resistant isolates in disk diffusion were able to produce AmpC β-lactamase by a phenotypic confirmatory test. The majority of the positive AmpC isolates were obtained from inpatients (30 isolates) ($P > 0.05$). Among the AmpC producing isolates, 31 isolates produced both ESBL and AmpC β-lactamase and 9 isolates carried AmpC alone. All of the positive AmpC isolates were MDR strains. The antibiotic resistance profile among positive and negative AmpC producing isolates is shown in Table 3. It was found

Table 3. Prevalence of antibiotic resistance among ESBL and AmpC producing isolates

Antibiotics	ESBL+ $n = 80$ No (%)	ESBL-n = 120 No (%)	P value	AmpC+ $n = 40$ No (%)	AmpC-n = 160 No (%)	P value
PRL	80 (100)	35 (29)	0.000	40 (100)	75 (47)	0.001
CAZ	80 (100)	12 (10)	0.000	40 (100)	52 (32.5)	0.000
CTX	80 (100)	8 (6.7)	0.000	36 (90)	52 (32.5)	0.000
ATM	80 (100)	1 (0.8)	0.000	34 (85)	47 (29.5)	0.003
CPM	80 (100)	2 (1.7)	0.000	33 (82.5)	49 (30.6)	0.000
FOX	45 (56.2)	15 (12.5)	0.000	40 (100)	20 (12.5)	0.000
PTZ	44 (54.2)	8 (6.7)	0.000	38 (95)	14 (8.8)	0.000
GEN	57 (70.8)	0 (0)	0.000	25 (62.5)	32 (20)	0.003
AN	32 (39.8)	0 (0)	0.000	19 (47.5)	13 (8.2)	0.005
CIP	60 (75)	5 (4.2)	0.000	30 (75)	35 (21.9)	0.000
LEV	55 (68.8)	5 (4.2)	0.000	30 (75)	30 (18.8)	0.000
IMI	33 (41.7)	5 (4.2)	0.000	32 (80)	6 (3.8)	0.000
ETP	28 (35.7)	0 (0)	0.000	22 (55)	6 (3.8)	0.000
SXT	66 (81.2)	22 (18.3)	0.000	35 (87.5)	53 (33.1)	0.005
NIT	35 (43.8)	15 (12.5)	0.000	28 (70)	22 (13.7)	0.002

Abbreviations: PRL, Piperacillin; CAZ, Ceftazidime; CTX, Cefotaxime; ATM, Aztreonam; CPM, Cefepime; FOX, Cefoxitin; PTZ, Piperacillin-tazobactam; GEN, Gentamicin; AN, Amikacin; CIP, Ciprofloxacin; LEV, Levofloxacin; IMI, Imipenem; ETP, Ertapenem; SXT, Trimethoprim/sulfamethoxazole; NIT, Nitrofurantoin.



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that the resistance to all tested antibiotics was significantly higher in positive AmpC isolates than negative AmpC isolates ($P < 0.01$).

Molecular detection of β-lactamase and integron genes

According to PCR results, the prevalence of ESBL genes including bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA-1} among the *K. pneumoniae* isolates was 49% ($n = 98$), 3.5% ($n = 7$), 42% ($n = 83$), and 37.5% ($n = 75$), respectively. Out of 200 isolates, 133 isolates harbored at least one of the beta-lactamase genes. In addition, ESBL combinations were observed include bla_{TEM}+bla_{CTX-M}+bla_{OXA-1} (27 isolates), bla_{TEM}+bla_{CTX-M} (7 isolates), bla_{CTX-M}+bla_{OXA-1} (7 isolates), bla_{TEM}+bla_{OXA-1} (5 isolates), bla_{TEM}+bla_{SHV} (2 isolate), bla_{SHV}+bla_{CTX-M} (2 isolate), and bla_{SHV}+bla_{CTX-M}+bla_{OXA-1} (2 isolate). Prevalence of the ESBLs genes among phenotypically ESBLs producing isolates was 64.6%, 4.2%, 100%, and 79.2% for bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA-1}, respectively. There was a direct correlation between the isolates collected from inpatients and the presence of bla_{CTX-M} ($P = 0.049$), whereas there was no significant relationship between the presence of bla_{TEM}, bla_{SHV}, or bla_{OXA-1} in outpatient and inpatient isolates.

Based on PCR results, 22.5% ($n = 45$) harbored at least one of the AmpC genes including bla_{DHA} and bla_{CIT}. Among the cefoxitin-resistant isolates ($n = 60$), 63.3% ($n = 38$) carried bla_{DHA} and 3.3% ($n = 2$) carried bla_{CIT} alone. In addition, 8.3% ($n = 5$) co-harbored bla_{DHA} and bla_{CIT}. Other AmpC genes were not detected in the isolates. There were no significant relations between the presence of the AmpC genes in outpatients and inpatients ($P > 0.05$).

DNA sequencing of AmpC genes showed 100% homology to the bla_{CMY-2} gene for the CIT family and 100% homology to the bla_{DHA-1} gene for the DHA family.

Out of total isolates, 45 isolates producing both ESBL and AmpC include TEM+CTX-M+OXA-1+DHA (15 isolates), OXA-1+CTX-M+DHA (12 isolates), TEM+DHA (6 isolates), TEM+CTX-M+CIT+DHA (3 isolates), TEM+OXA-1+DHA (3 isolates), TEM+CTX-M+DHA (2 isolates), OXA-1+CTX-M+CIT (2 isolates), and CTX-M+DHA+CIT (2 isolates). It was found that from all AmpC positive isolates, 80% ($n = 36$) produced CTX-M in addition to AmpC genes.

PCR results from integron genes showed that 43.5% ($n = 87$) carried class I integrons and all of them were MDR isolates. Moreover, the association between isolates carrying integrons and antibiotic resistance was significant for all of the antibiotics ($P < 0.05$). Class I integron was associated with all of the beta-lactamase genes, except SHV. In addition, there was a significant difference in the age group above 80 and the prevalence of *int*I1 gene.

Five amplicons were identified in class I integrons with different sizes, including 700 bp (32 isolates), 1,020 bp (17 isolates), 1,500 bp (19 isolates), 1,600 bp (10 isolates), and 1870 bp (13 isolates). Sequencing results showed eight different cassette arrays of class I integrons (Table 4). The *dfr* and *aad* cassettes conferring resistance to trimethoprim and aminoglycosides were detected in 85% and 60% of the isolates harboring class I integron, respectively. *DfrA5-dfrA30* gene cassette arrays were found as the most prevalent cassette (Table 4).

Table 4. Characterization of class I integrons and gene cassettes in the isolates

Integron type (No.)	CS (No = 77)	Size of cassettes (bp)	Inserted cassette (s)
I (10)	-	-	*No cassette*
II (10)	+	1,600	*dfrA17, aadA5*
III (3)	+	1,020	*aadA2*
VI (13)	+	1870	*dfrA12, orf, aadA2*
V (12)	+	1,500	*dfrA12, aadA2*
VI (7)	+	700, 1,020	*dfrA5, dfrA30, aadA2*
VII (25)	+	700	*dfrA5, dfrA30*
VIII (7)	+	1,020, 1,500	*aadA2, dfrA12, aadA2*

Notes: CS: Conserved segments.

Plasmid replicon typing

Out of 36 isolates carrying bla_{CTX-M} or bla_{CTX-M}+AmpC genes, IncFII plasmids were found as the most frequently carried replicons (22/36 isolates; 61%), followed by IncL/M (5/36; 13.9%), and IncA/C (3/36; 8.3%). In seven isolates, no replicon was found. The properties of the replicons and their associations with integron cassette arrays are shown in Table 5.

Resistance transfer determination

The conjugation experiment revealed four isolates were able to transfer the bla_{CTX-M} to *E. coli* K12. Two isolates could conjugatively transfer the bla_{DHA-1} gene to *E. coli* K12 by IncL/M. Among conjugative plasmids, IncL/M and IncA/C were carrying bla_{TEM} and bla_{OXA-1}. More details are shown in Table 6.

PFGE analysis

The PFGE analysis demonstrated the clonal diversity among the 21 selected *K. pneumoniae* isolates (Fig. 1). *K. pneumoniae* isolates were clustered into 15 PFGE clonal groups, according to the drawn dendrogram (K1-15). The PFGE results revealed that 10 isolates of the 21 *K. pneumoniae* tested were divided into four clusters, and 11 isolates were singletons (Fig. 1). Among the obtained clusters, C4 cluster with four isolates was the most common clone in the PFGE analysis (Fig. 1).

MLST analysis

To better understand the clonal relationship with higher 85% similarity in PFGE (10 isolates), MLST was carried out and analysis of the results revealed that the isolates were assigned to 2 different STs (ST11 and ST147) (Fig. 1). The main STs identified among the isolates were ST147 belonged to cluster K8, K10, K12 (8 isolates) and ST11 belonged to cluster K3 (2 isolates). The ST11 was only observed in one

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Table 5. The properties of the replicons and their associations with integron gene cassettes

Isolate No.	Beta-lactamases	AmpC	Inc group	*intI1* gene cassette
21	OXA-1	DHA-1	UT	*dfrA17, aadA5*
30	OXA-1	DHA-1	IncL/M	*dfrA12, orf, aadA2*
35	OXA-1	DHA-1	IncFII	*dfrA12, orf, aadA2*
42	OXA-1	DHA-1	IncFII	*dfrA5, dfrA30, aadA2*
58	OXA-1	DHA-1	IncFII	*dfrA12, orf, aadA2*
91	OXA-1	DHA-1	IncFII	*dfrA12, aadA2*
119	OXA-1	DHA-1	UT	*dfrA5, dfrA30, aadA2*
39	OXA-1	CMY-2	IncFII	*aadA2, dfrA12, aadA2*
51	TEM	DHA-1	IncFII	*dfrA5, dfrA30*
80	-	DHA-1, CMY-2	IncL/M	*aadA2, dfrA12, aadA2*
3	TEM, OXA-1	DHA-1	IncFII	*No cassette*
10	TEM, OXA-1	DHA-1	IncFII	*dfrA12, orf, aadA2*
31	TEM, OXA-1	DHA-1	IncA/C	*dfrA12, aadA2*
38	TEM, OXA-1	DHA-1	IncFII, IncL/M	*dfrA12, aadA2*
50	TEM, OXA-1	DHA-1	IncFII	*dfrA12, orf, aadA2*
57	TEM, OXA-1	DHA-1	IncFII	*dfrA12, aadA2*
75	TEM, OXA-1	DHA-1	IncFII	*dfrA12, orf, aadA2*
78	TEM, OXA-1	DHA-1	UT	*dfrA12, orf, aadA2*
110	TEM, OXA-1	DHA-1	UT	*dfrA12, aadA2*
84	TEM	DHA-1, CMY-2	IncA/C	*dfrA5, dfrA30*
112	TEM	DHA-1, CMY-2	IncFII	*dfrA5, dfrA30*
1	TEM	DHA-1, CMY-2	IncFII	*dfrA5, dfrA30*
4	TEM, OXA-1	DHA-1	UT	*dfrA12, aadA2*
7	TEM, OXA-1	DHA-1	IncFII	*dfrA12, aadA2*
13	-	DHA-1, CMY-2	IncL/M	*dfrA5, dfrA30*
17	TEM, OXA-1	DHA-1	IncFII	*dfrA12, aadA2*
25	TEM, OXA-1	DHA-1	UT	*dfrA12, orf, aadA2*
100	OXA-1	CMY-2	IncA/C	*aadA2, dfrA12, aadA2*
125	OXA-1	DHA-1	IncFII	*dfrA12, orf, aadA2*
138	TEM	DHA-1	IncFII	*dfrA5, dfrA30*
147	OXA-1	DHA-1	IncFII	*dfrA12, orf, aadA2*
155	TEM, OXA-1	DHA-1	IncFII	*dfrA5, dfrA30, aadA2*
176	OXA-1	DHA-1	UT	*dfrA17, aadA5*
182	OXA-1	DHA-1	IncL/M	*dfrA5, dfrA30*
190	TEM, OXA-1	DHA-1	IncFII	*No cassette*
197	OXA-1	DHA-1	IncFII	*dfrA5, dfrA30*

Notes: UT, Untypeable.

Table 6. Profile of antimicrobial resistance in the transconjugants and their parents

Isolate No.	Antimicrobial resistance phenotypes	Beta-lactamase genes	Inc groups
A	PRL, CAZ, CTX, ATM, CPM, FOX, PTZ, GEN, AN, CIP, LEV, IMI, ETP, SXT, NIT	bla_{CTX-M}, bla_{OXA-1}, bla_{DHA-1}	IncL/M
TA	PRL, CAZ, CTX, ATM, CPM, FOX, PTZ, ETP, SXT	bla_{CTX-M}, bla_{DHA-1}	IncL/M
B	PRL, CAZ, CTX, ATM, CPM, FOX, PTZ, GEN, AN, CIP, LEV, IMI, ETP, SXT, NIT	bla_{CTX-M}, bla_{OXA-1}, bla_{CMY-2}	IncFII
TB	PRL, CAZ, CTX, ATM, CPM, GEN, AN, IMI, ETP, SXT, NIT	bla_{CTX-M}, bla_{OXA-1}	IncFII
C	PRL, CAZ, CTX, ATM, CPM, FOX, PTZ, GEN, CIP, LEV, IMI, ETP, SXT, NIT	bla_{CTX-M}, bla_{TEM}, bla_{OXA-1}, bla_{DHA-1}	IncFII, IncL/M
TC	PRL, CAZ, CTX, ATM, CPM, FOX, PTZ, GEN, CIP, LEV, IMI, ETP, SXT	bla_{CTX-M}, bla_{TEM}, bla_{DHA-1}	IncL/M
D	PRL, CAZ, CTX, ATM, CPM, FOX, PTZ, CIP, LEV, IMI, SXT	bla_{CTX-M}, bla_{TEM}, bla_{DHA-1}, bla_{CMY-2}	IncA/C
TD	PRL, CAZ, CTX, ATM, CPM, FOX, PTZ, SXT	bla_{CTX-M}, bla_{TEM}, bla_{CMY-2}	IncA/C

Abbreviations: PRL, Piperacillin; CAZ, Ceftazidime; CTX, Cefotaxime; ATM, Aztreonam; CPM, Cefepime; FOX, Cefoxitin; PTZ, Piperacillin-tazobactam; GEN, Gentamicin; AN, Amikacin; CIP, Ciprofloxacin; LEV, Levofloxacin; IMI, Imipenem; ETP, Ertapenem; SXT, Trimethoprim/sulfamethoxazole; NIT, Nitrofurantoin.
Notes: T: Selected transcojugants. A, B, C, and D: Isolates that were able to perform conjugation.

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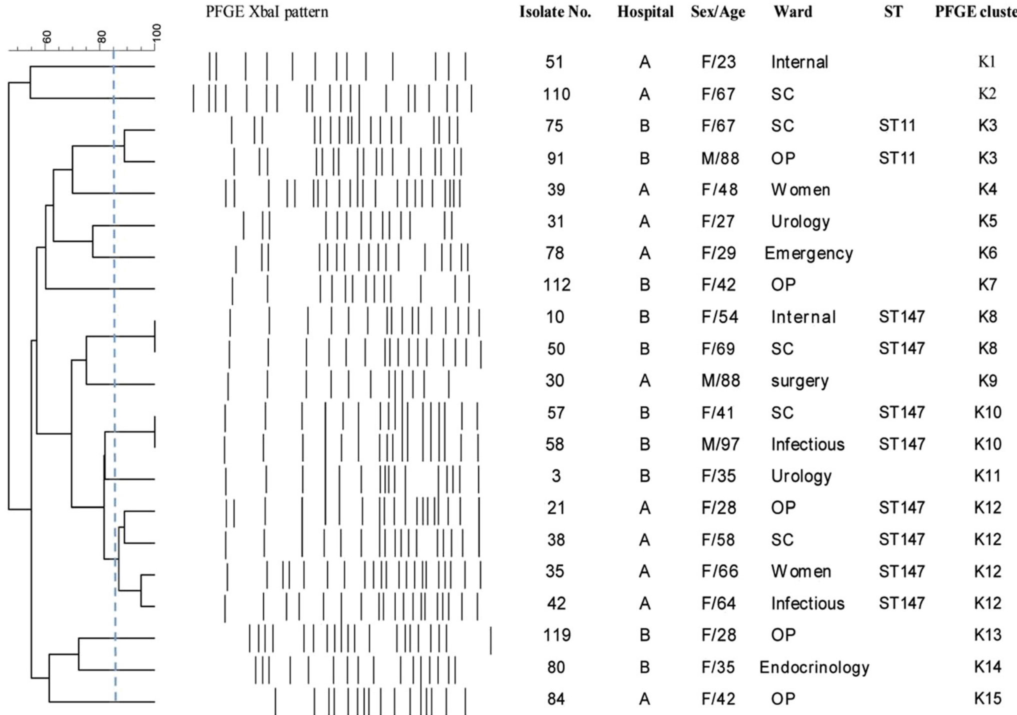
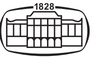


Fig. 1. Evaluation of genomic relatedness of the isolates using PFGE. Dendrogram was constructed based on UPGMA by using Dice coefficient with a 1.0% band position tolerance. The scale above the dendrogram shows percentage of similarity and the dotted line indicates 85% similarity. *Notes*: F, Female; M, Male; SC, Special care unit (ICU or CCU); OP, Outpatient; ST, Sequence types in MLST

hospital that was related to a hospitalized patient in ICU and one patient that was referred to the hospital (outpatient). ST147 was the most prevalent ST in the current study.

DISCUSSION

It is a global fact that drug-resistant pathogens causing nosocomial infections are associated with delays in proper diagnosis and treatment, thus posing serious challenges to effective treatment options [23, 24]. Increasing multidrug-resistant strains of *K. pneumoniae* as the second most etiologic agent of urinary tract infections has become a concern in the world [25, 26]. Therefore, a deeper understanding of the dissemination and frequency of bacterial resistance patterns in specific geographical locations will be useful to prescribe appropriate treatment for patients. The main goal of this study was to evaluate the antimicrobial resistance and current diversity of *K. pneumoniae* isolates in UTI samples in Tehran, Iran. A high prevalence of MDR *K. pneumoniae* isolates has been reported in other studies around the world [27, 28]. Based on current results, low resistance to ertapenem was observed following amikacin and imipenem. These results are in line with similar reports in Iran and other countries [29, 30]. Fortunately, resistance to carbapenems is still rare among *K. pneumoniae* isolates in Iran [31, 32]. On the other hand, all isolates were resistant to ampicillin and the high rate of ampicillin resistance can be related to the production of beta-lactamase enzymes which follows some reports from Iran and other countries [33, 34]. In our survey, the prevalence of ESBL-producing *K. pneumoniae* was 40% and most of the isolates were related to hospitalized patients especially in ICU. Our results were almost in correlation with Dotis et al. from Greek [35] but in contrast to Gürntke's reports from Germany [36]. However, higher rates for the prevalence of ESBL-producing *K pneumoniae* were detected in Iran. Feizabadi et al. demonstrated that the prevalence of ESBL among clinical *K. pneumoniae* isolates was 72.1%, which is much higher than our results [37]. It should be noted that the frequency of ESBL producing *K. pneumoniae* isolates varies based on the geographical area, different institutes, age of the population, and the patient's condition [38]. There are several reasons including prolonged treatment and regular use of urinary catheters during hospitalization that are related to the higher prevalence of ESBLs enzymes in hospitalized patients. In addition, co-resistance in ESBL producers isolates may be due to plasmids co-harboring different resistance genes carried by bacteria [16]. Our data showed a high prevalence of $bla_{\text{CTX-M}}$, $bla_{\text{OXA-1}}$, bla_{TEM}, and bla_{SHV} genes, respectively. Compared to other resistance genes, in this study, there was a direct relationship between the $bla_{\text{CTX-M}}$ gene and hospitalized patients. These data suggest that CTX-M-positive *K. pneumoniae* isolates probably originated from the hospital environment. There are numerous studies have reported $bla_{\text{CTX-M}}$ as the most prevalent ESBL in *K. pneumoniae* isolates [32, 39]. In this study, three isolates were positive for $bla_{\text{CTX-M}}$ gene by PCR but were negative for ESBL production by the phenotypic method which indicates molecular methods are more reliable than phenotypic methods for



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detecting ESBLs [40]. In addition, the significant association between the presence of $bla_{CTX\text{-}M}$ and AmpC genes in this study can be a concern, since it can lead to phenotypic misdiagnosis and limitation of treatment options [41].

The result of disk diffusion showed that among 36 cefoxitin-resistant isolates only 24 isolates were positive through the phenotypic AmpC test. This suggests that phenotypic detection of AmpC β-lactamases has poor specificity and is not advisable for routine detection of the β-lactamases. However, PCR results showed that 22.5% ($n = 45$) of isolates harbored at least one of the AmpC genes including bla_{DHA} and bla_{CIT}. Our results demonstrated the bla_{DHA} as the most prevalent AmpC gene. The results of similar studies conducted in Asia are consistent with the results of our study [42, 43]. In Europe, a study conducted by Mata et al. reported that *DHA-1* was the most frequent AmpC type in *K. pneumoniae* (62.5%), followed by *CMY-2* type [44]. However, our results are in contrast with two studies in Iran that reported *CIT* as the most prevalent AmpC gene [45, 46]. Other resistance mechanisms to cefoxitin such as reducing outer membrane permeability and producing carbapenemases may explain the cefoxitin-resistant isolates with negative AmpC confirmatory test [47].

In this study, AmpC and ESBL coproducing isolates showed a high frequency of co-resistance to cephalosporins, piperacillin-tazobactam, fluoroquinolones, and SXT. Co-resistance to the other antibiotic groups in both AmpC and ESBLs positive isolates has been reported by other studies that often lead to treatment failure [48, 49]. Although imipenem and ertapenem were known as the most effective agents against *K. pneumoniae* in the present study, a significant resistance pattern to these antibiotics was found among ESBL or AmpC producing isolates. The use of carbapenems in hospitalized patients that do not respond to the other types of antibiotics can be a reason for such a considerable resistance pattern.

Integrons as mobile elements are recognized to be the important source of transferable resistance genes and serve as reservoirs of antimicrobial resistance genes among microorganisms populations [50]. This study characterized class I integrons and their cassette arrays. Our results revealed that 87 (43.5%) of the isolates contained class I integrons and all of them were MDR strains. In accordance with our results, the association between the presence of class I integrons and the prevalence of MDR has been shown [51, 52]. Compared to non-ESBL strains, *K. pneumoniae*-producing ESBL has been demonstrated to have a higher rate of class I integrons [53]. Among isolates carrying integron 1 gene, 10 isolates harbored integrons without cassettes. This is probably due to the lack of a 3′ conserved section or strains carrying larger integrons that were not amplified by the PCR conditions utilized in this study [52].

In our study, *dfrA5* and *dfrA30* gene cassettes were found to be the most prevalent cassette arrays which encode dihydrofolate reductases enzymes. In a similar study, Firoozeh et al. reported *dfrA5, dfrA12,* and *dfrA30* gene cassettes as common cassettes from clinical isolates of *K. pneumoniae* in Kashan, Iran [54]. In other studies, the cassette arrays of class I integron in clinical isolates of *K. pneumoniae* were included *dfrA17*, *dfrA12, dfrA1*, *dfrA25*, and *dfrA27* genes in China [55, 56], and *dfrA17* and *dfrA12* among Gram-negative bacteria in the USA [57].

Based on plasmid replicon typing, three epidemic resistance plasmids including IncFII, IncL/M, and IncA/C were high prevalent, respectively. These results are in line with the current study by Aghamohammad et al. in Iran [58]. IncFII, IncA/C, IncL/M, IncN, and IncI1 plasmids have been identified in *Enterobacteriaceae* from different sources worldwide [59]. Many researchers have described the association between IncFII plasmid replicon and positive $bla_{CTX\text{-}M}$ enterobacterial isolates [60–62]. Previous reports have also characterized plasmids belonging to IncFII or IncL/M carrying $bla_{CTX\text{-}M}$ in *K. pneumoniae* strains [63, 64]. The widespread distribution of IncFII plasmids and class 1 integron-integrase gene in our study suggests that they may play an important role in increasing the prevalence of MDR *K. pneumoniae* isolates. On the other hand, the conjugative experiment revealed that plasmids carrying $bla_{DHA\text{-}1}$ and $bla_{CMY\text{-}2}$ belonged to IncL/M and IncA/C types. Association of IncA/C plasmids with bla_{CMY} gene has been previously reported in the USA, the United Kingdom, and Italy [65].

The PFGE and MLST analyses demonstrated the clonal diversity of our isolates that indicating the horizontal transfer of resistance genes between bacterial species through mobile elements such as plasmids that were in line with other reports [66, 67]. K8, K10, and K12 pulsotypes were associated with ST147 that carried the $bla_{OXA\text{-}1}$ and bla_{TEM} genes. ST147 has been accounted for several global nosocomial outbreaks [58] and was the most prevalent ST in our study. Solgi et al. also reported the presence of these lineages in infections caused by *K. pneumoniae* in Iran and suggest the sequence types are among the common STs in *K. pneumoniae* isolates in Iranian hospitals [68]. In the present study, all selected isolates were able to transfer $bla_{CTX\text{-}M}$, also two isolates transferred the $bla_{DHA\text{-}1}$ gene to *E. coli* K12 through conjugation. Finally, 21 isolates were categorized into 4 pulsotypes and 11 unique clusters in PFGE. MLST revealed ST147 and ST11 as identified sequence types and ST147 was the most prevalent ST in the current study. Therefore, *K. pneumoniae* can be considered as a major pathogen and important hazard for public health. Up-to-date treatment with combination therapy and/or new antimicrobial agents, for example, might be helpful against such drug-resistant organisms.

DECLARATIONS

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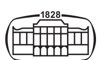
Conflicts of interest: The authors have no conflicts of interest to declare.

Ethical approval: The studies were performed according to the ethical safety guidelines of Pasteur Institute of Iran under



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Ethical Number: IR. PII.REC.1395.73. Written informed consent to collect the samples and perform the experiments was obtained from all patients.

Consent to participate: Written informed consent was obtained from the subjects included in this study. It guaranteed private handling of patient data for research purposes only.

Availability of data and material: Not applicable.

Code availability: Not applicable.

Author contributions: Shaghayegh Shahkolahi, Pegah Shakibnia, Shahla Shahbazi, Samira Sabzi and Farzad Badmasti: Methodology, Software, Formal analysis, Data Curation, Writing- original draft.

Mohammad Reza Asadi Karam: Conceptualization, Writing- original draft, Writing-review and editing, Investigation, Project administration, Resources, Validation, Supervision, Funding acquisition.

Mehri Habibi: Conceptualization, Methodology, Supervision, Writing-review and editing, Formal analysis, Investigation, Project administration, Validation, Funding acquisition.

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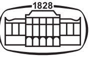

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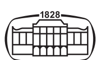

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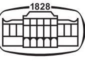

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