ANTIBIOTIC RESISTANCE, VIRULENCE AND GENETIC DIVERSITY OF *KLEBSIELLA PNEUMONIAE* IN COMMUNITY- AND HOSPITAL-ACQUIRED URINARY TRACT INFECTIONS IN IRAN

FATEMEH EGHBALPOOR¹, MEHRI HABIBI¹, OMID AZIZI², MOHAMMAD REZA ASADI KARAM^{1*} and SAEID BOUZARI¹

¹Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran ²Health Sciences Research Center, Torbat Heydariyeh University of Medical Sciences, Torbat Heydariyeh, Iran

(Received: 20 November 2018; accepted: 14 January 2019)

Klebsiella pneumoniae is among the most important causes of urinary tract infection (UTI). The aim of this study was to investigate the prevalence and correlation of antibiotic resistance with virulence characteristics and genetic diversity in K. pneumoniae isolated from UTIs in Iran. Phenotypic tests and antibiotic susceptibility were carried out on the isolates. Detection of the virulence and extendedspectrum β-lactamase (ESBL) genes was performed by polymerase chain reaction. Pulsed-field gel electrophoresis (PFGE) was used for exploring the genomic relatedness. Hemolysin, biofilm, and hypermucoviscosity formation were observed in 87.1%, 86.4%, and 12.1% of isolates, respectively. The antibiotic resistance rate of K. pneumoniae isolates ranged from 12.1% for meropenem to 100% for amoxicillin. The prevalence of virulence genes ranged from 1.4% for *cnf-1* to 100% for *mrkD*, fimH, kpn, and entB genes. In this study, 91.7%, 33.3%, and 4.2% of phenotypically ESBL-producers were positive for bla_{CTX-M} , bla_{TEM} , and bla_{SHV} genes, respectively. An association was observed between the presence of traT, fyuA, or cnf-1 genes with antibiotic resistance. Two clone types were obtained by PFGE that indicate different K. pneumoniae clones in community- and hospital-acquired UTIs. The findings of this study are valuable in development of treatment strategies against UTIs in Iran.

Keywords: urinary tract infections, *Klebsiella pneumoniae*, antibiotic resistance, ESBL, virulence factors, PFGE

^{*}Corresponding author; E-mail: m_asadi12@yahoo.com

Introduction

Urinary tract infections (UTIs) are among prevalent infections acquired both in community and in hospitals. It is estimated that 40% of women will have a UTI with a rate of 25% for recurrent UTIs [1]. Klebsiella pneumoniae is recognized as a common pathogen associated with both community- and hospital-acquired UTIs [2]. Its pathogenicity is related to expression of a variety of virulence factors including fimbriae, toxins, iron acquisition systems, lipopolysaccharide, and capsule [2]. Fimbriae type 1 and type 3 as mannose-sensitive (MSHA) and mannoseresistant hemagglutination pili (MRHA) play a major role in adherence and colonization of K. pneumoniae in the urinary tract [3]. FimH and MrkD composed the tip adhesin of types 1 and 3 pili in K. pneumoniae, respectively, and play relevant roles in biofilm formation [3]. Biofilms have known roles in pathogenesis of K. pneumoniae strains, including the resistance to immune responses and acquisition of antibiotic resistance [4]. Capsule is among the main virulence factors of K. pneumoniae isolates for protection against host immune system. K1 and K2 capsular serotypes containing the mucoviscosity-associated gene A (magA) were associated with severe UTI infections [5]. K. pneumoniae strains also have two potential toxins, a cytolytic pore forming toxin (α-hemolysin) and cytotoxicnecrotizing factor 1 (CNF1) that cause damage to tissues in the host [6].

Antibiotics are used routinely for therapy of UTIs caused by *K. pneumoniae* strains. To date, K. pneumoniae is known worldwide for rapid acquisition of resistance to different antibiotics, including resistance to β-lactams, fluoroquinolones, and carbapenems [7, 8]. Antibiotic resistance among the K. pneumoniae strains poses a great threat to public health and imposes a high cost to the UTI patients [9]. Resistance of these isolates to antibiotics is acquired by different mechanisms such as production of β-lactamase enzymes named as extendedspectrum β-lactamases (ESBLs) [10]. TEM, SHV, and CTX-M as the most common ESBL types confer resistance to all β-lactam antibiotics, except for carbapenems, and β-lactamase inhibitors. Furthermore, ESBL-producing isolates usually carry other resistance genes that confer resistance to other classes of antibiotics, such as aminoglycosides and quinolones [10, 11]. Unfortunately, many physicians prescribe that antibiotics before the results of antibiotic sensitivity can be evaluated by the laboratories. Thus, the choice of effective antibiotics against UTIs caused by K. pneumoniae should be based on determination the causative agent and knowledge of the resistance patterns of the isolate [12].

K. pneumoniae is recognized as one of the most important opportunistic pathogens in UTIs; however, there are only a few studies about pathogenicity and correlation between the virulence factors with antibiotic resistance in these isolates in Iran. Thus, the objectives of this study were to investigate the prevalence and

correlation of the virulence factors with antibiotic resistance in *K. pneumoniae* isolated from outpatients and inpatients in Tehran, Iran. Then, the clonal relatedness among the *K. pneumoniae* isolates was evaluated by pulsed-field gel electrophoresis (PFGE) technique.

Methods

Collection of K. pneumoniae isolates from UTI patients

From July to December 2017, 140 K. pneumoniae isolates were collected from the urine samples of outpatients (n = 80) and inpatients (n = 60) in several hospitals in Tehran, Iran. Patients were informed about the project and provided the informed consent. The exclusion criteria in this study were the presence of fungi, mixed cultures, and age less than 18 years. The urine samples were collected from the clean catch mid-stream urine of the patients. After collection of the urine with colony count $\geq 10^5$ CFU/ml, identification of K. pneumoniae isolates in these samples was performed on the basis of standard microbiological and biochemical tests.

Phenotypic determination of virulence factors

Hemagglutination assay. K. pneumoniae isolates were screened for type 1 (MSHA) and type 3 fimbriae (MRHA). Briefly, bacterial suspensions and human erythrocytes of blood group O (3%) were added to the 96-well microtiter plates in the presence and absence of D-mannose 2%. The plates were incubated at room temperature and reported as MRHA when agglutination was observed in the presence of mannose and MSHA when it was not occurred with mannose. The wells containing erythrocytes in the presence and absence of mannose were applied as negative controls and Escherichia coli ATCC 25922 as positive control for MRHA phenotype.

Hemolysin production. The phenotypic ability of the K. pneumoniae isolates in production of α -hemolysin was evaluated in culture media. The isolates were cultured in 5% sheep blood agar (Merck, Germany) and incubated overnight at 37 °C. Production of a clear zone of lysed erythrocytes around the colonies was considered as hemolysin producer isolate.

Biofilm formation testing. The ability of the *K. pneumoniae* isolates to produce biofilm was monitored by a microtiter plate test. Briefly, *K. pneumoniae* isolates were subcultured in lysogeny broth, diluted at 1:100 ratio, and incubated for 48 h. After several washes, 0.5% of crystal violet was added to plates and incubated for 15 min at room temperature. The plates were washed and a mixture of 33% acetic

acid and 80% ethanol was added to plates to dissolve the crystal violet and plates read at absorbance 590 nm with ELISA reader (BIO-TEK, USA). The *K. pneumoniae* isolates were classified as strong biofilm producers [$(2.5 \le \text{optical density (OD)}]$, moderate producers ($1.5 \le \text{OD} < 2.5$), weak producers ($0.7 \le \text{OD} \le 1.5$), and non-biofilm producers isolates ($0.7 \le \text{OD} \le 1.5$), and non-biofilm producers isolates ($0.7 \le \text{OD} \le 1.5$). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* strains were used as positive controls and *E. coli* DH5 α as a negative control.

Hypermucoviscosity (HMV) test. The string test was applied to evaluate the HMV phenotype of the isolates [13]. The K. pneumoniae isolates were cultured on 5% sheep blood agar plates and incubated for 18–24 h at 37 °C. When an inoculation loop was touched onto the surface of the colonies and slowly raised, the formation of a viscous string of at least 5 mm in length was considered as a HMV-positive phenotype.

Antibiotic susceptibility test. Antimicrobial susceptibility patterns of the isolates to different antibiotics were evaluated using the disk diffusion method using Mueller–Hinton agar (Merck). The tested antibiotics (Mast, Co., Merseyside, UK) were amoxicillin (10 µg), ticarcillin (75 µg), ampicillin–sulbactam (10 + 10 µg), cephalotin (30 µg), cephalexin (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), meropenem (10 µg), nalidixic acid (30 µg), norfloxacin (10 µg), gentamicin (10 µg), tobramycin (10 µg), and trimethoprim–sulfamethoxazole (SXT; 30 µg). The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) recommendations [14]. The *E. coli* ATCC 25922 was used as control strain. Isolates that were resistant to at least three different classes of antimicrobial agents were determined to be multidrug-resistant (MDR) phenotypes.

Screening of ESBL isolates. The K. pneumoniae isolates were screened for ESBL production by combined disk method according to CLSI guidelines [14]. The phenotypic confirmatory test was performed by ceftazidime (30 μ g) alone, combined with clavulanic acid (10 μ g) as well as cefotaxime (30 μ g) alone, and combined with clavulanic acid (10 μ g) disks. E. coli ATCC 25922 and K. pneumoniae ATCC 700603 were applied as negative and positive controls, respectively.

DNA extraction and polymerase chain reaction (PCR) detection of virulence and ESBL genes. PCR was applied to detect the virulence genes encoding adhesins (fimH and mrkD), fimH-like adhesin (Kpn), entrobactin biosynthesis (entB), yersiniabactin iron receptor (fyuA), serum resistance-associated outer membrane lipoprotein (traT), magA, α -hemolysin (hlyA), and cnf-1. Furthermore, the presence of bla_{TEM}, bla_{SHV}, and bla_{CTX-M} in the ESBL-producing isolates was confirmed by PCR. All specific primers (Genfanavaran, Iran) are listed in Table I. PCR reactions contained 1× buffer, 0.1 mM dNTPs, 2.5 mM MgCl₂,

Table I. Primers used to amplify virulence and ESBL genes in K. pneumoniae isolates

Target	Primer sequence $(5'-3')$	Annealing temperature (°C)	Size (bp)
fimH-1	F: ATGAACGCCTGGTCCTTTGC	55	688
	R: GCTGAACGCCTATCCCCTGC		
mrkD	F: CCACCAACTATTCCCTCGAA	52	240
	R: ATGGAACCCACATCGACATT		
Kpn	F: GTATGACTCGGGGAAGATTA	55	626
	R: CAGAAGCAGCCACCACACG		
entB	F: ATTTCCTCAACTTCTGGGGC	57	371
	R: AGCATCGGTGGCGGTGGTCA		
fyuA	F: GCGACGGGAAGCGATGATTTA	55	547
	R: TAAATGCCAGGTCAGTCACT		
tratT	F: GGTGTGGTGCGATGAGCACAG	63	290
	R: CACGGTTCAGCCATCCCTGAG		
magA	F: GTGCTCTTTACATCATTGC	59	1,282
	R: GCAATGGCCATTTGCGTTAG		
hlyA	F: AACAAGGATAAGCACTGTTCTGGCT	62	1,177
	R: ACCATATAAGCGGTCATTCCCGTCA		
cnf1	F: AGATGGAGTTTCCTATGCAGGAG	56	498
	R: CATTCAGAGTCCTGCCCTCATTATT		
bla_{TEM}	F: ATGAGTATTCAACATTTCCG	53	931
	R: CCAATGCTTAATCAGTGAGC		
bla_{SHV}	F: CTTTACTCGCTTTATCG	53	867
	R: TCCCGCAGATAAATCAC		
$bla_{\mathrm{CTX-M}}$	F: TCTTCCAGAATAAGGAATCCC	51	593
	R: CCGTTTCCGCTATTACAAAC		

Note: ESBL: extended-spectrum β-lactamase; F: forward; R: reverse.

50 pmol of each primer, and 0.5 U Taq polymerase (CinnaGen, Iran) in a total volume of 25 μ l. The used programs were an initial denaturation (94 °C for 5 min), followed by 30 cycles at denaturation (94 °C for 1 min), annealing (specific temperature for 1 min; Table I) and extension (72 °C for 1 min), and a final step (72 °C for 5 min).

PFGE analysis. PFGE was performed on 45 selected isolates to determine genomic diversity of the isolates. Briefly, these isolates were cultured on trypticase soy agar and then prepared bacterial plugs lysed with lysis buffer. The plugs were digested with restriction enzyme XbaI (Fermentas, Lithuania) for about 150 min at 37 °C. Then, electrophoresis of the plugs was performed in agarose 1% (Ultrapure, Invitrogen, USA) with CHEF-DR III system (Bio-Rad, USA). The stained gels with ethidium bromide were observed with Gel Doc. The obtained patterns were analyzed using GelCompare II, version 6.5 software (Applied Maths, St Martens-Latem, Belgium). Dendrogram drawing and clonal associations between the genotypes were performed with average linkage method and analyzed with unweighted pair group method analysis [15].

Statistical analysis

Statistical data analysis was performed using SPSS software version 19.0 for Windows (IBM, Chicago, USA). Among the statistical tests, χ^2 , two-tailed Fisher's exact, and analysis of variance tests were used to assess and compare the relationships between various virulence factors in *K. pneumoniae*. Moreover, pair gene relationships were assessed using Phi coefficients. The *p* value <0.05 was considered for all statistical accounts.

Results

Prevalence of UTI in relationship with profile of patients

Among the UTI patients included in this study, the rate of UTI in females (78.6%) was higher than males (21.4%). The age range of patients was 18–85 years. The frequency of *K. pneumoniae* in different age groups was 18–40 (44.3%), 41–60 (37.8%), and 61–85 (17.9%) years. Distribution of the inpatient isolates according to the hospital settings was urology (31.6%), intensive care unit (ICU; 25%), gynecology (18.4%), coronary care unit (CCU; 15%), and surgery (10%).

Phenotypic characteristics of K. pneumoniae isolates

Phenotypic virulence characteristics. It was found that 87.1% of the isolates had the ability to produce hemolysin. The hemolysin activity was found in 93.3% and 82.5% of isolates collected from inpatients and outpatients, respectively. Furthermore, the distribution of hemolysin-positive isolates in different hospital settings was ICU (100%), CCU (100%), surgery (100%), urology (89.5%), and gynecology (81.8%). Furthermore, 22.2% and 77.8% of the non-hemolysin producer isolates were recovered from inpatients and outpatients, respectively.

According to our results, 86.4% of *K. pneumoniae* produced biofilm that were classified into strong (24%), moderate (54.5%), and weak biofilm producers (21.5%). In addition, 91.7% and 82.5% of biofilm producer isolates were collected from inpatients and outpatients, respectively. All isolates that were collected from ICU, CCU, and gynecology wards had the ability to form strong to moderate biofilm formation. The isolates that had no ability in biofilm production were collected from age group 18-40 (79%), 41-60 (10.5%), and 61-85 (10.5%) years. It was found that among 122 hemolysis-positive isolates, altogether 110 (90.2%) had the ability for biofilm formation (p = 0.004).

The HMV phenotype was found in 17 (12.1%) of isolates in which 15 (88.2%) and 2 (11.8%) of them were isolated from inpatients and outpatients, respectively (p = 0.001). Based on our investigations, all K. pneumoniae isolates showed the presence of type 1 (MSHA) and type 3 fimbriae (MRHA).

Antibiotic susceptibility of K. pneumoniae isolates. According to the disk diffusion results, the susceptibility of K. pneumoniae isolates to antibiotics was meropenem (87.9%), norfloxacin (77.1%), gentamicin (75.7%), tobramycin (75.7%), nalidixic acid (75%), ampicillin–sulbactam (66.4%), ceftazidime (65%), cefotaxime (62.1%), SXT (61.4%), cephalotin (60.7%), cephalexin (60.7%), and ticarcillin (12.9%). Furthermore, all isolates were resistant to amoxicillin. As shown in Table II, 53 of the isolates with co-resistance to amoxicillin and ticarcillin were the most frequent resistance pattern (R1), whereas 12 isolates showed single patterns (data not shown).

The distribution of antibiotic resistance according to the outpatients and inpatients is also shown in Table III. Among the antibiotics tested, there was a significant difference between the resistance to all antibiotics in inpatients and outpatients groups (p < 0.05; Table III).

According to the results, 33 (23.6%) of K. pneumoniae isolates were classified as MDR isolates. Out of 33 MDR isolates, 14 (42.4%) were completely resistant to all antibiotics investigated. MDR in females was higher than males (26 vs. 7), but these differences were not statistically significant (p > 0.05).

Pattern name	Resistance patterns	No. of isolates
R1	AMX, TC	52
R2	AMX	18
R3	AMX, TC, CL, KF, CTX, CAZ, SAM, SXT, NOR, MEM, GEN, TB, NAL	14
R4	AMX, TC, SXT	10
R5	AMX, TC, CL, KF, CTX, CAZ, SAM, SXT	6
R6	AMX, TC, CL, KF, CTX, CAZ, SXT	6
R7	AMX, TC, CL, KF, CTX, CAZ, SAM, SXT, NOR, GEN, TB, NAL	6
R8	AMX, TC, CL, KF, CTX, CAZ, SXT, NOR, GEN, TB, NAL	3
R9	AMX, TC, CL, KF, CTX, CAZ, SAM, GEN, TB	3
R10	AMX, TC, SAM, SXT, GEN, TB, NAL	2
R11	AMX, TC, CL, KF, CTX, CAZ, SAM, NOR, NAL	2
R12	AMX, TC, CL, KF, CTX, CAZ, SAM, SXT, NOR, NAL	2
R13	AMX, TC, SAM	2
R14	AMX, TC, CTX, CAZ, CL, KF, SAM, NOR, MEM, GEN, TB, NAL	2

Table II. Antibiotic resistance patterns of *K. pneumoniae* isolates

Note: AMX: amoxicillin; TC: ticarcillin; CRO: ceftriaxone; CAZ: ceftazidime; CL: cephalexin; KF: cephalotin; SAM: ampicillin–sulbactam; SXT: trimethoprim–sulfamethoxazole; NOR: norfloxacin; GEN: gentamicin; TB: tobramycin; NAL: nalidixic acid; MEM: meropenem.

		` /	of antibiotic	Total no. (%) of	
Antibiotic list		Inpatients $(n = 60)$	Outpatients $(n = 80)$	positive isolates $(n = 140)$	p value
β-lactams	Ticarcillin	57 (95.0%)	65 (81.2%)	122	0.020
	Cephalotin	37 (61.7%)	18 (22.5%)	55	0.000
	Cephalexin	37 (61.7%)	18 (22.5%)	55	0.000
	Ceftazidime	36 (60.0%)	15 (18.8%)	51	0.000
	Cefotaxime	38 (63.3%)	15 (18.8%)	53	0.000
β-lactam/ β-lactamase inhibitors	Ampicillin– sulbactam	38 (63.3%)	9 (11.2%)	47	0.000
Carbapenem	Meropenem	15 (25.0%)	2 (2.5%)	17	0.000
Quinolones	Nalidixic acid	31 (51.7%)	4 (5.0%)	35	0.000
	Norfloxacin	28 (46.7%)	4 (5.0%)	32	0.000
Aminoglycosides	Tobramycin	28 (46.7%)	6 (7.5%)	34	0.000
	Gentamicin	28 (46.7%)	6 (7.5%)	34	0.000
Sulfonamides	Trimethoprim— sulfamethoxazole	31 (51.7%)	22 (27.5%)	53	0.005

Table III. Distribution of antibiotic resistance according to the outpatients and inpatients

Furthermore, 51.5% of MDR isolates were in the age groups of 41–60, 30.3% in 18–40, and 18.2% in 61–85 years. Twenty-nine isolates of MDR isolates were collected from inpatients and four isolates from outpatients. In addition, the distribution of MDR isolated from inpatients in different hospital wards was ICU (31%), urology (24.2%), gynecology (20.7%), CCU (13.8%), and surgery (10.3%).

The ESBL phenotypic screening by combined disk test showed that among 140 isolates, 48 isolates (34.3%) were ESBL producers. Among the ESBL producers, 35 (72.9%) belonged to inpatients and 13 (27.1%) to outpatients (p = 0.0001). Table IV demonstrates the antimicrobial resistance pattern of ESBL-producing isolates for applied antibiotics. All ESBL-positive isolates exhibited resistance to ticarcillin, cephalotin, cephalexin, and cefotaxime. Furthermore, the ESBL-positive isolates exhibited the lowest resistance to meropenem (33.3%; Table IV).

Distribution of virulence genes among K. pneumoniae isolates. According to our results, fimH and mrkD adhesins, siderophore receptor gene entB, and fimH-like adhesin (kpn) were detected in 100% of the isolates. The prevalence rate of traT, iron receptor gene fyuA, and magA capsular gene was 60%, 37.9%, and 3.6%, respectively. Toxins α -hemolysin (hlyA) and cnf-1 were present in 7.1% and 1.4% of isolates, respectively. The prevalence and distribution of different virulence genes according to the inpatients and outpatients is shown in Table V. It was found that isolates from inpatients had more virulence genes as compared to the isolates

Table IV. Prevalence of antibiotic resistance and virulence genes among ESBL-positive and -negative isolates

		% (n)		
Antibiotic list	ESBL+ $(n = 48)$	ESBL- (n = 92)	Total $(N = 140)$	p value
Ticarcillin	48 (100)	73 (80.4)	122 (87.1)	0.000
Cephalotin	48 (100)	7 (7.6)	55 (39.3)	0.000
Cephalexin	48 (100)	7 (7.6)	55 (39.3)	0.000
Cefotaxime	48 (100)	5 (5.4)	53 (37.9)	0.000
Ceftazidime	47 (97.9)	2 (2.2)	49 (35.0)	0.000
SXT	38 (79.2)	16 (17.6)	54 (38.6)	0.000
SAM	38 (79.2)	9 (9.8)	47 (33.6)	0.000
Meropenem	16 (33.3)	1 (1.1)	17 (12.1)	0.000
Tobramycin	31 (64.6)	3 (3.3)	34 (24.3)	0.000
Gentamicin	31 (64.6)	3 (3.3)	34 (24.3)	0.000
Nalidixic acid	31 (64.6)	4 (4.3)	35 (25.0)	0.000
Norfloxacin	30 (62.5)	2 (2.2)	32 (22.9)	0.000
Virulence genes				
traT	32 (66.7)	52 (56.5)	84 (60.0)	0.279
fyuA	25 (52.1)	28 (30.4)	53 (37.9)	0.010
magA	2 (4.2)	3 (3.3)	5 (3.6)	1.000
hlyA	7 (14.6)	3 (3.3)	10 (7.1)	0.032
cnf-1	2 (4.2)	0 (0)	2 (1.4)	_

from outpatients and this difference was significant about cnf-1, magA, and traT genes (p < 0.05; Table V). Furthermore, it was observed that magA gene was statistically more frequent among HMV-producing isolates compared with non-HMV producer isolates (p = 0.001).

Correlation between virulence characteristics and antimicrobial resistance. The correlation of virulence characteristics with ESBL production and antibiotic resistance is shown in Tables IV and VI, respectively. The correlation between biofilm

Table V. Distribution of virulence genes according to the inpatients and outpatients

			tive isolates in UTI tients			
Virulence genes		Inpatients $(n = 60)$	Outpatients $(n = 80)$	No. (%) of positive isolates	p value	
Toxin genes	hlyA	6 (10%)	4 (5%)	10 (7.1%)	0.326	
_	cnf1	2 (3.3%)	0 (0)	2 (1.4%)	_	
Protectin/invasion	magA	5 (8.3%)	0 (0)	5 (3.6%)	_	
genes	traT	46 (76.7%)	38 (47.5%)	84 (60.0%)	0.001	
Iron gene	fyuA	26 (43.3%)	27 (33.8%)	53 (37.9%)	0.292	

Note: UTI: urinary tract infection.

Table VI. Correlation of virulence characteristics with antibiotic resistance in the isolates

					7	Antibiotic resistance (number)	istance (nun	lber)				
Virulence factors	TC	KF	CT	CTX	CAZ	MEM	SAM	NAL	NOR	SXT	GEN	TB
Hemolysis	106	48	48	48	45	17	42	33	30	44	52	31
Biofilm	106	51	51	*05	*47*	17	42	32	31	47	59	59
HMV	15	13*	13*	13*	13*	5*	11**	17*	10*	12*	*	*∞
hlyA	6	*	*8	*	*	0	5	_	7	7	4	4
cnf-I	7	7	2	2	7	0	7	7	7	2	7	7
magA	3	2	7	2	2	-	2	2	2	7	2	7
traT	73	38	38	64	32	13	36*	5 0*	23	32	79*	26*
fyuA	47	78*	28*	28*	25*	4	20	13	13	21	13	13

Note: TC: ticarcillin; CTX: cefotaxime; CAZ: ceftazidime; CL: cephalexin; KF: cephalotin; SAM: ampicillin-sulbactam; SXT: trimethoprim-sulfamethoxazole; NOR: norfloxacin; GEN: gentamicin; TB: tobramycin; NAL: nalidixic acid; MEM: meropenem; HMV: hypermucoviscosity. $^*p < 0.05$ (significant correlation between the virulence factor and antibiotic resistance).

production and antimicrobial resistance was found to be statistically significant for cefotaxime and ceftazidime antibiotics (p < 0.05). In addition, isolates harboring HMV were more resistant to all of the antibiotics, except ticarcillin (p < 0.05). Our results showed that there was no significant difference between resistance to the antibiotics and hemolysin production (p > 0.05; Table IV). According to Table IV, isolates harboring traT gene were more resistant to aminoglycosides (p < 0.05), nalidixic acid (p = 0.049), and ampicillin–sulbactam (p = 0.006). Furthermore, isolates harboring fyuA and hlyA genes showed higher resistance to the first- and third-generation cephalosporins (p < 0.05).

Genotypic detection of ESBLs. PCR amplification on phenotypically ESBL-producing isolates (n = 48) showed that 44 (91.7%), 16 (33.3%), and 2 (4.2%) isolates were positive for $bla_{\rm CTX-M}$, $bla_{\rm TEM}$, and bla_{SHV} genes, respectively. Of the 48 phenotypically ESBL-producing isolates, 22.9% and 4.2% of the isolates were shown to have simultaneously $bla_{\rm TEM} + bla_{\rm CTX-M}$ and $bla_{\rm SHV} + bla_{\rm CTX-M}$ genes, respectively. The prevalence of $bla_{\rm CTX-M}$ and $bla_{\rm TEM}$ alone in the phenotypically ESBL-positive isolates was 64.5% and 4.2%, respectively. There were only two ESBL-producing isolates negative for ESBL genes. It was found that the prevalence of $bla_{\rm CTX-M}$ and $bla_{\rm TEM}$ was significantly higher in inpatients as compared to the outpatient isolates (p < 0.05).

Genotypic virulence patterns in K. pneumoniae isolates. In this study, 12 virulence patterns (KP patterns) of K. pneumoniae were obtained by different combinations of virulence genes (Table VII). All isolates indicated multiple virulence markers and contained four to seven virulence genes. Three isolates showed unique patterns, whereas KP1 with the presence of fimH, mrkD, kpn, entB, and traT was the most prevalent profile among the isolates (37.8%; Table VII).

Pattern	fimH	mrkD	kpn	entB	traT	fyuA	magA	hlyA	cnf-1	No. (%) of isolates
Kp1	+	+	+	+	+	_	-	_	_	53 (37.8%)
Kp2	+	+	+	+	_	_	_	_	_	26 (18.6%)
Kp3	+	+	+	+	_	+	_	_	_	24 (17.1%)
Kp4	+	+	+	+	+	+	_	_	_	21 (15.0%)
Kp5	+	+	+	+	_	_	_	+	_	3 (2.2%)
Kp6	+	+	+	+	+	_	+	_	_	3 (2.2%)
Kp7	+	+	+	+	_	+	_	+	_	3 (2.2%)
Kp8	+	+	+	+	+	+	+	_	_	2 (1.4%)
Kp9	+	+	+	+	+	+	_	+	_	2 (1.4%)
Kp10	+	+	+	+	+	_	_	+	+	1 (0.7%)
Kp11	+	+	+	+	+	+	_	_	+	1 (0.7%)
Kp12	+	+	+	+	+	-	-	+	_	1 (0.7%)

Table VII. Common virulence patterns identified among the K. pneumoniae isolates

Genomic analysis of K. pneumoniae isolates by PFGE. Totally, 45 K. pneumoniae isolates were considered for PFGE analysis, based on the virulence patterns, antibiotic resistance, and epidemiological data. These isolates were clustered into 41 clonal groups, based on the depicted dendrogram (C1–C41). The isolates showed 9–21 bands in PFGE that most of them had 16 bands (Figure 1). In accordance with

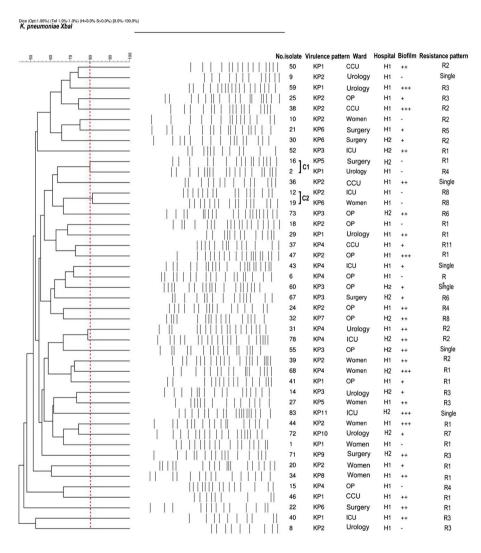


Figure 1. XbaI-digested DNA from 45 *K. pneumoniae* isolates in PFGE. Dendrogram was constructed based on UPGMA using Dice coefficient with a 1.0% band position tolerance, and the scales above the dendrogram indicate the percentage of similarity. The dotted line indicates 80% similarity

the similarity coefficient of 80%, only two clone types (C1 and C2) with two isolates in each were observed. Isolates in C1 were collected from the hospitalized patients in urology and surgery wards of two different hospitals. Isolates in C2 belonged to women hospitalized in ICU and gynecological ward and both of them were recovered in one hospital.

Discussion

Clinicians need to know about virulence properties and antimicrobial resistance patterns of pathogens causing UTI for a better understanding of these infections. In this study, we evaluated correlations between virulence properties and antimicrobial resistance of the *K. pneumoniae* isolates in UTI patients.

In agreement with the other reports, a high prevalence of virulence genes fimH, mrkD, kpn, and entB was observed in K. pneumoniae isolates [2, 16] that indicated their important role in pathogenesis of these isolates. It was found that all isolates harboring magA were isolated from inpatients that in accordance with other studies suggest significant role of MagA in pathogenesis of K. pneumoniae in complicated or severe UTIs [17]. To the best of our knowledge, there is no study reporting the presence of CNF1 in K. pneumoniae isolated from UTIs, whereas in this study, cnf1 was detected in two isolates that were recovered from hospitalized patients. The cnf1-positive isolates were associated with ESBL, strong biofilm production, and harbored fimH, mrkD, kpn, entB, hlyA, fyuA, and traT genes; moreover, these isolates were sensitive only to meropenem. These characteristics suggest that K. pneumoniae harboring cnfl toxin genes have the potential to become high-risk isolates in hospitals as they are capable to develop MDR phenotype. Similar to our findings, a high frequency rate of biofilm formation was reported in K. pneumoniae isolated from different countries [2, 18]. This may be related to the significant prevalence of factors implicating in biofilm formation such as type 1 or type 3 fimbriae and EntB [2, 19]. In this study, biofilm formation was similar in K. pneumoniae isolated from inpatient and outpatient cases, which suggests role of biofilm formation in bacterial colonization in both inpatients and outpatients [20]. In this study, the isolates collected from inpatients were capable to form a moderate and strong biofilm compared to outpatients that can be associated with long hospitalization and challenges for treatment of inpatients. This study in accordance with the results of Soto et al. [21] showed that isolates containing hemolysin produced biofilm in moderate to strong that suggests that colonization with hemolytic and biofilm producer isolates is more likely to develop into UTIs. Furthermore, all isolates that had ability to forming HMV were positive for hemolytic and biofilm production and it has been suggested that

this ability increases colonization of *K. pneumoniae*. Other recent studies also identified that the HMV-producing isolates had higher biofilm than non-HMV isolates [22, 23].

β-lactams, cephalosporins, and SXT are among the recommended antibiotics for treatment of UTIs [24]. However, according to this study and others, it is recommended that the use of these antibiotics, especially β-lactams, should be limited [25, 26]. The frequent and misuse of these antibiotics has led to the high resistance levels. On the contrary, our results and others showed the high effectiveness of carbapenems, quinolones, and aminoglycosides against K. pneumoniae to be considered as effective treatment agents, especially in complicated UTIs [24, 25]. Despite this, there are some studies that reported significant rate of resistance to these antibiotics in K. pneumoniae [27, 28]. The variable results could be related to the differences in geographical area, UTI type, sampling, and social habits of the communities [29].

To date, infections caused by MDR *K. pneumoniae* have become a major challenge for treatment, resulting in increased mortality rates and excessive costs for patients [30]. MDR was observed in about 24% of the isolates that was lower than studies in Nepal [31], Ethiopia [29], and China [32]. It is possible the excessive use of antibiotics and lack of appropriate strategies for control of UTIs increase the rate of MDR isolates in the societies [29].

In this study, 34.5% of isolates produced ESBL that was lower than the rate of ESBL in other geographical regions of Iran (70% and 97%) [33, 34]. The studies showed that the prevalence of ESBL in *K. pneumoniae* was higher than 10% for half of the countries in different geographical regions. These frequency rates were variable from 0% in Iceland to 91% in Romania [35]. In this study, the frequency rate of ESBLs among inpatient isolates was significantly higher than among outpatient isolates. This could be due to the extensive use of different classes of antibiotics in inpatients that increases the possibility of the ESBL acquisition as compared to the outpatients [36]. Furthermore, high antibiotic resistance especially MDR phenotype was found among ESBL-positive isolates as compared to non-ESBL isolates, which can lead to serious difficulties in treatment of these patients.

In this study, 46 (95.8%) of phenotypically ESBL-positive isolates indicated the presence of the ESBL genes. The absence of the ESBL genes in two of the phenotypically ESBL-positive isolates suggests that ESBL production in these isolates might be related to other classes of ESBLs, which requires further studies [37]. In accordance with our findings, a high prevalence of CTX-M is reported among the ESBL-positive strains from Asia, America, and Africa [35, 38, 39]. Furthermore, in agreement with the present study, an increased shift from TEM or SHV to CTX-M genotype was shown in Europe [35, 38]; whereas in addition to CTX-M, the high frequency of TEM and SHV genotypes was reported in Isfahan,

Iran [39]. Different concurrent presence of ESBL genes is also reported from Iran and other countries [35]. For example, on the contrary to our findings, the simultaneous presence of bla_{SHV} , bla_{CTX-M} , and bla_{TEM} genes was observed in a study in Iran (37%) [33].

Some associations were found between the presence of virulence characteristics and antibiotic resistance. In this regard, a correlation between the presence of traT, fyuA, or cnf-1 genes with antibiotic resistance was observed. It was also found that all resistant isolates to meropenem produced biofilm or isolates with HMV property showed higher tendency to antibiotic resistance. Furthermore, all ESBL-positive isolates were biofilm producers that moderate and strong biofilm production was detected in 58.3% and 33.3% of ESBL producers, respectively. The prevalence of HMV phenotype was higher among ESBL-producing isolates, where 12 (25%) of ESBLs exhibited HMV compared to the non-ESBLs (5.4%) (p = 0.002). ESBL-positive isolates tended to harbor some of the virulence genes such as fyuA and hlyA more than ESBL-negative isolates (p < 0.05). It was also found that the isolates with $bla_{\rm TEM}$ genotype significantly harbored fyuA (p = 0.048) and hlyA virulence genes. These correlations suggest that the co-presence of the virulence and resistance genes on same mobile genetic elements may enhance their spread by horizontal gene transfer [40].

In this study, 41 patterns in PFGE were observed that demonstrated the high genetic variability of *K. pneumoniae* isolates in Tehran, Iran. This diversity suggests that the isolates are obtained from different clones and may indicate the good hygienic conditions in these hospitals. There were only two pulsotypes that isolates in clone type C2 were collected from one hospital and with same antibiotic resistance patterns (R8). This suggests that the isolates in C2 may originate from one clone in this hospital and there is a risk of spreading the clone in the hospital environments.

In conclusion, a significant rate of virulence factors and antibiotic resistance was observed in *K. pneumoniae* isolates. Furthermore, the correlations were observed between the antibiotic resistance and virulence characteristics in these isolates that could increase their ability in pathogenicity in the urinary tract. These findings and others indicate that improvement in strategies of antibiotic therapy and control of UTIs caused by *K. pneumoniae* should be performed before the emerging of MDR strains for which there are no effective antibiotics.

Acknowledgements

This work was financially supported by Pasteur Institute of Iran. FE and MH contributed equally to this work and should be considered as co-first authors.

Conflict of Interest

The authors declare no conflict of interest.

References

- Mann, R., Mediati, D. G., Duggin, I. G., Harry, E. J., Bottomley, A. L.: Metabolic adaptations of uropathogenic *E. coli* in the urinary tract. Front Cell Infect Microbiol 7, 241 (2017).
- 2. El Fertas-Aissani, R., Messai, Y., Alouache, S., Bakour, R.: Virulence profiles and antibiotic susceptibility patterns of *Klebsiella pneumoniae* strains isolated from different clinical specimens. Pathol Biol (Paris) **61**, 209–216 (2013).
- 3. Paczosa, M. K., Mecsas, J.: *Klebsiella pneumoniae*: Going on the offense with a strong defense. Microbiol Mol Biol Rev **80**, 629–661 (2016).
- 4. Schroll, C., Barken, K. B., Krogfelt, K. A., Struve, C.: Role of type 1 and type 3 fimbriae in *Klebsiella pneumoniae* biofilm formation. BMC Microbiol **10**, 179 (2010).
- Chang, L., Bastian, I., Warner, M.: Survey of *Klebsiella pneumoniae* bacteraemia in two South Australian hospitals and detection of hypermucoviscous phenotype and magA/rmpA genotypes in *K. pneumoniae* isolates. Infection 41, 559–563 (2013).
- Blum, G., Falbo, V., Caprioli, A., Hacker, J.: Gene clusters encoding the cytotoxic necrotizing factor type 1, Prs-fimbriae and α-hemolysin form the pathogenicity island II of the uropathogenic *Escherichia coli* strain J96. FEMS Microbiol Lett 126, 189–195 (1995).
- Paterson, D. L., Bonomo, R. A.: Extended-spectrum β-lactamases: A clinical update. Clin Microbiol Rev 18, 657–686 (2005).
- Kumar, V., Sun, P., Vamathevan, J., Li, Y., Ingraham, K., Palmer, L., Huang, J., Brown, J. R.: Comparative genomics of *Klebsiella pneumoniae* strains with different antibiotic resistance profiles. Antimicrob Agents Chemother 55, 4267–4276 (2011).
- Derakhshan, S., Najar Peerayeh, S., Bakhshi, B.: Association between presence of virulence genes and antibiotic resistance in clinical *Klebsiella pneumoniae* isolates. Lab Med 47, 306–311 (2016).
- Lina, T. T., Khajanchi, B. K., Azmi, I. J., Islam, M. A., Mahmood, B., Akter, M., Banik, A., Alim, R., Navarro, A., Perez, G., Cravioto, A., Talukder, K. A.: Phenotypic and molecular characterization of extended-spectrum beta-lactamase-producing *Escherichia coli* in Bangladesh. PLoS One 9, e108735 (2014).
- Barguigua, A., El Otmani, F., Talmi, M., Bourjilat, F., Haouzane, F., Zerouali, K., Timinouni, M.: Characterization of extended-spectrum β-lactamase-producing *Escherichia* coli and *Klebsiella pneumoniae* isolates from the community in Morocco. J Med Microbiol 60, 1344–1352 (2011).
- 12. Dromigny, J., Nabeth, P., Claude, J. P. G.: Distribution and susceptibility of bacterial urinary tract infections in Dakar, Senegal. Int J Antimicrob Agents 20, 339–347 (2002).
- 13. Yu, W. L., Ko, W. C., Cheng, K. C., Lee, H. C., Ke, D. S., Lee, C. C., Fung, C. P., Chuang, Y. C.: Association between *rmpA* and *magA* genes and clinical syndromes caused by *Klebsiella pneumoniae* in Taiwan. Clin Infect Dis **42**, 1351–1358 (2006).

- Clinical and Laboratory Standards Institute: Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. CLSI Document M100-S25. Clinical and Laboratory Standards Institute, Wayne, PA, 2015, pp. 1–236.
- 15. Solgi, H., Badmasti, F., Giske, C. G., Aghamohammad, S., Shahcheraghi, F.: Molecular epidemiology of NDM-1- and OXA-48-producing *Klebsiella pneumoniae* in an Iranian hospital: Clonal dissemination of ST11 and ST893. J Antimicrob Chemother **73**, 1517–1524 (2018).
- Ranjbar, R., Memariani, H., Sorouri, R., Memariani, M.: Distribution of virulence genes and genotyping of CTX-M-15-producing *Klebsiella pneumoniae* isolated from patients with community-acquired urinary tract infection (CA-UTI). Microb Pathog 100, 244–249 (2016).
- Cubero, M., Grau, I., Tubau, F., Pallarés, R., Dominguez, M., Liñares, J., Ardanuy, C.: Hypervirulent *Klebsiella pneumoniae* clones causing bacteraemia in adults in a teaching hospital in Barcelona, Spain (2007–2013). Clin Microbiol Infect 22, 154–160 (2016).
- 18. Barreto, S., Zambrano, M., Araque, M.: Phenotypic variations of susceptibility in *Klebsiella pneumoniae* strains of nosocomial origin and their association with biofilm formation. Investigacion Clinica **50**, 221–229 (2009).
- 19. Vuotto, C., Longo, F., Balice, M. P., Donelli, G., Varaldo, P. E.: Antibiotic resistance related to biofilm formation in *Klebsiella pneumoniae*. Pathogens 3, 743–758 (2014).
- Jacobsen, S. A., Stickler, D., Mobley, H., Shirtliff, M.: Complicated catheter-associated urinary tract infections due to *Escherichia coli* and *Proteus mirabilis*. Clin Microbiol Rev 21, 26–59 (2008).
- Soto, S., Smithson, A., Martinez, J., Horcajada, J., Mensa, J., Vila, J.: Biofilm formation in uropathogenic *Escherichia coli* strains: Relationship with prostatitis, urovirulence factors and antimicrobial resistance. J Urol 177, 365–368 (2007).
- 22. Shah, R. K., Ni, Z. H., Sun, X. Y., Wang, G. Q., Li, F.: The determination and correlation of various virulence genes, ESBL, serum bactericidal effect and biofilm formation of clinical isolated classical *Klebsiella pneumoniae* and hypervirulent *Klebsiella pneumoniae* from respiratory tract infected patients. Polish J Microbiol **66**, 501–508 (2017).
- Wacharotayankun, R., Arakawa, Y., Ohta, M., Tanaka, K., Akashi, T., Mori, M., Kato, N.: Enhancement of extracapsular polysaccharide synthesis in *Klebsiella pneumoniae* by RmpA2, which shows homology to NtrC and FixJ. Infect Immun 61, 3164–3174 (1993).
- 24. Yang, Q., Zhang, H., Wang, Y., Xu, Z., Zhang, G., Chen, X., Xu, Y., Cao, B., Kong, H., Ni, Y., Yu, Y., Sun, Z., Hu, B., Huang, W., Wu, A., Feng, X., Liao, K., Luo, Y., Hu, Z., Chu, Y., Lu, J., Su, J., Gui, B., Duan, Q., Zhang, S., Shao, H., Badal, R. E.: Antimicrobial susceptibilities of aerobic and facultative Gram-negative bacilli isolated from Chinese patients with urinary tract infections between 2010 and 2014. BMC Infect Dis 17, 192 (2017).
- Al Benwan, K., Al Sweih, N., Rotimi, V. O.: Etiology and antibiotic susceptibility patterns
 of community- and hospital-acquired urinary tract infections in a general hospital in
 Kuwait. Med Princ Pract 19, 440–446 (2010).
- Mishra, M. P., Sarangi, R., Padhy, R. N.: Prevalence of multidrug resistant uropathogenic bacteria in pediatric patients of a tertiary care hospital in Eastern India. J Infect Public Health 9, 308–314 (2016).
- 27. Navaneeth, B., Belwadi, S., Suganthi, N.: Urinary pathogens' resistance to common antibiotics: A retrospective analysis. Tropical doctor **32**, 20–22 (2002).

- 28. Das, R., Chandrashekhar, T., Joshi, H., Gurung, M., Shrestha, N., Shivananda, P.: Frequency and susceptibility profile of pathogens causing urinary tract infections at a tertiary care hospital in western Nepal. Singapore Med J 47, 281–285 (2006).
- Alemu, A., Moges, F., Shiferaw, Y., Tafess, K., Kassu, A., Anagaw, B., Agegn, A.: Bacterial
 profile and drug susceptibility pattern of urinary tract infection in pregnant women at
 University of Gondar Teaching Hospital, Northwest Ethiopia. BMC Res Notes 5, 197 (2012).
- 30. Hou, X.-H., Song, X.-Y., Ma, X.-B., Zhang, S.-Y., Zhang, J.-Q.: Molecular characterization of multidrug-resistant *Klebsiella pneumoniae* isolates. Braz J Microbiol **46**, 759–68 (2015).
- 31. Chander, A., Shrestha, C. D.: Prevalence of extended spectrum beta lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* urinary isolates in a tertiary care hospital in Kathmandu, Nepal. BMC Res Notes **6**, 487 (2013).
- 32. Li, B., Yi, Y., Wang, Q., Woo, P. C., Tan, L., Jing, H., Gao, G. F., Liu, C. H.: Analysis of drug resistance determinants in *Klebsiella pneumoniae* isolates from a tertiary-care hospital in Beijing, China. PLoS One 7, e42280 (2012).
- 33. Mahmoudi, S., Pourakbari, B., Rahbarimanesh, A., Abdolsalehi, M. R., Ghadiri, K., Mamishi, S.: An outbreak of ESBL-producing *Klebsiella pneumoniae* in an Iranian referral hospital: Epidemiology and molecular typing. Infect Disord Drug Targets (2018).
- 34. Feizabadi, M. M., Delfani, S., Raji, N., Majnooni, A., Aligholi, M., Shahcheraghi, F., Parvin, M., Yadegarinia, D.: Distribution of bla_{TEM}, bla_{SHV}, bla_{CTX-M} genes among clinical isolates of Klebsiella pneumoniae at Labbafinejad Hospital, Tehran, Iran. Microb Drug Resist 16, 49–53 (2010).
- 35. Coque, T. M., Baquero, F., Canton, R.: Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe. Euro Surveill **13**, 19044 (2008).
- Al Mously, N., Al Arfaj, O., Al Fadhil, L., Mukaddam, S.: Antimicrobial susceptibility patterns of ESBL *Escherichia coli* isolated from community and hospital-acquired urinary tract infections. J Health Spec 4, 133 (2016).
- 37. Ahmed, D., Wahid, S. U. H., Sadique, T., Sultana, N., Islam, M., Halim, F., Islam, N., Hossain, A.: Recurrent urinary tract infection due to co-infection with extended spectrum β-lactamase-producer uropathogenic *Escherichia coli* and enteroaggregative *E. coli*. JMM Case Rep 1, 1–5 (2014).
- Barguigua, A., El Otmani, F., Talmi, M., Reguig, A., Jamali, L., Zerouali, K., Timinouni, M.: Prevalence and genotypic analysis of plasmid-mediated beta-lactamases among urinary Klebsiella pneumoniae isolates in Moroccan community. J Antibiot (Tokyo) 66, 11–16 (2013).
- Moghadampour, M., Rezaei, A., Faghri, J.: The emergence of bla_{OXA-48} and bla_{NDM} among ESBL-producing Klebsiella pneumoniae in clinical isolates of a tertiary hospital in Iran. Acta Microbiol Immunol Hung 65, 335–344 (2018).
- Qin, X., Hu, F., Wu, S., Ye, X., Zhu, D., Zhang, Y., Wang, M.: Comparison of adhesin genes and antimicrobial susceptibilities between uropathogenic and intestinal commensal *Escherichia coli* strains. PLoS One 8, e61169 (2013).