

Antibiotic Resistant, Virulence-associated Genes, Biofilm and Efflux Pump Gene Expression and Molecular Typing of Klebsiella Pneumoniae Strains Recovered from Clinical Samples

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Research

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Abstract

Background

Multidrug-resistant (MDR) *Klebsiella pneumoniae* strains are one of the most important life-threatening nosocomial pathogens. In the current study, antibiotic resistant, **virulence-associated genes**, gene expression of efflux pumps and biofilm genes as well as molecular typing of *K. pneumoniae* strains were investigated. A total of 505 clinical specimens were collected from hospitalized patients and *K. pneumoniae* strains were isolated by standard microbiological methods. Antibiotic resistant profile, prevalence of virulence associated genes, biofilm and efflux pump genes were investigated. The gene expression analysis of biofilm and efflux pump genes were analysed quantitative Real Time PCR. Moreover, molecular typing of *K. pneumoniae* strains using Repetitive element sequence-based PCR (rep-PCR) technique was also carried out.

Results

One hundred *K. pneumoniae* strains out of 500 clinical samples were isolated and the highest prevalence of resistance was observed against ciprofloxacin (75%), Trimethoprim-sulfamethoxazole (73%) and Nitrofurantoin (38%). Virulence associated genes including *entB*, *traT* and *rmpA* were found in 80%, 62% and 48%, respectively. Gene prevalence for biofilm association gene including *mrkA*, *fimH* and *mrkD* were 42% for all genes. The *AcrAB*, *TolC* and *mdtK* efflux pump genes were observed in 41%, 33% and 26%, respectively. In addition, most MDR strains formed biofilm, as well as, *AcrAB* efflux pump and *mrkA* biofilm gene expression was up-regulated in MDR *K. pneumoniae* strains and a significant statistically association was also observed between MDR strains and high expression of efflux pump and biofilm genes. In addition, the *K. pneumoniae* strains differentiated into 11 different genetic clusters by rep-PCR analysis.

Conclusions

High prevalence of resistance, presence of diverse virulence factors and high level of efflux pump and biofilm gene expression in diverse clones of *K. pneumoniae* strains pose an important public health issue.

Background

Klebsiella pneumoniae is a Gram-negative pathogen belonging to the Enterobacteriaceae family [1]. *K. pneumoniae* can cause various infections such as urinary tract infections, pneumonia and bloodstream infections and it is considered as an important nosocomial pathogen in Iran [2]. *K. pneumoniae* has recently attracted the attention of researchers due to the increased severity of infections, antibiotic resistance and treatment deficiencies [3, 4]. The increase in multidrug-resistant (MDR) *K. pneumoniae* in recent years is mainly due to the indiscriminate use of antibiotics to treat and prevent infections caused by this bacterium [5]. *K. pneumoniae* uses a variety of mechanisms to resist antibiotics [6]. One of the important mechanisms for creating the MDR is efflux pump systems and biofilm formation capacity [7]. Efflux pumps are protein based structure which capable extrude the different toxic substances out of cells and the AcrAB-TolC efflux pump system which belongs to the Resistance Nodulation Division (RND) efflux pump which is an important cause of MDR of *K. pneumoniae* strains occurrence [8–11]. The AcrAB-TolC efflux pump is composed a periplasmic component (AcrA), a transporter located in the inner membrane (AcrB) and an outer membrane compartment (TolC). The AcrAB-TolC efflux pump plays an important role in developing resistance to various antibiotics such

quinolones, tetracycline, and chloramphenicol in MDR strains of *K. pneumoniae* [12]. The ability to form biofilms in *K. pneumoniae* strains, allows it to protect against the host immune system and antibiotics in MDR isolates and there are a lot of biofilm related genes including mrk (type 3 fimbriae), fimH-1 (type 1 fimbrial adhesion) [13, 14]. Studies show that the efflux pump plays an important role in antibiotic resistance and biofilm formation [15]. There are several studies showed a correlation of *K. pneumoniae* antibiotic resistance, efflux pump and biofilm formation ability [16]. In the study of Subramanian et al, 2012, indicated that 80% of biofilm forming isolates from 100 clinical samples showed an MDR phenotype [17]. Various Virulence factors such as those encoding regulators of mucoid phenotype A (rmpA), bacteriocin biosynthesis [enterobactin (entB), and serum resistance-associated outer membrane lipoprotein (traT) play an important role in the pathogenicity of *K. pneumoniae* strains [18].

Molecular typing of *K. pneumoniae* strains can be used to prevent infection in the hospital and also to find the dominant genotype among the isolates can be useful in finding the source of infection and applying prevention protocols [19, 20]. Due to the high prevalence of MDR strains of *K. Pneumoniae* in Iran, the aim of this study was to investigate the antibiotic resistance profile and distribution of virulence genes in *K. Pneumoniae* strains, efflux pump and biofilm gene expression, as well as, molecular typing of *K. pneumoniae* strains using Repetitive element sequence-based PCR (rep-PCR).

Methods

Bacterial isolates and identification

A total of 505 clinical specimens including urine, blood, sputum and cerebrospinal fluid (CSF) were collected from hospitalized patients were admitted to Baghiatallah and Imam Khomeini hospitals in Iran from January 2018 to July 2019. The *K. pneumoniae* strains were identified using conventional microbiological tests including catalase, MR-VP (methyl red – Voges Proskauer), lysine iron agar, Kligler agar, phenylalanine agar, urea agar, SIM (sulfide, indole, motility), blood agar, and MacConkey agar [21]. The isolated strains were stored at -20 °C in brain heart infusion broth containing 20% glycerol for further investigation.

Antimicrobial susceptibility test

Antibiotic susceptibility of *K. pneumoniae* strains was evaluated according to the Clinical and Laboratory Standards Institute (CLSI) standard based on to Kirby-Bauer disk diffusion method [22]. The antimicrobial susceptibility assays to 17 antibiotics were performed using commercially available antibiotics including ceftazidime (30 µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), tobramycin (10 µg), gentamicin (10 µg), streptomycin (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), Imipenem (10 µg), co-trimoxazole (1.25/23.75 µg), chloramphenicol (30 µg), Amoxicillin/Clavulanic acid (AMC, 20/10 µg), Meropenem (10 µg), clindamycin (30 µg), polymixin B (10 µg), tetracycline (30 µg) (MAST, Group Ltd., Merseyside, UK). The plates were incubated at 37 °C for 24 hours and subsequently, the inhibition zone diameters were recorded in millimeter and interpretation was done based on CLSI. *K. pneumoniae* ATCC 13883 was used as a control in this study. Multidrug-resistant (MDR) strains were identified based on their resistance to at least one drug in three different groups of antibiotics.

Phenotypic detection of efflux pump

The phenotypic and qualitative detection of the efflux pump in *Klebsiella pneumoniae* strains was performed by Cartwheel method. Briefly, the plates of Muller Hinton Agar culture media containing ethidium bromide were prepared and the culture medium was divided into 8 parts and the bacteria in 0.5 McFarland turbidity concentration were streaked on plates. After 24 h of incubation at 37 °C, the plates were studied under UV transilluminator. The strains that had efflux pumps did not show emission of fluorescence [23].

Phenotypic detection of biofilm formation

Phenotypic detection of biofilm formation was performed using Congo red agar test. Briefly, the *K. pneumoniae* strains was cultured in Brain Heart Infusion agar enriched with 5% (w/v) sucrose and Congo red based on Freeman et al. 1989. The *K. pneumoniae* strains which formed biofilm exhibited a dry dark crystalline colonies and considered as exopolysaccharides producers [24].

Quantitative detection of biofilm

Quantitative biofilm detection test was performed by plate microtiter method in 96 house plate. In summary, first 100 µl of 24-hour culture of strains with OD = 0.1 were added into wells. After 24 h incubation, each well was washed twice with PBS and then, stained with crystal violet for 15 min.

Finally, the stained cells were solubilized in 33% (v/v) acetic acid and their adsorption was read at 570 nm OD 570 nm. Based on the type of biofilm, the strains of biofilm former were divided into three categories: strong biofilm, moderate biofilm, weak biofilm and negative biofilm producers. In addition, the standard strain *K. pneumoniae* ATCC 13833 and LB broth was considered as a positive and negative control, respectively [25].

Molecular detection of virulence associated genes

The genomic DNA of the strains was extracted by the DNA extraction kit (Bioneer, Korea) according to the manufacturer's protocol. The frequency of *entB*, *Trat* and *mpA* virulence gene, *mdtk*, *tolC* and *AcrAB* efflux pump, *fimH-1*, *mrkA* and *mrkB* biofilm associated genes were detected by PCR. The PCR conditions were as follow: Initial denaturing at 94 °C for 5 min followed by 30 cycles, each cycle contained 1 min at 94 °C for denaturation, 30 second for annealing (Table 1) and 60 S for extension steps and finally one cycle for final extension at 72 °C for 10 min. The primer of target genes are given in Table 1.

Table 1
The primer sequences of target genes which used in this study.

Target gene	Primer sequence (5' to 3')	Annealing Temperature (°C)	Reference
<i>AcrAB</i>	F ATCAGCGGCCGGATTGGTAAA R CGGGTTCGGGAAAATAGCGCG	53	[26]
<i>TolC</i>	F ATCAGCAACCCCGATCTGCGT R CCGGTGACTTGACGCAGTCCT	51	[26]
<i>MdtK</i>	F GCGCTTAACTTCAGCTCA R GATGATAAATCCACACCAGAA	43	[26]
<i>mrkA</i>	F ACGTCTCTAACTGCCAGGC R TAGCCCTGTTGTTTGCTGGT	55	[16]
<i>mrkD</i>	F CCACCAACTATTCCCTCGAA R ATGGAACCCACATCGACATT	43	[27, 28]
<i>fimH</i>	F GCCAACGTCTACGTTAACCTG R ATATTTACGGTGCCTGAAAA	43	[26]
<i>entB</i>	F CTGCTGGGAAAAGCGATTGTC R AAGGCGACTCAGGAGTGGCTT	49	[26]
<i>traT</i>	F GGTGTGGTGCGATGAGCACAG R CACGGTTCAGCCATCCCTGAG	55	[28]
<i>rmpA</i>	F ACTGGGCTACCTCTGCTTCA R CTTGCATGAGCCATCTTTCA	53	[29]
<i>16S rRNA</i>	F AGCCGACCTGAGAGGGTGA R TCTGGACCGTGTCTCAGTTCC	55	[30]

Gene expression analysis of *AcrAB* and *mrkA* gene

AcrAB efflux pump and *mrkA* biofilm gene expression analysis was done using quantitative Real Time PCR method in *K. pneumoniae* strains. Briefly, 100 µl of each bacterium was added into 96 well plate and incubated for 18 h at 37 °C aerobically. Then, each well was washed using PBS and adherent cells were scraped off using LB broth. Subsequently, total RNA of collected strains were extracted using an RNA extraction kit (Qiagen, USA) according to instruction protocol.

The extracted RNA was converted to complementary DNA (cDNA) by a cDNA synthesis kit (Fermentase, Lithuania) based on manufacturer's instructions. In order to perform the Real Time PCR, each cDNA was used as a template in 20 µl final volume containing 2 µl cDNA, 10 pmol of each primer (Table 1), and 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems) using Bioneer Real-Time PCR equipment (Korea). The *16S rRNA* was used as a housekeeping gene to normalize the levels of mRNA expression and the relative expression of AcrAB efflux pump gene was calculated using $\Delta\Delta C_T$ method.

Molecular typing of MDR *K. pneumoniae* strains

Molecular typing of *K. pneumoniae* strains was done using the Repetitive element sequence-based PCR (rep-PCR) method. The rep-PCR was performed using two following primers: Forward: REP1 5'- III ICG ICG ICA TCI GGC-3' Reverse: REP2 5'- ICG ICT TATCIG GCC TAC-3' as described previously. The PCR amplification products were visualized using electrophoresis on 1.5% agarose gel and stained with safe red. Finally, the banding patterns and size were determined via Image Lab 4.0. The obtaining results were analyzed by gel compare II software using Dice correlation coefficient and the UPGMA method [31].

Statistical Analysis

All tests of this study are repeated three times and one way ANNOVA test was used for statistical analysis. In addition, $P < 0.05$ was also considered significant.

Results

Isolation and antimicrobial susceptibility pattern of *K. pneumoniae* strains

A total of 100 *K. pneumoniae* isolates were collected from 505 clinical samples. The 100 *K. pneumoniae* strains were recovered from specimens of urine (n=70), blood (n=20), Sputum (n=6) and cerebrospinal fluid (CSF, n=4) based on routine microbiological methods. Among 100 clinical strains of *K. pneumoniae*, 75 (75%), 73 (73%) and 68 (68%) of strains were resistant to ciprofloxacin, Trimethoprim-sulfamethoxazole and Nitrofurantoin, respectively and 28 (28%), 52 (52%) strains were susceptible to streptomycin and imipenem, respectively (Table 2). In addition, 92 (92%) strains revealed MDR phenotypes and most MDR strains were resistant to imipenem, meropenem and beta-lactam antibiotics. The studied strains were categorized into 25 antimicrobial resistant patterns (Table 2).

Phenotypic detection of biofilm formation and efflux pump

Phenotypic detection of biofilm formation was done using Congo red agar test and our results showed that 77% (77 strains) of isolates exhibited black colonies, which presumably indicate biofilm formation (Fig 1A). The other strains formed white colonies which reflecting no biofilm formation. Moreover, the Cartwheel results showed that 90% (90 isolates) of strains had efflux pumps (Fig 1B).

Table 2
Antimicrobial sensitivity, biofilm formation and efflux pump patterns of *K. pneumoniae* strains.

Isolates NO.	Antibiotic resistant pattern	Anti-biotype (AB)	MDR	Biofilm former	Mean OD 570	Phenotypic efflux pump
10, 17, 23, 73, 92, 98	CTX, CAZ, CRO, FOX, NA, CP, FM, TE, S, GM	AB1	+	+	2.63±0.14	+
15, 20	CTX, CAZ, CRO, FOX, NA, CP, FM, TMP	AB2	+	-	0.025±0.03	+
1, 6, 13, 25	CTX, CAZ, CRO, FOX, TMP, GM, S	AB 3	+	+	2.13±0.19	+
4, 12, 21, 74, 77, 94	CAZ, FOX, FM, TE, TMP, S	AB 4	+	+	3.52±0.21	+
18, 35, 49	CTX, CAZ, CRO, FOX, FM, TMP	AB 5	+	+	0.76±0.06	+
3, 16, 22	CTX, CAZ, CRO, NA, CP	AB 6	+	+	0.38±0.08	+
8, 45, 75	CTX, CAZ, CRO, FOX, NA, CP, FM, TE	AB 7	+	+	2.69±0.17	+
5, 41, 76	CTX, CAZ, CRO, FOX, NA, CP, AMC, CF, S, FM, TMP, IMP, GM, MEN	AB 8	+	+	3.4±0.12	+
19, 31, 72	CTX, CAZ, CRO, NA, CP, FM, TMP, GM, S	AB 9	+	+	1.8±0.07	-
7	AMC, S, CF, TMP	AB 10	+	+	0.17±0.16	+
9, 28, 87	FM, CF, AMC, K, GM, TMP, CP, AN, FOX, CTX, CRO, CAZ	AB 11	+	+	0.81±0.007	+
24, 26, 40	S, CF, AMC, FM, IPM, TMP, MEN, NA, CP	AB 12	+	-	0.16±0.003	+
14, 39	CL, TE, PB, AN, TMP, GM, K, S, FM, AMC, CF	AB 13	+	+	0.15±0.006	+
41, 59, 61, 93	AMC, CF, FM, PB, CL, TE	AB 14	+	+	0.94±0.007	-
36, 43	CP, TE, NA, FM, MEN, TMP, CF, AMC	AB 15	+	+	0.16±0.006	+
53, 81, 96	CF, AMC, MEN, TMP, IPM, CP, NA	AB 16	+	+	0.20±0.13	+
27, 30, 62, 88	FM, AMC, CF, IPM, AN, MEN, GM, TMP, CP, TE, NA, CL	AB 17	+	+	0.19±0.001	+
2, 11, 60, 78, 100	CF, AMC, TMP, CP, TE, NA	AB 18	+	+	0.13±0.006	+
50, 80, 85, 86	AMC, FM, CF, CP, TE, NA, MEN, IPM, TMP, GM, AN	AB 19	+	+	2.13±0.003	+

48, 52, 58, 65	AMC, S, CF, FM, IPM, MEN, CP, NA	AB 20	+	+	3.82±0.009	+
33, 47, 71, 56, 83, 69	CF, K, FM, AMC, GM, MEN, IPM, AN, TMP, NA, TE, CP	AB 21	+	+	0.19 ±0.007	+
34, 42, 99	AMC, CF, FM, IMP, MEN, TMP, CP, NA, TE	AB 22	+	+	1.45±0.008	-
44, 50, 54, 7, 66, 79, 97, 63, 64, 70, 82	CAZ, CRO, FOX, K, FM, AMC, CF, AN, TMP, GM, IMP, MEN, CP, TE, NA	AB 23	+	-	0.02±0.002	+
29, 32, 51, 55, 57, 68, 84	CP, TE, NA, TMP, GM, IMP, MEN, AMC, FM, CF, CAZ, CTX, CRO, FOX	AB 24	+	-	0.029±0.001	+
30, 89, 90, 91, 37, 38, 46, 95	CF, AMC	AB 25	-	+	0.56±0.001	+
CF: Chloramphenicol, AMC: Amoxicillin-clavulanic acid, FOX: Cefoxitin, CRO: Ceftriaxone, CTX: Cefotaxime, CAZ: Ceftazidime, FM: Nitrofurantoin, MEN: Meropenem, IMP: Imipenem, GM: Gentamycin, TMP: Trimethoprim-sulfamethoxazole, NA: Nalidixic acid, TE: Tetracycline, CP: Ciprofloxacin, S: Streptomycin, K: Kanamycin, CL: Clindamycin, PB: Polymixin B, S: Streptomycin,						

Quantitative biofilm production

The results of quantitative biofilm production test is shown in Table 2 as mean OD 570 values. According to OD values among 77% biofilm producers, 50 isolates (71%) were categorized strong biofilm former (OD> 0.204), 16 (20%) as moderate biofilm former (0.102<OD<0.204) and 11 isolates (14%) as weak biofilm former (0.0551<OD<0.102). There are a significant between MDR phenotype, biofilm formation and efflux pump among *K. pneumoniae* strains (P<0.05). Moreover, there was a significant correlation between biofilm formation in isolates recovered from urine comparing to other type specimens (P<0.05).

Frequency of biofilm, efflux pump and virulence associated genes

The prevalence of biofilm, efflux pump and virulence associated genes are given in Table 3. The *mrkA*, *mrkD* and *fimH* genes encoding type 1 and type 3 fimbrial adhesion engage in biofilm formation were present in all biofilm former strains. Moreover, the efflux pump genes including *AcrAB*, *ToIC* and *mdtK* were observed 41 (41%), 33 (33%) and 26 (26%) strains, respectively. The *AcrAB* was more prevalent in *K. pneumoniae* strains comparing to other efflux pump genes. In addition, the *AcrAB* efflux pump gene was more prevalent in urine samples comparing to other clinical specimens.

In addition, the virulence related genes including enterobactin biosynthesis gene (*entB*), outer membrane protein coding gene (*traT*) and mucoid phenotype A (*rmpA*) was seen in 80 (80%), 62 (62%) and 48 (48%) strains,

respectively. As reported in Table 3, analysis of selected genes showed that biofilm was more pronounced among virulence associated gene positive than among negative strains. The entB virulence gene was detected in all blood, CSF and sputum isolates. There were 8 virulence profiles (V1-V8) Based on virulence detected gene and V1 was the most prevalent virulence type.

Table 3
Biofilm, efflux pump and virulence association genes among *K. pneumoniae* strains.

Isolate NO	Biofilm association genes			Efflux pump genes			Virulence association gene			Virulence gene Type	Type of samples
	<i>mrkA</i>	<i>mrkD</i>	<i>fimH</i>	<i>AcrAB</i>	<i>TolC</i>	<i>mdtK</i>	<i>entB</i>	<i>TraT</i>	<i>mpaA</i>		
1	+	+	+	-	+	-	+	+	-	V1	Urine
2	+	+	+	+	+	+	+	+	+	V2	Urine
3	+	+	+	-	+	-	+	+	+	V2	Urine
4	+	+	+	+	-	-	+	+	+	V2	Urine
5	+	+	+	+	-	+	-	+	-	V3	Urine
6	+	+	+	-	-	+	+	+	-	V1	Urine
7	+	+	+	-	+	-	+	-	+	V4	Urine
8	+	+	+	+	-	+	+	-	+	V4	Urine
9	+	+	+	-	-	-	-	+	+	V5	Urine
10	+	+	+	-	+	+	+	+	-	V1	Urine
11	+	+	+	+	+	-	+	+	-	V1	Urine
12	+	+	+	-	-	-	+	+	-	V1	Urine
13	+	+	+	-	+	-	+	+	-	V1	Urine
14	+	+	+	-	-	-	-	+	-	V3	Urine
15	+	-	-	+	+	-	+	-	+	V4	Urine
16	-	+	+	-	-	+	+	+	+	V2	Urine
17	+	+	+	-	-	+	+	+	+	V2	Urine
18	+	+	+	+	+	+	+	+	+	V2	Urine
19	-	+	+	-	-	-	-	+	-	V3	Urine
20	+	-	-	+	-	-	+	+	-	V1	Urine
21	+	+	+	-	-	-	+	+	-	V1	Urine
22	+	+	+	+	+	-	-	+	-	V3	Urine
23	+	+	+	-	-	-	+	+	-	V1	Urine
24	+	+	+	+	-	-	-	-	+	V6	Urine
25	+	+	+	-	+	-	+	+	+	V2	Urine
26	+	+	+	-	+	-	-	+	+	V5	Urine
27	+	+	+	-	-	-	-	+	-	V3	Urine

28	-	+	+	-	+	-	+	+	-	V1	Urine
29	-	-	-	+	-	-	+	+	+	V2	Urine
30	+	-	-	-	-	-	-	+	+	V5	Urine
31	+	+	+	+	-	-	+	+	+	V2	Urine
32	+	-	-	+	-	+	-	-	-	V7	Urine
33	-	+	+	+	-	+	+	+	-	V1	Urine
34	+	+	+	-	+	-	+	+	-	V1	Urine
35	-	+	+	-	-	-	-	+	-	V3	Urine
36	-	+	+	+	-	-	+	+	-	V1	Urine
37	+	-	-	+	-	-	-	+	-	V3	Urine
38	+	-	-	+	-	-	+	+	-	V1	Urine
39	+	+	+	+	+	-	+	+	-	V1	Urine
40	+	+	+	-	-	+	+	-	-	V8	Urine
41	+	+	+	-	-	-	+	-	+	V4	Urine
42	+	+	+	-	-	-	+	-	+	V4	Urine
43	+	+	+	-	-	+	+	-	+	V4	Urine
44	+	+	+	+	+	-	-	-	+	V6	Urine
45	+	+	+	+	+	-	+	+	+	V2	Urine
46	+	+	+	+	+	-	+	+	+	V2	Urine
47	+	+	+	-	-	+	+	+	-	V1	Urine
48	+	+	+	-	-	-	+	-	-	V8	Urine
49	+	+	+	-	+	+	-	-	-	V7	Urine
50	+	-	-	+	-	+	+	-	+	V4	Urine
51	-	+	+	-	+	-	+	+	+	V2	Blood
52	+	+	+	+	+	-	+	+	+	V2	Urine
53	+	+	+	+	-	+	+	+	+	V2	Sputum
54	+	+	+	-	+	-	+	-	+	V6	Blood
55	+	+	+	-	-	-	+	-	+	V4	Blood
56	+	+	+	+	-	+	+	-	+	V4	Blood
57	+	+	+	-	-	-	-	-	+	V4	Urine
58	+	+	+	-	+	+	+	+	-	V1	Blood

59	+	+	+	-	-	-	-	+	-	V3	Urine
60	+	+	+	-	+	+	+	+	-	V1	Urine
61	+	+	+	-	-	-	+	+	+	V2	Blood
62	+	+	+	-	-	-	+	+	+	V2	CSF
63	+	+	+	-	+	+	-	-	+	V6	Urine
64	+	+	+	-	+	+	+	-	+	V4	Sputum
65	+	+	+	-	-	-	+	-	+	V4	Blood
66	+	+	+	-	-	-	+	-	-	V8	Sputum
67	+	+	+	-	+	-	-	-	-	V7	Urine
68	+	+	+	-	+	-	+	+	-	V1	Blood
69	+	+	+	+	-	-	+	+	-	V1	Urine
70	+	+	+	+	-	-	+	+	+	V2	Blood
71	+	+	+	+	-	-	-	-	-	V7	Urine
72	+	+	+	-	-	-	+	-	-	V8	CSF
73	+	+	+	+	-	-	+	-	+	V4	Urine
74	+	+	+	-	-	-	-	+	-	V1	Urine
75	+	+	+	+	+	-	+	+	-	V3	Blood
76	+	+	+	+	+	-	+	-	+	V4	Urine
77	-	+	+	-	-	-	+	+	-	V1	Blood
78	+	+	+	-	-	-	+	-	-	V8	Urine
79	+	+	+	-	-	-	+	-	-	V8	Blood
80	+	+	+	+	-	-	+	-	-	V8	Blood
81	+	+	+	-	-	-	+	+	+	V2	CSF
82	+	+	+	+	-	-	+	-	-	V8	Urine
83	+	+	+	-	-	-	+	-	-	V8	Blood
84	+	+	+	-	-	-	+	+	-	V1	Urine
85	+	+	+	+	-	-	+	-	-	V8	Blood
86	-	+	+	+	-	-	+	-	-	V8	Sputum
87	+	+	+	-	-	+	+	+	-	V1	Blood
88	-	+	+	-	-	+	+	-	-	V8	Blood
89	+	-	-	+	-	-	+	-	+	V4	Urine

90	+	-	-	+	-	-	+	+	+	V2	Sputum
91	+	-	-	+	-	-	+	+	+	V2	Urine
92	+	+	+	-	-	-	+	-	-	V8	Urine
93	+	+	+	-	-	+	+	-	-	V8	Blood
94	-	+	+	-	-	-	+	-	+	V4	Urine
95	+	-	-	+	-	-	+	+	-	V1	Urine
96	+	+	+	-	+	+	+	+	+	V2	Blood
97	+	+	+	+	-	-	+	+	-	V1	Urine
98	+	+	+	-	-	-	+	+	+	V2	CSF
99	+	+	+	+	-	-	+	+	+	V2	Blood
100	+	+	+	-	-	+	+	-	-	V8	Sputum

***AcrAB* efflux pump and *mrkA* biofilm gene expression**

The eight MDR (1, 6, 10, 13, 17, 18, 23 and 25 isolates NO.) and non-MDR (30, 37, 38, 46, 89, 90, 91 and 95 isolates NO.) selected *K. pneumoniae* strains were used for *AcrA* and *mrkA* gene expression analysis. The results of Real Time PCR showed that *AcrA* and *mrkA* gene were up-regulated significantly in MDR isolates comparing to non-MDR isolates. There was a significant relationship between MDR isolates, *AcrA* and *mrkA* gene expression ($P < 0.05$) (Figure 2).

Rep-PCR typing

According to the dendrogram, Repetitive element sequence-based PCR (rep-PCR) revealed 11 distinct patterns of *K. pneumoniae* isolates (Figure 3). The 11 rep genotypes were designed rep1 to rep11. The rep type 4-7 were the most common and, followed by type rep 1, 9 and 3, which consisted non-MDR isolates (Group I: 30, 95, 90, 91, Group C: 37, 89, Group J: 38 and 46 isolates NO). The rep 5 type was unique and each contained one strain exclusively. Based on statistical correlation tests, the rep 2, 4, 5, 6 and 7 significantly correlates with MDR strains and virulence patterns ($P < 0.05$). The strains showed high similarity which may suggest that those isolates consist a clonal lineage ($P < 0.05$).

Discussion

Multidrug-resistant (MDR) *K. pneumoniae* strains are an important cause of several life-threatening infections, worldwide [32]. The extensive use of antimicrobial agents led to a high prevalence of MDR *K. pneumoniae* strains [33]. The increasing rate of *K. pneumoniae* strains resistant to multiple antimicrobials is a global public

health problem [34]. In this study, the prevalence of MDR *K. pneumoniae* isolates was 92%. The high rate of MDR strains was also shown in other studies. Moreover, 48% and 47% of *K. pneumoniae* strains were resistant to imipenem and meropenem, respectively. From the results, it can be concluded that there has been a significant increase in carbapenem resistant *K. pneumoniae* isolates in Iran. It seems that the production of carbapenemase and metallo-beta-lactamase have an important role in carbapenems resistance [35]. Our results is supported by some previous reports. Manjula et al, indicted 90.2% of isolates were MDR and the majority of MDR strains were resistant to a high range of antibiotics including penicillin, cephalosporin, fluoroquinolone, aminoglycoside, and sulfonamide [36].

One of the possible reason for high rate of antimicrobial resistance is lack of strict policies for use of antibiotics in Iran. Another mechanism of multidrug resistant is efflux pumps which used by *K. pneumoniae* strains [37]. The efflux pumps could reduce the intracellular concentration of antibiotics which is as an important cause of bacterial survival [38]. In our study, the AcrAB efflux pump is most common efflux pump in *K. pneumoniae* strains comparing to *mdtK* and it was significantly correlated with MDR phenotype. Our results are consistent with other reports which indicated that the multidrug efflux pump system (AcrAB-TolC) in *K. pneumoniae* strains is responsible for antibiotics especially fluoroquinolones such as ciprofloxacin, tetracycline and beta-lactam antibiotics in MDR isolates.

In addition, 77% of *K. pneumoniae* strains were biofilm former and 89% of biofilm former were MDR. Until now, it has been shown a significant correlation between MDR phenotype and the biofilm forming ability of *K. pneumoniae* strains [39]. In addition, the relationship between antibiotic resistance and biofilm formation has also been studied in strains grown under the antibiotic dose especially in sub-minimum inhibitory concentration [40]. There was a significant relationship between strains of *K. pneumoniae* isolated from urine and biofilm formation. Most of urine originate strains exhibited strong biofilm capacity. In the current study, *K. pneumoniae* strains isolated from clinical specimens harbored high prevalence of efflux pump, biofilm and virulence associated genes. The virulence associated genes were also dominant in MDR strains. Type 1 fimbriae (fimH-1) and Type 3 fimbrial adhesion (*mrkA* and *mrkD*) are the most common bacteria cells adhesive agent which can mediate the binding of *K. pneumoniae* strains to endothelial and epithelial cells of urinary tracts cause to urinary tract infections [41, 42]. Type 3 fimbrial adhesion plays an important role in the biofilm formation of *K. pneumoniae* strains, however, their exact role in the biofilm formation remains unclear [43]. The results of Nirwati et al, 2019 study on biofilm formation in *K. pneumoniae* strains isolated from clinical samples showed that 85.63% of the strains were biofilm and that is more than the results of our study [44]. In our study, the fimH, *mrkA* and *mrkD* were detected in all types of urine, blood and CSF isolates especially in urine isolates. The enterobactin biosynthesis gene (*entB*), serum resistance-associated outer membrane lipoprotein (*traT*) and regulators of mucoid phenotype A (*rmpA*) were detected in 80%, 62% and 48% of *K. pneumoniae* strains. The *traT* gene encodes an outer membrane protein which plays an important role in conjugation and inhibition of complement cascade and act as invasins [45]. Molecular typing of bacteria is a very suitable method for differentiation of microbial pathogens and among the typing methods, rep-PCR technique is widely used for genotyping of bacterial strains. [46]. In our study, out of 100 *K. pneumoniae* strains, rep-PCR could differentiated into 11 distinct patterns and most MDR strains were put in the same patterns. Our data confirmed the Lai et al results which showed pathogenic *K. pneumoniae* strains are heterogeneous, because of variation in genome sequences [47]. Our results showed the correlation of MDR strains with rep-PCR patterns. However, the rep-PCR revealed no statistically significant correlation with virulence type. In addition, the rep-PCR results showed that

the same rep-type in two studied hospitals indicated that the same clonal distribution of *K. pneumoniae* in two hospitals. Our findings can help to interpretation of MDR *K. pneumoniae* outbreaks associated with specific patterns in the future.

Conclusion

In our study, we reported the high prevalence of MDR *K. pneumoniae* strains with resistance to multiple antimicrobial agents, the ability to formed biofilm and presence of efflux pump, biofilm and virulence associated genes which can be a great barrier to treatment of *K. pneumoniae* related infections. Moreover, high genetic similarity of MDR strains in hospitals showed clonal dissemination of *K. pneumoniae* strains that requiring control tools. However, further studies are needed to investigate other epidemiological aspects of the *K. pneumoniae* strains.

Abbreviations

RND: Resistance Nodulation Division

MDR: multidrug-resistant

rep-PCR: Repetitive element sequence-based PCR

CSF: cerebrospinal fluid

MR-VP: (methyl red – Voges Proskauer)

CLSI: Clinical and Laboratory Standards Institute

Declarations

Availability of data and materials

All quantitative data generated or analysed during this study are included in this published article.

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Contributions

R. R conceive and designed the experiments. A. A performed the experiments, analyzed the results, done the statistical analyses and wrote the manuscript. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

Ethics approval for this study was obtained from Baqiyatallah University of Medical Sciences, Tehran, Iran permitted this study with number: IR.BMSU.REC.1397.056. Written informed consent was obtained from all patients participating in this study.

Consent for publication

Not applicable.

Conflict of Interest

The authors declare that there are no conflict of interest.

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Figures

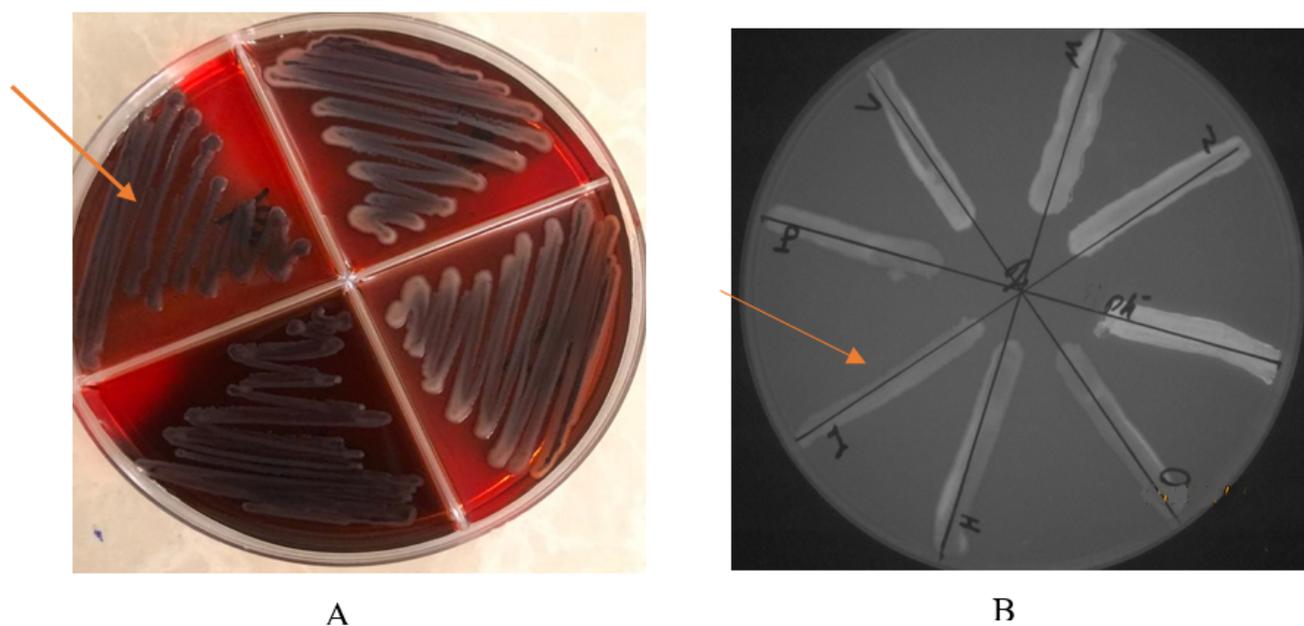


Figure 1

Congo red agar (A) and Cartwheel tests (B) for detection of biofilm producers and efflux pump system in *K. pneumoniae* strains. As can be observed the biofilm forming bacteria exhibited the black appearance in colony (Arrow). Moreover, the isolates had efflux pump didn't show fluorescent emission under UV transilluminator (Arrow).

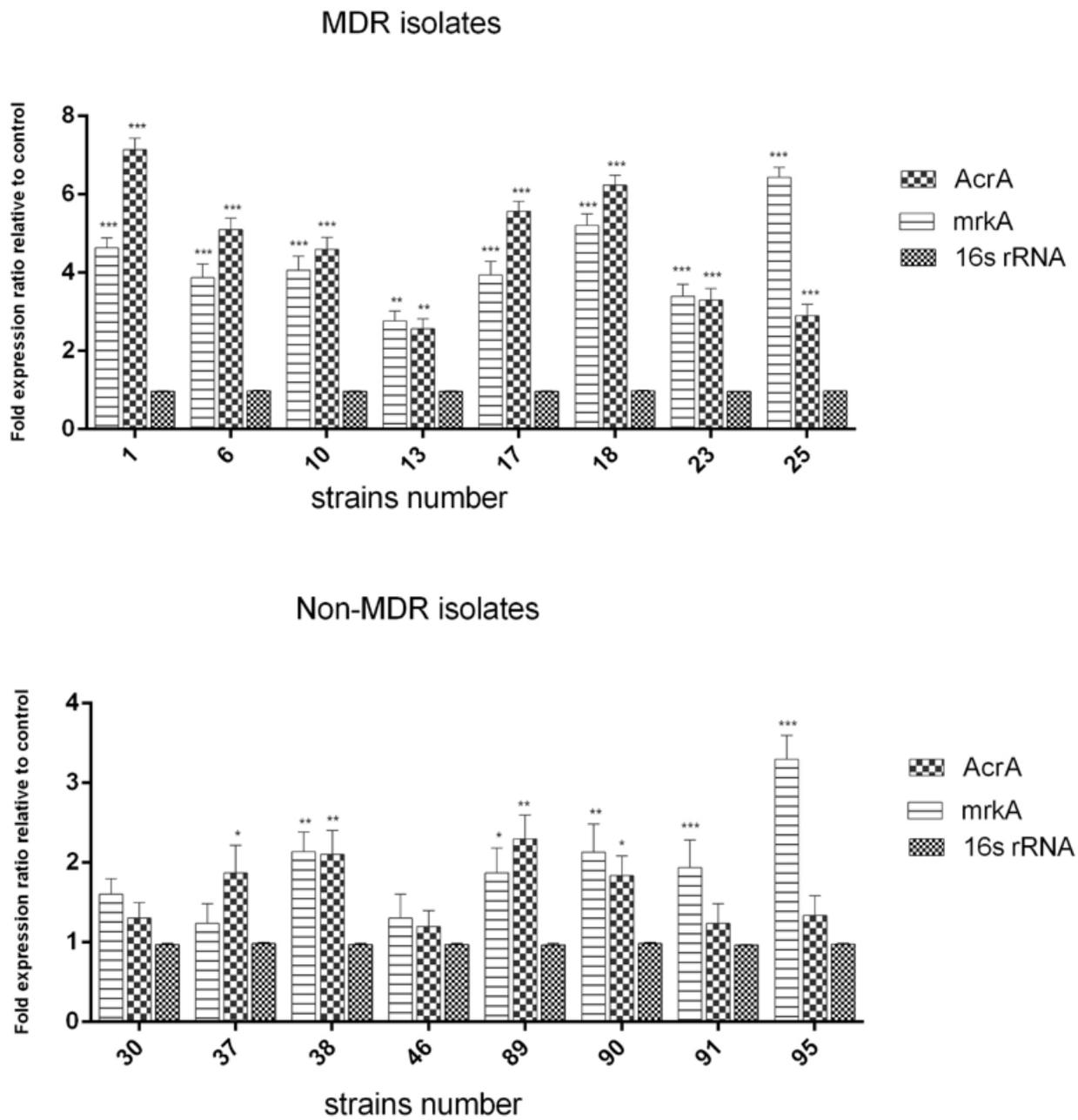


Figure 2

AcrA and mrkA relative gene expression among MDR and non-MDR strains as fold difference between AcrA, mrkA gene and 16S rRNA gene. Data are mean SD of three independent experiments. Significant difference specified as *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

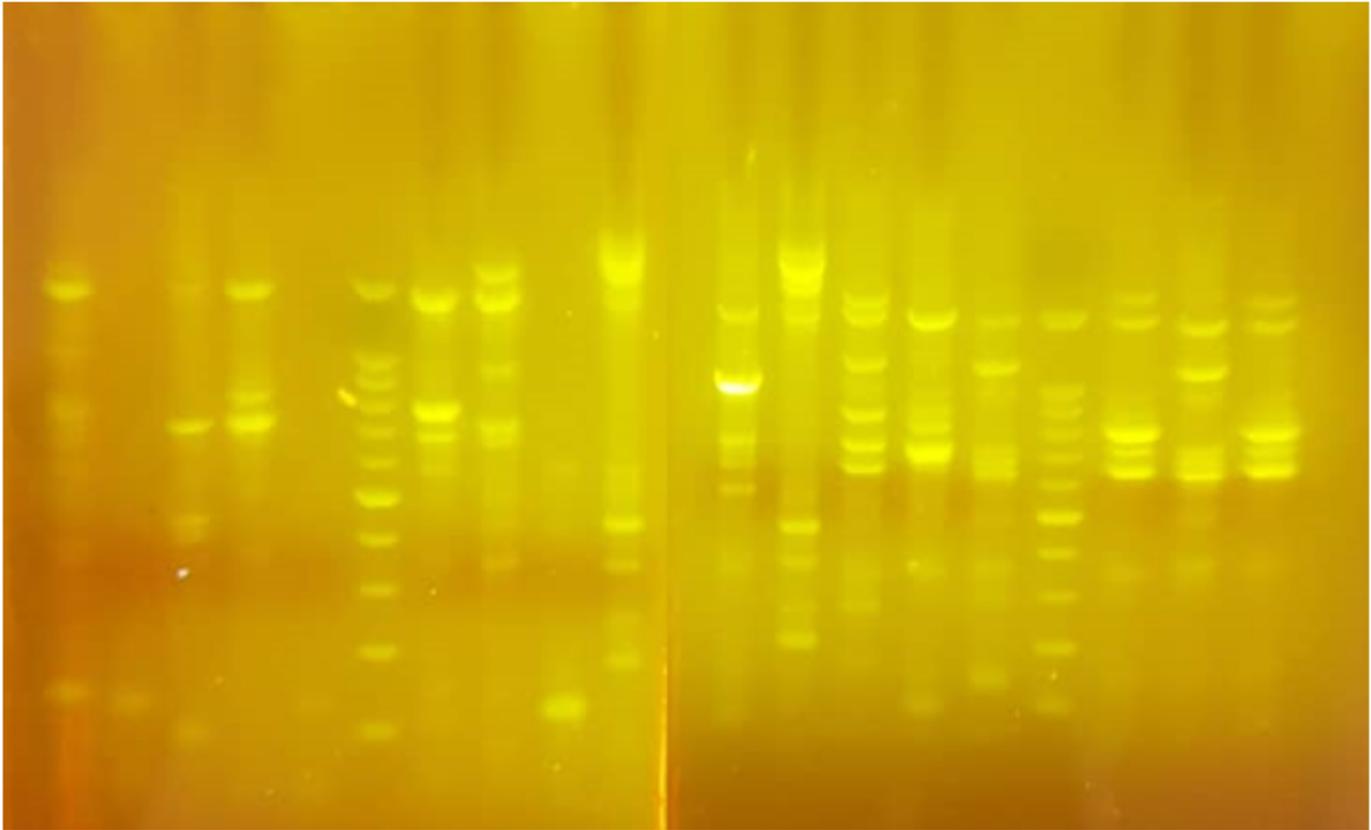


Figure 3

The rep-PCR fingerprints of *K. pneumoniae* strains.

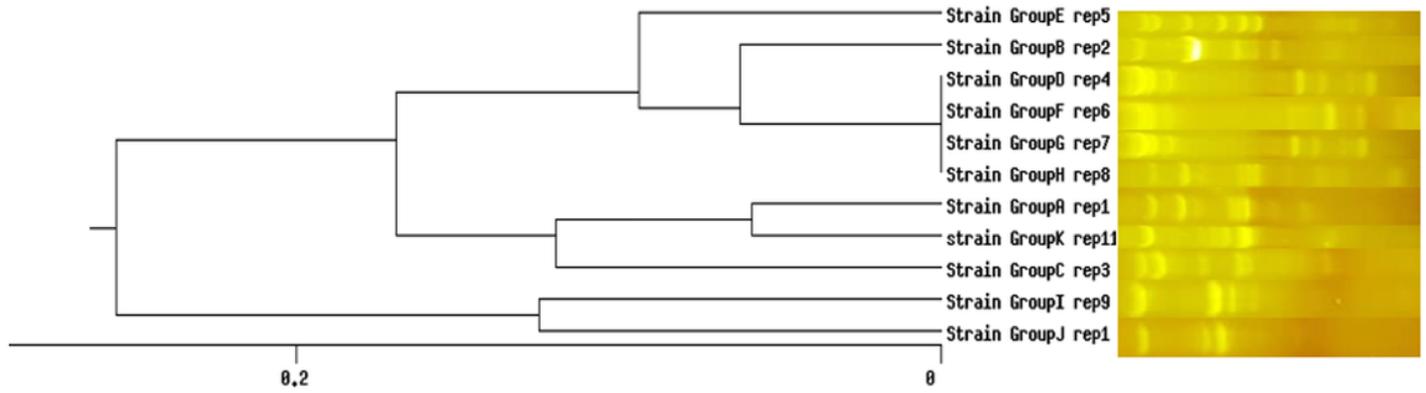


Figure 4

The 11 rep types identified by Rep-PCR in *K. pneumoniae* isolates using the UPGMA based on Dice similarity.