

Virulence Factors, Antibiotic Resistance patterns, and Molecular Types of Clinical Isolates of *Klebsiella Pneumoniae*

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Research

Keywords: *Klebsiella pneumoniae*, antibiotic resistance, ESBL, virulence genes, molecular typing

Posted Date: March 26th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-331542/v1>

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Version of Record: A version of this preprint was published at Expert Review of Anti-infective Therapy on October 28th, 2021. See the published version at <https://doi.org/10.1080/14787210.2022.1990040>.

Abstract

Background *Klebsiella pneumoniae* is armed with a wide range of antibiotic resistance mechanisms which mostly challenges effective treatment. Due to this fact, the aims of the current study were to identify the clinical strains of *K. pneumoniae* as well as to determine their phenotypes and molecular characterization related to antimicrobial resistance and virulence genes. Methods In this investigation, specimens from a hospital and different laboratories located in Shahr-e-Qods, Tehran, Iran were collected during a period of nine-month (December 2018 to August 2019). The isolated strains of *K. pneumoniae* were then identified through standard microbial and biochemical assays. Additionally, disk diffusion, combined disk, modified Hodge test and PCR were performed for antibiotic resistance of the strains and virulence genes profiling, respectively. The molecular typing was accomplished by ERIC-PCR. Results Eighty-four isolates of *K. pneumoniae* were identified and subjected to the study. Fifty-two percent of the isolated strains of *K. pneumoniae* were detected as multidrug resistant (MDR) pathotypes with the highest resistance to ceftriaxone (65%) and the lowest resistance to colistin (23%). Twenty-seven (52%) out of 52 (100%) MDR pathotypes of isolated *K. pneumoniae* were identified as ESBL producers. According to Modified Hodge Test (MHT) results, out of 24 resistant strains of isolated *K. pneumoniae* to imipenem and meropenem, 15 pathotypes (62.5%) were detected as KPC producers. The gene of blaCTX (encoding carbapenemase) with 96% ranked first, while the blaKPC gene with the prevalence of 71% ranked second among ESBL producers. The aminoglycoside resistance gene of Aac6-Ib showed the highest frequency with the prevalence percentage of 90%. The virulence genes of mrkD (94%) and magA (11%) were the highest and lowest among isolates, respectively. According to ERIC-PCR results the isolated strains of *K. pneumoniae* were divided into four clusters in which the cluster 4 was predominant group. Conclusions The high prevalence of antibiotic resistance and virulence genes in conjunction with a significant relationship between the strains reveals a high pathogenic capacity of the isolated pathotypes of *K. pneumoniae*. These findings emphasize the choose of more effective antibiotic regimens for treatment of infections caused by *K. pneumoniae*. Keywords: *Klebsiella pneumoniae*, antibiotic resistance, ESBL, virulence genes, molecular typing.

Introduction

K. pneumoniae is a gram-negative, non-motile bacillus of the Enterobacteriaceae family with a polysaccharide capsule, which is substantial in pathogenesis and its ability to prevent phagocytosis [1]. This bacterium is an opportunistic pathogen and one of the most common causes of nosocomial infections ranging from pneumonia, meningitis, liver abscess, urinary tract infection (UTIs) and wound infection, to bacteremia and sepsis [2, 3]. *K. pneumoniae* pathogenesis is associated to the presence of certain virulence genes that encode virulence factors and allow it to attack the mammalian immune system and lead to a variety of diseases [4]. Through years, frequent use and contact with antibiotics in hospitals have created antibiotic-resistant *K. pneumoniae* strains, limiting available treatment options for medical intervention against *Klebsiella* infections, and developing many problems for medical staff and patients [5]. In the absence of correct and complete knowledge of antibiotic resistance mechanisms in

bacteria, particularly *K. pneumoniae*, not only is the treatment extremely challenging, but it can inadvertently assist in the intensifying drug resistance.

Antibiotic resistance in gram-negative bacteria occurs due to enzymatic and non-enzymatic mechanisms [6,7]. Enzymatic pathways are imposed by the expression of antibiotic inactivating enzymes, while non-enzymatic pathways develop mainly because of gene mutations which result in resistance due to changes in efflux pumps, membrane permeability or target molecules [8]. Genes encoding resistance enzymes can be derived either from the bacterium itself or from Miniature Inverted Transposable Elements (MITEs) such as the plasmid encoding beta-lactamases or aminoglycoside-modifying enzymes [9,10]. The presence of genes encoding beta-lactamase and carbapenems enzymes are the most important mechanisms of antibiotic resistance in *K. pneumoniae* [11]. Extended-Spectrum Beta-Lactamases (ESBLs) hydrolyze many beta-lactam antibiotics and therefore pose major problems in the treatment of dangerous bacterial infections [12].

Resistance in *K. pneumoniae* isolates are mainly associated with ESBLs, as these enzymes lead to multidrug resistance (MDR) [13]. ESBL enzymes can inactivate broad-spectrum cephalosporins, monobactams, and penicillin such as class A beta-lactamases (*TEM-1*, *TEM-2*, and *SHV-1*), which form resistance to ampicillin, amoxicillin, and first-generation cephalosporins. Mutations in these genes trigger resistant to third generation cephalosporins [14, 15]. In addition to penicillin and cephalosporins, *CTX-M* enzymes are also a group of ESBLs which impose resistance to oxyimino -beta-lactams [16]. Another group of beta-lactamases commonly found in *K. pneumoniae* isolates is *K. pneumoniae* carbapenems (KPCs), which inflict resistance to carbapenem antibiotics [17]. A carbapenems identified in the accessory genome of *K. pneumoniae* is New Delhi Metallo-Beta-Lactamase 1 (NDM-1), a class B Metallo-beta-lactamase (MBL) encoded by the plasmid, and other examples of b-lactamases include carbapenems (*bla*_{KPC} and *bla*_{OXA48}) [18]. It could be worth mentioning that resistance to aminoglycosides is related to overexpression of drug metabolizing enzymes such as aminoglycoside N-6-acetyltransferase-Ib AAC (6)-Ib as well as 16S rRNA methyltransferases such as *ArmA* and *RmtB* [19]. Virulence-associated genes include coding regulators of mucoid phenotype A (*rmpA*), type 1 and type 3 adhesins (*fimH-1*, *mrkD*), aerobactin (iron siderophore) synthase (*iucC*), bacteriocin biosynthesis [enterobactin (*entB*) and yersiniabactin (*irP-1*)], and serum-dependent outer membrane lipoprotein (*traT*), which due to the number and function of these genes, they will play a significant role in the pathogenicity of *K. pneumoniae* strains isolated from nosocomial infections [20, 21].

The increasing prevalence of antibiotic resistance in pathogens of human and animal populations is now one of the most significant global health issues. Therefore, due to the development of resistance to antibiotics in *K. pneumoniae* and its various mechanisms, investigation of resistance pattern may lead to appropriate prescription of antibiotics, which results in more rapid improvement of related nosocomial infections [22, 23]. Additionally, this could in turn help inhibit new resistance patterns, by omitting the antibiotics with borderline sensitivity from therapeutic regimens in certain regions. Thus, the current study aimed to evaluate the phenotypic and genotypic nature of antibiotic resistance and virulence genes in *K.*

pneumoniae strains isolated from clinical specimens in Shahr-e-Qods hospital. The relationship between the strains was also investigated by PCR (ERIC-PCR).

Materials And Methods

Bacterial strains

In the present study, during a 9-month period from December 2018 to August 2019, clinical samples suspected to *K. pneumoniae* infections were collected from the patients admitted to the 12th Bahman Hospital and other laboratories in Shahr-e-Qods, Tehran, Iran.

Bacterial culture

Following initial culture on Eosin-methylene blue agar (EMB), blood agar, and MacConkey agar media, the samples were incubated for 24 hours at 37 °C. The resultant bacterial colonies were examined by different diagnostic and biochemical tests, including Triple Sugar Iron (TSI), Simon citrate, urease and Methyl Red Voges Proskauer (MRVP), and then cultured on Sulfur-Indole-Motility (SIM) medium for further biochemical analysis. All identified *K. pneumoniae* strains were stored in skim milk agar stock for further analysis. [24].

Antibiotics susceptibility testing

Disc fusion (Kirby-Bauer) method was used for the evaluation of antibiotic resistance by culturing the isolates on the Mueller-Hinton agar medium. Using following antibiotic discs: including tetracycline (30 µg), imipenem (10 µg), gentamicin (10 µg), colistin (10 µg), amikacin (30 µg), ceftazidime (20 µg), cefotaxime (30 µg), ceftriaxone (30 µg), nitrofurantoin (32 µg), aztreonam (30µg), fosfomycin (32 µg), azithromycin (15 µg), and piperacillin/tazobactam (100.10 µg) .The newly cultured isolated colonies (16-24 hours) were dissolved in sterile saline to obtain the desired homogeneous suspension in comparison to McFarland 0.5 standard unit. Then, the suspension was cultured on Mueller-Hinton agar whole surfaces and the antibiotic discs were placed at regular intervals (7 discs per plate and 2 plates per microbe were used). After incubating the plates for 18-24 hours at 37 °C, the diameter of the growth inhibition zone was investigated. The diameters of the antibiogram growth inhibition zones were measured according to the CLSI guidelines and the strains were classified based on how they reacted to each drug into three groups: sensitive, semi-sensitive, and resistant. The minimum inhibitory concentration (MIC) for colistin was also measured by Vitek systems [25-26].

ESBL screening by Combined Disk Test (CDT)

K. pneumoniae isolates strains resistance to third generation cephalosporin, suspected as ESBLs producers were tested by the Combined Disc Test (CDT). Using 0.5 McFarland turbidity standards, a proper bacterial suspension was prepared from newly grown (16-24 hours) colonies of isolates and cultured on Mueller-Hinton agar using a sterile cotton swab. Afterward, discs containing ceftazidime (30 µg), cefotaxime (30 µg) and cefepime (30 µg), and ceftazidime + clavulanic acid (30 µg/ 10 µg),

cefotaxime (30 µg) + clavulanic acid (30 µg/10 µg) and cefepime (30 µg) + clavulanic acid (30 µg/10 µg) were used. The antibiotic discs were placed on the Mueller- Hinton agar and the plates were incubated at 37 °C for 18-24 hours. After incubation, the diameter of the growth inhibition zone around the cephalosporin discs with and without clavulanic acid was measured and compared. An increase in the inhibition zone diameter of > 5mm was proved as ESBLs production [27].

Modified Hodge Test (MHT) for detection of carbapenemase (KPC)-producing isolates

The modified Hodge Test (MHT) assay is a simple phenotypic test to detect the presence of the carbapenemase enzyme in bacteria [28]. Thus, based on a 0.5 McFarland turbidity standard, a suspension of *E. coli* ATCC 25922 was prepared in 5 ml of Mueller-Hinton broth or saline. The *E. coli* ATCC 25922 suspension was then diluted 1:10 by adding 0.5 ml of it to 4.5 ml of Mueller-Hinton broth or saline. Next, a bacterial grass culture was performed from the diluted solution on Mueller-Hinton agar and dried for 3-5 minutes at room temperature. A disc of meropenem (10 µg) or ertapenem (10 µg) was then placed in the center of the plate. In a straight line, from the edge of the disc to the edge of the plate, the desired isolate was cultured. The same procedure was repeated for the other isolate in another direction. Three organisms were examined on each plate. Incubation was performed overnight at 35 °C for 24 hours. Then, the presence of cloverleaf was examined at the intersection of the studied organism and *E. coli* 25922 in the growth inhibition zone, indicating a positive result [29, 30].

DNA extraction

The bacterial genome was extracted using the boiling method. The bacteria were firstly cultured in 10 ml of BHI broth medium and incubated overnight. After examining the turbidity of tubes at 600 nm, proper OD was ≥ 4 for genome extraction. The culture media were transferred to 2 ml microtubes and centrifuged at 9000 rpm for 10 minutes. Then, the supernatant was rinsed out and 400 mL of distilled water was added, followed by boiling for 15 minutes at 100 °C in a Bain-Marie bath. The microtubes were centrifuged again for 10 minutes at 6000 rpm, and 100 µl of the supernatant, known as the extracted genome, was collected using a sampler and transferred to a new microtube. Finally, the DNA adsorption rate was investigated using a spectrophotometer at 260 nm, and extracted genomes was stored at -20 °C [31].

Molecular detection of antibiotic resistance and virulence genes by Polymerase Chain Reaction (PCR)

In this study, the detection of genes involved in antibiotic resistance and virulence, including *bla*_{CTX-M}, *bla*_{SHV}, *bla*_{TEM}, *bla*_{KPC}, *bla*_{NDM}, *aac* (6')-Ib, *armA*, *IrP-1*, *rmpA*, *magA*, *mrkD* were investigated using PCR. All the primers used in this study are listed in Table 1. The PCR protocols were performed according to the manufacturer's instructions (Amplicon 2X). The PCR products were loaded on 1% agarose gel and the green bands were observed by UV light and it was analyzed using a gel documentation system [23]. Lane M was contained DNA marker (100 bp DNA ladder) for a faster identification of PCR products or amplicons. Then, the electrophoresis was performed with a potential difference of 80 volts for one hour and the stained gels were inspected using a Bio-Rad Gel Doc EZ Imager (Bio-Rad, VIC, AUS).

Table 1. Sequences of primers used in the study.

Target genes	Primer Sequence (5' → 3')	Amplicon size (bp)	Tm °C	Reference
<i>bla</i> _{CTX-M}	For: TTTGCGAGTGCAGTACCAGTAA Rev: CTCCCCTGCCGGTTTTATC	519	56	59
<i>bla</i> _{SHV}	For: TTA ACTCCCTGTTAGCCA Rev: GATTTGCTGATTTGCCCC	796	57	60
<i>bla</i> _{TEM}	For: ATGAGTATTCAACATTTCCG Rev: CTGACAGTTACCAATGCTTA	867	55	60
<i>bla</i> _{KPC}	For: ATG TCACTGTATCGCCGTCT Rev: TTTTCAGAGCCTTACTGCCC	893	58	61
<i>bla</i> _{NDM}	For: GGTTTGGCGATCTGGTTTTTC Rev: CGGAATGGCTCATCACGATC	1113	55	62
<i>aac</i> (6')-Ib	For: ATGACTGAGCATGACCTTG Rev: AACCATGTACACGGCTGG	484	56	63
<i>ArmA</i>	For: CCGAAATGACAGTTCCTATC Rev: GAAAATGAGTGCCTTGGAGG	846	58	64
<i>Irp-1</i>	For: TGAATCGCGGGTGTCTTATGC Rev: TCCCTCAATAAAGCCCACGCT	238	60	55
<i>RmpA</i>	For: ACTGGGCTACCTCTGCTTCA Rev: CTTGCATGAGCCATCTTTCA	531	58	65
<i>MagA</i>	For: GGTGCTCTTTACATCATTGC Rev: GCAATGGCCATTTGCGTTAG	1238	56	66
<i>MrkD</i>	For: CCACCAACTATTCCCTCGAA Rev: ATGGAACCCACATCGACATT	226	56	55

Molecular typing

The molecular relationship and genetic diversity of the isolates was determined by ERIC-PCR using the primers ERIC-F (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC-R (5'-AAGTAAGTGA CTGGGGTGAGCG-3'). ERIC-PCR reactions were prepared in a volume of 25 µl, including 1 µl of each primer (final concentration 2 pmol / µl), 12.5 µl of Master Mix (Applied Biosystem), 9.5 µl of deionized water, and 4 µl of pattern DNA. At the beginning of the ERIC-PCR reaction to denature the template DNA strands, 95 °C was applied for 5

min and then the target DNA sequences were proliferated during 30 cycles including denaturation at 92 °C for 30 s; annealing at 52 °C for 1min; extension at 65 °C for 8 min; Also, a final extension step at 65 °C for 8 min and final storage at 4 °C. The ERIC-PCR reaction products were evaluated on 1.5% agarose gel [32,33]. Comparison of ERIC-PCR patterns was performed. The presence of a band with the number 1 and the absence of a band with the number 0 were encoded in a matrix. Analysis of ERIC-PCR typing results was done according to the number and weight of bands observed in the product electrophoresis of each sample. Finally the Amplification of the products which had been run, was drawn with the UPGMA algorithm on these websites: /http://insilico.ehu.es/dice_upgma, <http://genomes.urv.cat/UPGMA/index.php> [34,35].

Results

Bacterial strains and antibiotic susceptibility profile

Based on the primary culture results of the samples, 84 specimens were identified as *K. pneumonia*. The results of antimicrobial susceptibility tests indicated the highest resistance against ceftriaxone (65%), cefotaxime (64%) and Fosfomycin (60%), respectively. The lowest levels of resistance were observed for colistin (23%), nitrofurantoin (25%), imipenem (28%) and azithromycin (32%), respectively. The Table 2 represents the complete results of all antibiotic disk tested in this study. More than 50% of the strains were multidrug resistance (MDR).

Table 2. Antibiotic susceptibility pattern of *K. pneumoniae* isolates based on CLSI (n = 84).

Sensitive no (%)	Semi-sensitive no	Resistant no (%)	Antibiotics
50(59)	5	29(34)	Amikacin
26(30)	7	51(60)	Fosfomycin
34(40)	4	46(54)	Aztreonam
54(64)	1	29(34)	Tetracycline
28(33)	1	55(65)	Ceftriaxone
31(36)	4	49(58)	Ceftazidime
29(34)	1	54(64)	Cefotaxime
57(67)	0	27(32)	Azithromycin
64(76)	0	20(23)	colistin
55(65)	5	24(28)	Imipenem
56(66)	7	21(25)	Nitrofurantoin
44(52)	2	38(45)	Gentamicin
42(50)	3	36(42)	Piperacillin / Tazobactam

Phenotypic detection of ESBL and carbapenemase producing K. pneumoniae

The results of the combined disk test indicated that out of 52 third generation cephalosporin resistant isolates, 27 (52 %) were ESBL positive. On the other hand, the results of MHT test revealed that, out of 24 samples resistant to imipenem and meropenem, 15 (62.5%) *K. pneumoniae* isolates were carbapenems-positive with the ability to produce carbapenems enzymes.

Virulence factors and antibiotic resistance-associated genes

Out of 84 samples, 81 isolates (96%), 79 isolates (94%) and 77 isolates (91%) had *bla*_{CTX-M}, *bla*_{SHV} and *bla*_{TEM} genes respectively. Which most of these genes were observed in all resistance isolates. Additionally, our results showed that among the genes involved in KPC resistance, *bla*_{KPC} was positive in 60 isolates (71%), of which 34 cases were among 36 resistant samples. The *bla*_{NDM} gene was also detected in 50 strains (60%), so that 33 of the 36 resistant samples had *bla*_{NDM} gene. Our data indicated that *bla*_{KPC} and *bla*_{NDM} genes had almost the same incidence among resistant *K. pneumoniae*, and there was no significant difference in their frequency (Fig. 1-2).

Examination of the genes involved in aminoglycoside resistance resulted in the detection of the *Aac6-Ib* gene in 76 isolates (90%), so that out of 36 resistant isolates, 34 samples were positive. Furthermore, the

armA gene was identified in 60 samples (71%), where 30 cases of which were among 36 resistant strains. We found that the *Aac6-1b* and *armA* genes had a high frequency among resistant specimens, while *Aac6-1b* was meaningfully ample than the *armA* gene (Table 3, Fig. 1).

Findings related to virulence genes showed that 50 isolates (60%) were positive for *Irp-1* gene, which was 34 out of 36 resistant samples. 70 samples (83%) were positive for *rmpA* gene, in this way, out of 36 resistant samples, 32 positive isolates were found. Moreover, in terms of the *magA* gene, 10 samples (11%) were positive with 6 cases related to the resistant strains. The *mrkD* gene was also positive in 79 samples (94%), so that out of 36 resistant isolates, 31 samples were positive. Our findings demonstrated that *mrkD*, *rmpA*, *Irp-1* and *magA* genes had the highest frequency among resistant strains, respectively, where *mrkD* was significantly more abundant compared to the other tested genes (Table 3, Fig. 1-2).

Table 3. The frequency of virulence and antibiotic resistance genes in the tested samples.

Genes	All isolates (n=84) (%)	Resistant isolates(n=36) (%)
ESBL resistance	—	
<i>bla</i> _{CTX-M}	(81)96%	35<96%
<i>bla</i> _{SHV}	(79)94%	35<96%
<i>bla</i> _{TEM}	(77)91%	35<96%
KPC resistance	—	
<i>bla</i> _{KPC}	(60)71%	(34)94%
<i>bla</i> _{NDM}	(50)60%	(33)91%
Aminoglycoside resistance	—	
<i>aac(6')-1b</i>	(76)90%	(34)94%
<i>armA</i>	(60)71%	(30)83%
Virulence genes	—	
<i>mrkD</i>	(79)94%	(31)86%
<i>rmpA</i>	(70)83%	(32)88%
<i>Irp-1</i>	(50)60%	(34)94%
<i>magA</i>	(10)11%	(6)16%

ERIC-PCR differentiated the isolates into four clusters, G1- G4, with 70% similarity (Figure 3). In the strains that were studied, the maximum number, 40, belonged to the G4 cluster, and the minimum, 8, belonged to the G3 cluster. Ten of the studied strains were in G2, and 26 strains in the G1 cluster (Figure 3, 4).

Discussion

The present survey revealed that the picture of the antibiotic resistance, virulence and genetic relationship between clinical strains of *K. pneumoniae* recovered from the patients admitted to the 12th Bahman Hospital and other laboratories in Shahr-e-Qods. *K. pneumoniae* causes several types of infections in humans, including respiratory, bloodstream and urinary tract infections (UTIs), which are commonly seen in hospitalized or immunocompromised patients [36,37]. These infections are often treated with beta-lactams and other effective antibiotics against *Enterobacteriaceae*. Nevertheless, the antibiotic-resistant and highly pathogenic species of *K. pneumoniae* are rapidly spreading around the world [38]. Bacterial resistance depends vastly on populational and geographical factors. Thus, the body of different people can provide different environments for the bacteria to grow, multiply and be affected by the drugs [39]. Therefore, the study of bacterial resistance in a specific population can provide an appropriate overview of effective drugs for the healthcare staff to provide an effective antibiotic regimen to ensure improved recovery of patients.

Our findings demonstrated that out of 84 isolates identified as *K. pneumoniae*, more than 50% of the samples had multi-drug resistance, with the highest resistance against ceftriaxone, cefotaxime and Fosfomycin, respectively. Notably, in the tested isolates we observed a high susceptibility to colistin, nitrofurantoin, azithromycin, imipenem, and tetracycline. In line with the present study, Rocha *et al.*, [40] reported that *K. pneumoniae* ESBL-positive isolates sampled from ICU patients showed the highest resistance to ceftriaxone. Kim *Det al.*, [41] found that the resistance rates of *Klebsiella pneumoniae* to cefotaxime, cefepime, and carbapenem were 38-41%, 33-41%, and <0.1-2%, respectively, from 2013 to 2015. According to them, all isolates are sensitive to imipenem. The highest susceptibility was reported to gentamicin and piperacillin/tazobactam, respectively. In a different report compared to our results, the highest sensitivity was observed against ceftriaxone, ciprofloxacin and gentamicin, respectively. However, in the present study, the highest resistance was to ceftriaxone [42].

Moreover, our results of the combined disk test indicated that out of 52 resistant samples, 27 (52%) cases were positive for ESBL, almost corroborating evidence with the findings of a study by Rupinder *et al.*, [43] who stated that ESBL production was observed in 48% of *E. coli*, 44% of *K. pneumoniae* and 50% of *P. aeruginosa* isolates in a tertiary hospital in Patiala, Punjab. While according to studies conducted in Iraq, 81.39% of *K. pneumoniae* isolates were ESBL producers [44]. This discrepancy between the reported frequencies can be due to the geographical diversity, the type of strains and the isolation under study or other different reasons.

The results of the genes involved in ESBL resistance showed that the prevalence of *bla*_{CTX-M}, *bla*_{SHV} and *bla*_{TEM} genes out of 84 samples were 81 (96%), 79 (94%) and 77(91%), respectively. Therefore, the highest

frequency was related to *bla*_{CTX-M} gene. In the study of Pishtiwan *et al.*, [45] the frequency of *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} genes was 64.7%, 35.2% and 41.1%, respectively. Also, according to Ugbo *et al.*, [46] the prevalence of *bla*_{SHV}, *bla*_{TEM} and *bla*_{CTX-M} was identified to be 55%, 35% and 45% respectively. These values were lower than the percentages of the present study and these results disagreed with the reports of studies by Bajpai *et al.*, [47] in which the gene that predominated was *bla*_{TEM} (48.7%), followed by *bla*_{TEM} (7.6%) and *bla*_{SHV} (5.1%). It is worth mentioning that the genotypic approach can be the method of choice for distinguishing ESBL-producing strains from Enterobacteriaceae because phenotypic tests for ESBL detection only confirm ESBL production.

Analysis of the detection of KPC resistance genes showed that *bla*_{KPC} (71%) was ampler than *bla*_{NDM} (60%) without any significant differences in resistant species. Liu *et al.*, [48] stated that among the tested isolates of *K. pneumoniae*, *bla*_{NDM} and *bla*_{KPC} genes had the highest frequency, respectively. This discrepancy may be attributed to genetic variations among strains associated with human populations and antibiotic regimens. In accordance with our data, Xiufeng *et al.*, [49] reported that *bla*_{KPC} and *bla*_{NDM} were highly detected in carbapenems-resistant *K. pneumoniae* samples isolated from a Chinese hospital.

In the case of genes involved in aminoglycoside resistance, the *Aac6-Ib* and *armA* genes had a frequency of 90% and 71%, respectively, while among the resistant samples, *Aac6-Ib* was the most frequently detected gene. Aligned with our results, Cirit *et al.*, [50] also reported *Aac6-Ib* to have the highest frequency among the genes involved in aminoglycoside resistance in nosocomial *K. pneumoniae* isolates. A study of highly aminoglycoside-resistant *K. pneumoniae* and *Klebsiella oxytoca* from an inpatient in Okinawa, Japan, with no known history of travelling overseas was accomplished. Genome sequencing analysis showed that these isolates harbored *armA*, which encodes a 16S rRNA methylase, *ArmA*, that confers pan-aminoglycoside resistance [51]. In a study conducted by Li *et al.*, [52] in China, out of 223 isolates of *K. pneumoniae*, 13 isolates (5.8%) contained *armA* and 8 isolates (3.6%) contained *rmtB*. 110 isolates of *K. pneumoniae* were phenotypically resistant and after PCR, 11.8% of the isolates contained *armA* gene and 7.3% of the strains contained *rmtB* gene. These values are lower than our findings about the *armA* gene.

According to the results of the modified Hodge test, out of 24 samples resistant to imipenem and meropenem, 15 (62.5%) samples were positive for carbapenems. Examination of virulence genes showed that among 84 samples, *mrkD*, *rmpA*, *Irp-1* and *magA* genes showed the frequency of 94%, 83%, 60% and 11%, respectively; among 36 resistant samples, *mrkD*, *rmpA*, *Irp-1* and *magA* genes have the highest frequency, respectively, while *mrkD* was significantly more abundant compared to the other genes. Based on these data, *mrkD* is evidently highly associated with multidrug resistance. In line with our findings, Liu *et al.*, [53] identified *mrkD* as the most common virulence gene with the prevalence of 100%. In 2004, an initial study of the *magA* gene was performed by Fang *et al.*, [54] who identified the gene as a virulence factor in the pathogenesis of *K. pneumoniae*. In this study, the *magA* gene was observed in 52 invasive strains (liver abscesses) and 15 non-invasive strains. El Fertas-Aissani *et al.*, [55] obtained the opposite result from the present report. In this regard none of the studied strains carried the *magA* gene. They also

examined the *mpA* gene, which contained 3.7% of the 54 strains of *K. pneumoniae* isolated from different clinical specimens, which is a lower percentage than the present study. In another study, Liu *et al.*, [48] reported that *mpA* and *magA* were the most abundant genes among the 117 isolates of *K. pneumoniae*, respectively, and Fu *et al.*, [56] observed the same frequency of genes in their study.

Since the presence of some virulence factors can be involved in the pathogenicity of bacteria, knowledge of the existence of these factors and their prevalence can be a good way to identify and treat the studied strain. The phylogenetic tree was drawn based on the UPGMA algorithm and the genetic relationship between the isolates was identified, which the isolates were differentiated into four clusters, G1- G4, with 70% similarity. Moreover, in the strains that were studied, the maximum number, 40, belonged to the G4 cluster, and the minimum, 8, belonged to the G3 cluster.

According to the research of Ferreira *et al.*, [57] the dendrogram obtained from ERIC-PCR results showed a genetic relationship between 25 *K. pneumoniae* studied. In their study, the clusters were determined using the method (UPGMA) and dice's similarity coefficient. Based on this, it was found that although the bacteria were isolated from different patients, *K. pneumoniae* in the bloodstream had a high genetic relationship with each other. In studies by Firmo *et al.*, [34] ERIC-PCR analysis of 16 isolates of *K. pneumoniae* showed that only three isolates (18.8%) (K4-R3, K6-R3 and K7-R3) present a single band pattern, which is 100% genetically similar, i.e. there was a clonal relationship. The other 13 isolates of *K. pneumoniae* presented heterogeneous profiles with a maximum of 40% similarity. El-Badawy *et al.*, [58] showed that most isolates have different origins by genotyping *K. pneumoniae* isolates using ERIC-PCR method. 32 isolates belonged to 18 different single roots, indicating that the prevalence of *K. pneumoniae* in different parts of the hospital was due to poor infection control.

Conclusion

The results of the present study revealed that the high prevalence of resistance strains of *K. pneumoniae*. The highest resistance was observed to ceftriaxone, cefotaxime and Fosfomycin and the lowest resistance was reported against colistin, nitrofurantoin, emipenem and azithromycin, respectively. Furthermore, the *bla*_{CTX}, *bla*_{KPC}, *Aac6-Ib* and *mrkD* seemed to be the most highly associated genes with multidrug resistance. The high prevalence of antibiotic resistance and virulence genes in conjunction with a significant relationship between the strains reveals a high pathogenic capacity of the isolated pathotypes of *K. pneumoniae*.

Our findings demonstrated a direct relationship between the frequency of the genes involved in the development of virulence and resistance and also can provide a highly effective model for physicians of relevant medical centers to prescribe more suitable antibiotic regimens aimed at improved clinical efficiency and faster recovery of patients. These findings emphasize the choose of more effective antibiotic regimens for treatment of infections caused by *K. pneumonia*.

Declarations

Acknowledgement

Thanks to guidance and advice from "Clinical Research Development Unit of Baqiyatallah Hospital". Research reported in this publication was supported by Elite Researcher Grant Committee under award number 982964 from the National Institutes for Medical Research Development (NIMAD), Tehran, Iran.

- **Ethics approval and consent to participate**

This study was approved based on ethic code: IR.NI.1398.172

- **Consent for publication**

The authors have confirmed that informed consent was obtained from all patients.

- **Availability of data and materials**

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

- **Competing interests**

The authors declare that they have no competing interests in this section.

- **Funding**

Research reported in this publication was supported by Elite Researcher Grant Committee under award number [982964] from the National Institutes for Medical Research Development (NIMAD), Tehran, Iran.

- **Authors' contributions**

MA performed laboratory analysis and drafted all sections of the text. PB revised the first round of manuscript. RR designed the study and revised the manuscript. All authors had full access to all of the data (including statistical reports and tables) in the study and can take responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final manuscript.

- **Acknowledgements**

Thanks to guidance and advice from "Clinical Research Development Unit of Baqiyatallah Hospital", Tehran, Iran.

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Figures

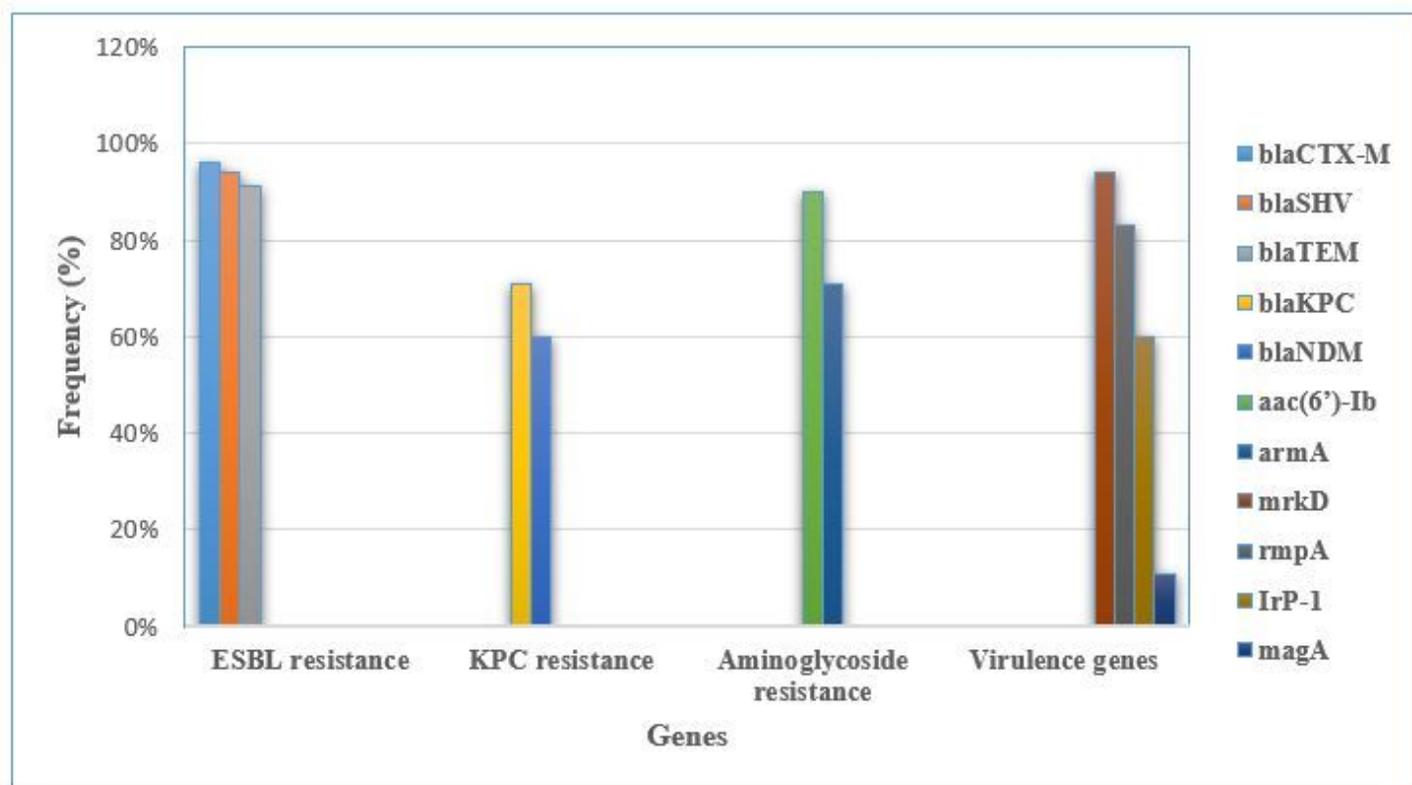


Figure 1

The frequency of virulence and antibiotic resistance genes in resistant strains of *K. pneumoniae*.

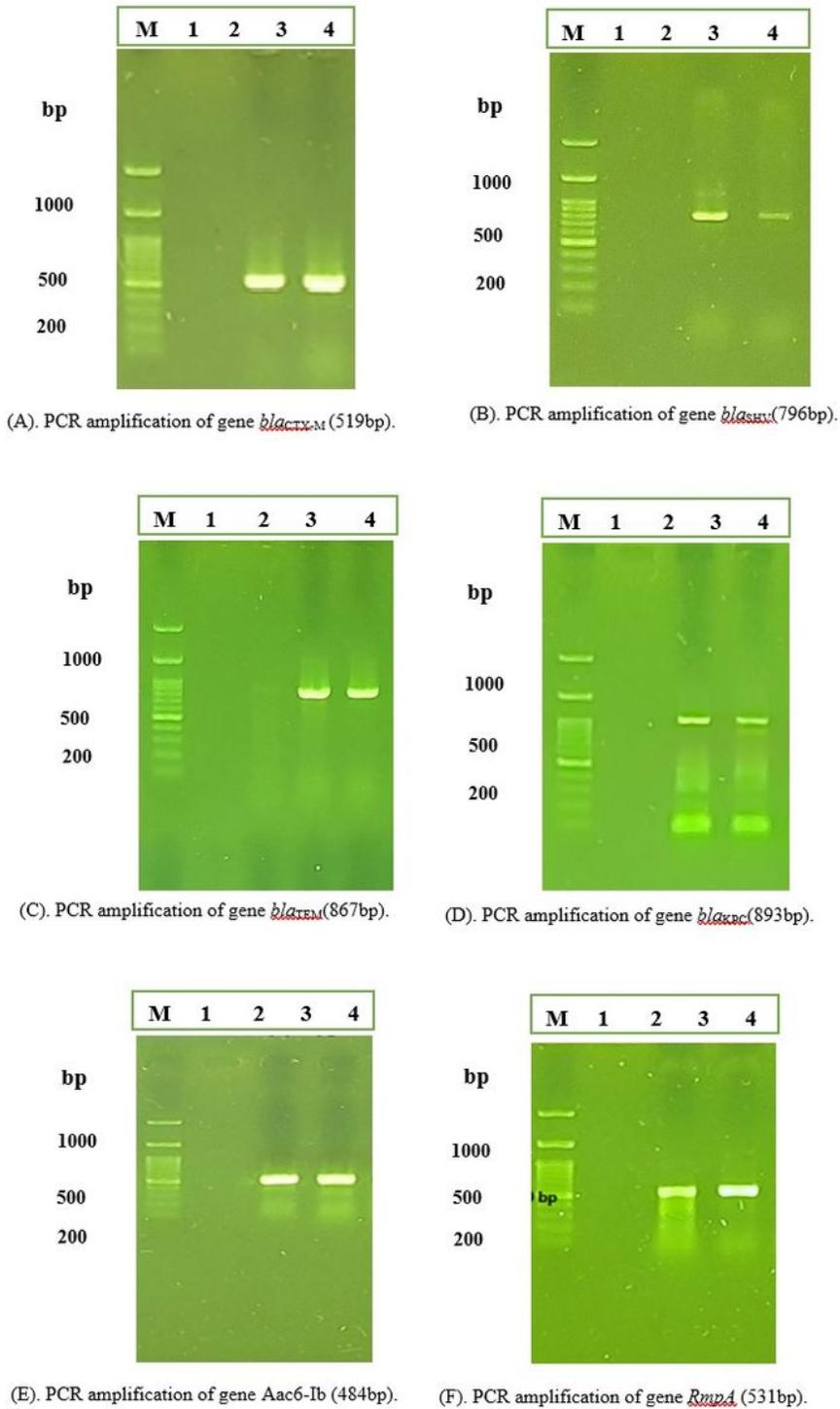


Figure 2

PCR amplification of some representative genes in some isolates of *k. pneumoniae*. Lane M: 100 bp ladder, next numbered lanes amplified with diagnostic *bla_{CTX-M}* (A), *bla_{SHV}* (B), *bla_{TEM}* (C), *bla_{KPC}* (D), *aac (6')-Ib* (E) and *rmpA* (F) genes, show negative results at lanes 1,2 and positive results at lanes 3,4 with: 519, 796, 867, 893, 484 and 531bp, respectively.

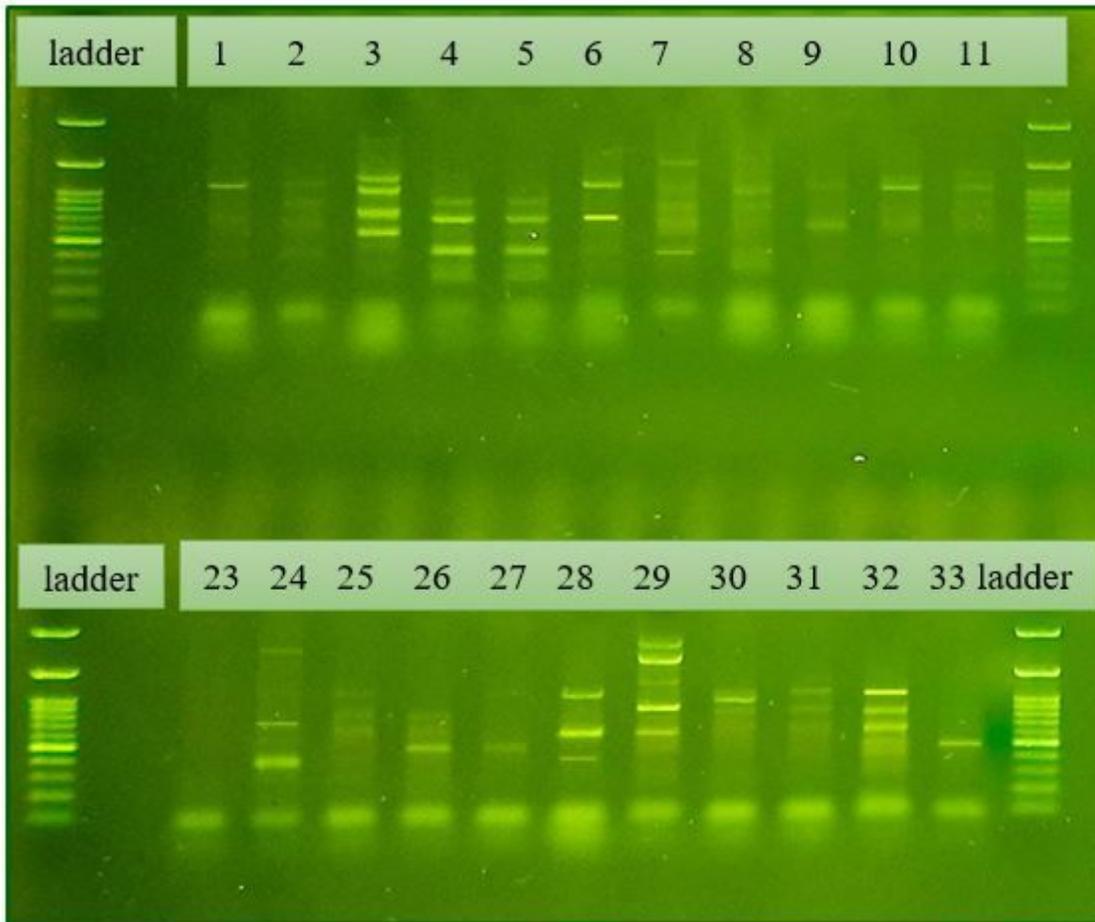


Figure 3

ERIC-PCR Phylogenetic analysis of some representative isolates.

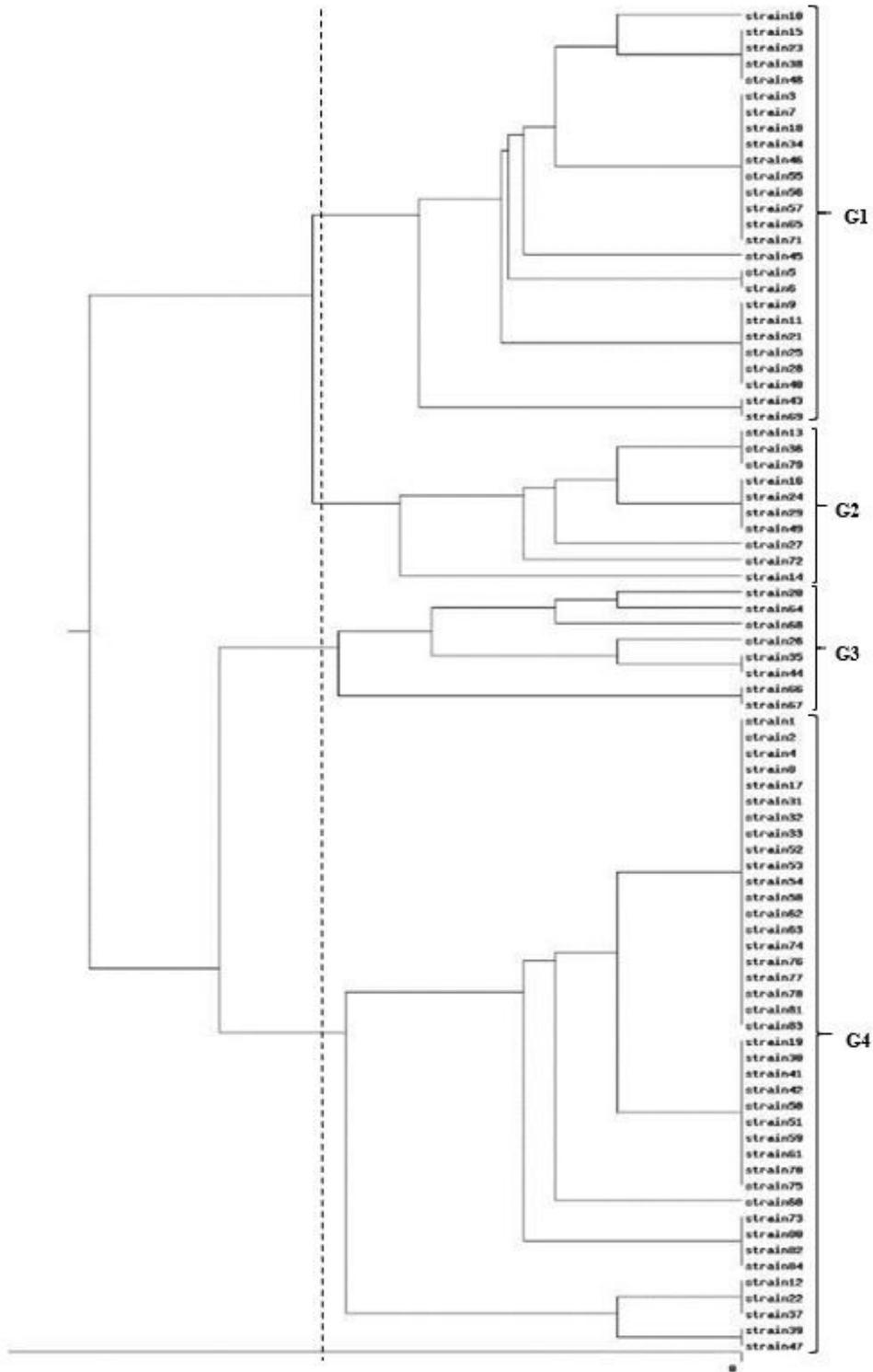


Figure 4

Dendrogram obtained by the UPGMA clustering technique, illustrating the genetic similarity of *K. pneumoniae* isolates by (ERIC) genotyping.