Original Article

Dissemination of Multidrug-Resistant, Class I and II Integrons and Molecular Typing of CTX-M-producing *Klebsiella pneumoniae*

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Abstract

Introduction: *Klebsiella pneumoniae* (*K. pneumoniae*) is an important opportunistic pathogen causes serious community and hospital-acquired infections, which is highly resistant to antibiotics. We aimed to determine the frequency of multidrug resistant (MDR) and molecular typing of clinical isolates of *K. pneumoniae*. **Methodology:** One hundred isolates of *K. pneumoniae* were collected from clinical samples in three general hospitals in Kermanshah. The antimicrobial susceptibility and extended-spectrum beta-lactamases (ESBL) production of isolates were determined using disk diffusion and combined disk methods, respectively. The bla_{CTX-M} gene, class I and II integrons were detected using polymerase chain reaction. The bla_{CTX-M} positive isolates were selected for genotyping using pulsed-field gel electrophoresis (PFGE). **Results:** MDR phenotype was observed in 56% of isolates. The 40% of isolates were ESBL positive and 35 isolates contained bla_{CTX-M}. Class I and II of integrons were detected in 50 (89.2%) and 39 (69.6%) of MDR isolates, respectively. PFGE patterns of *K. pneumoniae* bla_{CTX-M} positive isolates indicated 19 clusters (X_{1-19}) with different genotype patterns. **Conclusions:** The study findings highlight the concern of circulating MDR strains of *K. pneumoniae* with bla_{CTX-M} and class I and II integrons in Kermanshah hospitals. The presence of integrons among isolates may facilitate the spread of new resistance genes in this bacterium. Therefore, surveillance for the spread of MDR strains of this bacterium is recommended in hospitals.

Keywords: *Extended-spectrum beta-lactamases, integron, Klebsiella pneumoniae, pulsed-field gel electrophoresis*

Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is an important nosocomial pathogen with the potential of causing severe morbidity and mortality, particularly in intensive care units, surgical wards and among pediatric patients.[1,2] Hospital-associated factors, including mechanical ventilation, catheterization, parenteral nutrition, and lengthy hospitalization have been identified as the risk factors for *K. pneumoniae* infections.[3] This bacterium is one of the most prevalent agents of nosocomial infections with multidrug-resistant (MDR) characteristics.[4,5]

The role of integrons is very crucial in the spread and assemblage of resistant genes in pathogenic bacteria. The presence of integrons among Gram-negative bacteria has been increasingly reported worldwide.[6] Integrons are genetic features that contain gene cassettes transferable to other mobile genetic elements such as plasmids in the bacterial genome. Over recent years and following the widespread use of the broad-spectrum beta-lactam antibiotics, outbreaks of infections caused by extended-spectrum beta-lactamase producing *K. pneumoniae* have been widely reported throughout the world.[1,2,7] The resistance of this bacterium to third-generation cephalosporins was first described in 1983[8] and since then have been widely reported worldwide.[1,2] The prevalence of extended-spectrum beta-lactamases (ESBLs) among bacteria is a serious alarm since the majority of them are multiresistant. The surveillance of local dissemination of resistant strains of bacteria, in particular among hospital environments has become an important epidemiological tool to control infection. Study the bacterial genotypic homology can provide a better understanding of sources and dissemination patterns of *K. pneumoniae* infections.[8]

Various methods for bacterial genotyping have been developed using different molecular techniques. However,

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Pulsed-Field Gel Electrophoresis (PFGE) has been widely used as a standard method for *K. pneumoniae* typing.[9,10] In this method, only relatively major genetic events can result in changes of fingerprinting patterns.[11] Given the spread of multidrug resistance strains of *K. pneumoniae* in our region, study the Molecular typing of isolates can provide a better view of bacterial dissemination. We aimed to determine the frequency of MDR and molecular typing of clinical isolates of *K. pneumonia*e bla_{CTX-M} positive.

Methodology

Bacterial isolates

This descriptive cross-sectional study was performed on 100 available and nonduplicate isolates of *K. pneumoniae*. They were collected during 11 months (2012 and 2013) of an outbreak among hospitalized patients in three general hospitals (Imam Khomeini, Taleghani and Imam Reza) in Kermanshah. The isolates were from patients admitted in Kermanshah hospitals and no extra charges or procedures were imposed on the patients for this study. All relevant medical ethics were considered in this study.

All isolates were identified by bacteriological and biochemical tests[12] followed by confirmation using API 20 E Kit according to the manufacturer's instructions, and results were interpreted using API 20 E V4.1 identification software (biomerieux, France).

Antibiotic susceptibility test

Antimicrobial susceptibility testing for 15 antibiotics from nine different antibiotic categories was carried out by disk diffusion method on Mueller Hinton Agar (Merck, Germany) according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI).[13] The antibiotics tested were ampicillin (10 μg), cefazolin (30 μg), gentamicin (10 μg), tobramycin (10 μg), cefotaxime (30 μg), ceftriaxone (30 μg), ceftazidime (30 μg), cefpodoxime (10 μg), azetronam (30 μg), ertapenem (10 μg), imipenem (10 μg), meropenem (10 μg), pipracilin-tazobactam (100/10 μg), ciprofloxacin (30 μg), and co-trimoxazole (30 μg) (Mast Group, UK). MDR was defined as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories.[14]

Extended-spectrum beta-lactamases Phenotypic confirmatory test

Phenotypic confirmatory test was performed using combination disk method according to the CLSI recommendations.[13] In this method, cefotaxime (30 μg) and ceftazidime (30 μg) alone and in combination with clavulanic acid (10 μg) were used. If the inhibited zone diameter increased ≥5 mm for either antimicrobial agents in combination with clavulanic acid it was considered phenotypic positive for ESBL.[13] *K. pneumonia*e ATCC 700603 was used as a positive control and *E. coli* ATCC 25922 was used as a negative control.

Polymerase chain reaction amplification

The bacterial genome was extracted by boiling method and used as DNA template for polymerase chain reaction (PCR).[15] The DNA of ESBL producing isolates was targeted for the bla_{CTX-M}, however PCR amplification of Class I and II integrons was carried out on DNA of MDR isolates using the specific primers (SinaClon, Iran) listed in Table 1.[16-18] The amplified products were visualized by ethidium bromide stained 1% agarose gel under ultraviolet (UV) transilluminator (Bio-Rad, USA).

Pulsed-field gel electrophoresis

The clonal relatedness between bla_{CTX-M} positive isolates was investigated by pulsed-field gel electrophoresis. PFGE was carried out according to a previously described protocol with some modifications.[19] *K. pneumonia*e isolates and *Salmonella enterica* serovar Braenderup H9812 (As DNA marker) genome were digested with 20U of *Xba*I (Fermentas, Lithuania). After *Xba*I digestion of bacterial genomes, they were loaded into a 1% Low electroendoosmosis agarose (Merck, Germany). Electrophoresis was performed using a CHEF MAPPER apparatus (Bio-Rad, USA) at 14°C for 22 h. The following conditions were used for electrophoresis: initial switch time, 5 s; final switch time, 35 s; included angle, 120°; voltage gradient, 6 V/cm; ramping factor, linear. The gels were stained by ethidium bromide and visualized under UV light using Gel Doc apparatus (Bio-Rad, USA).

Table 1: Primers used in this study

Primer	Sequence (5́-3́)	Annealing temperature (°C)	Expected amplicon size (bp)	Reference
bla_{CTX-M}	F: TTTGCGATGTGCAGTACCAGTAA	51	544	[16]
	R: CGATATCGTTGGTGGTGCCATA			
Integron I	F: CAGTGGACATAAGCCTGTTC	55	160	[17]
	R: CCCGAGGCATAGACTGTA			
Integron II	F: TTGCGAGTATCCATAACCTG	55	280	[17]
	R: TTACCTGCACTGGATTAAGC			
5cs-3cs	F: GGCATCCAAGCAGCAAG	55	Variable	[18]
	R: AAGCAGACTTGACCTGA			

F: Forward; R: Reverse

Software analysis

The DNA fragment patterns were analyzed using Gelcompar II version 6.6 software (Applied maths, Belgium). The Dice coefficient was used to calculate similarities, and the unweighted paired group method based on the average linkages was used for cluster analysis. A cluster of isolates was defined to include all isolates with ≥80% similarity of their DNA patterns according to the Tenover's criteria.[11]

Statistical analysis

Data were recorded and entered into an Excel file. Statistical analyses were performed using SPSS software (version 16). Variables were analyzed using Chi-square test. A $P < 0.05$ was set as the statistical significance of all analyses. Simpson's Index of Diversity (D value) was calculated using equation.

$$D = 1 - \left(\frac{\sum n(n-1)}{N(N-1)}\right)$$

Results

The clinical samples tested included urine (n = 54, 54%), burn (n = 15, 15%), respiratory tract secretions (n = 15, 15%), and others (blood, wound, and ascitic fluid) (n = 16, 16%). They were collected form 59 (59%) female and 41 (41%) male with the average age of 39.5 ± 2.26 years old.

The antibiotic resistance of isolates is presented in Figure 1. Resistance to most antibiotics except carbapenem was significantly higher in ESBL producing isolates (P = 0.001). MDR and ESBL production were 56% and 40%, respectively. Among isolates, the highest prevalence of ESBL was in ICU (35.9%) and burn ward (26.4%). Forty (71.4%) were MDR isolates which of them ESBL producer. Class I and II integrons were found in 89.2% and 69.6% MDR isolates, respectively. In 35 isolates (62.5%) both class I and II genes were present. The bla_{CTX-M} was found in 35% of isolates. Gene cassette was detected at 100% of MDR isolates.

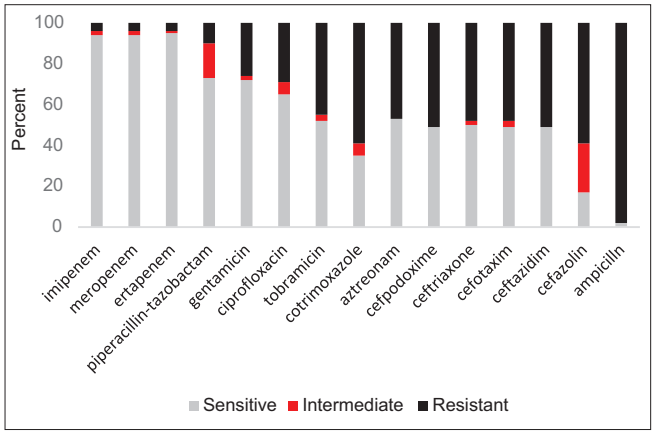


Figure 1: Antibiotic susceptibility of nosocomial isolates of *Klebsiella pneumoniae* measured by disk diffusion

A significant relationship was detected between bla_{CTX-M} and Class II integron (P = 0.039). As well as a significant association was revealed between bla_{CTX-M} with resistance to cefotaxime (P = 0.001), ceftriaxone (P = 0.001), ceftazidime (P = 0.005), cefpodoxime (P = 0.005), and aztreonam (P = 0.001).

According to the DNA fingerprinting of isolates, 19 clusters (X_{1-19}) were differentiated which included 10 clones and 9 unique clusters [Figure 2 and Table 2].

The ICU isolates were from respiratory tract secretions and urine samples, and infectious ward were from wound and blood samples. Cluster numbers from X_{11} to X_{19} each one had a unique genotype and the majority of their strains (>50%) were from the infectious ward. The Simpson's diversity index for the isolates was 0.9603.

Discussion

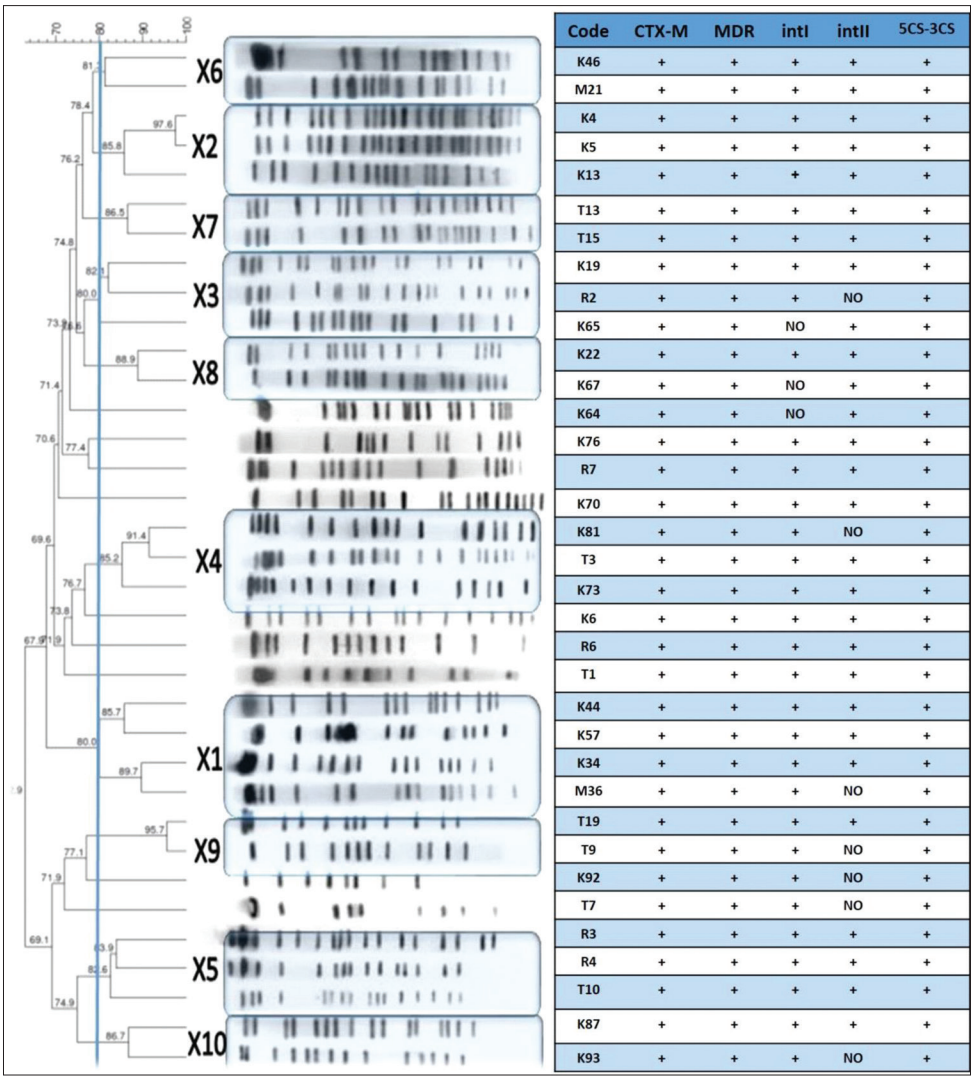
The increasing prevalence of clinical MDR isolates has been associated with higher morbidity and mortality rates. The rate of MDR among *K. pneumoniae* isolates in the present study is similar to previous research results reported.[20] However, resistance to carbapenems is still low and therefore, this group of antibiotics is effective against *K. pneumoniae* isolates.

Plasmid-mediated ESBLs have been found more frequently in *K. pneumoniae* strains than in other Enterobacteriaceae species.[21] Integrons associated ESBL genes, in isolates of *K. pneumoniae*, suggesting that the genetic mobile structures harbouring them are widespread. Our results indicated that the rate of ESBL-producing isolates of *K. pneumoniae* was high which may reflect the dissemination of resistant genes in hospitals. This is consistent with the previous research results that showed the ESBL-producing isolates were more common among hospitalized patients more likely exposed to antimicrobial agents such as third generation

Table 2: The distribution of clones (with at least 2 isolates) among hospital wards

Cluster number	Number of isolates	Hospitals					
		A			B	C	
		Burn	ICU	Infectious	ICU	ICU	Surgery
X1	4	1	2	1			
X2	3	1	1	1			
X3	3	2	1				
X4	3		1	1		1	
X5	3				2		1
X6	2	1	1				
X7	2					1	1
X8	2	2					
X9	2	2					
X10	2		2				
Sum	26	9	8	3	2	2	2

A: Imam Khomeini; B: Taleghani; C: Imam Reza; ICU: Intensive Care Unit



Code	CTX-M	MDR	intI	intII	5CS-3CS
K46	+	+	+	+	+
M21	+	+	+	+	+
K4	+	+	+	+	+
K5	+	+	+	+	+
K13	+	+	+	+	+
T13	+	+	+	+	+
T15	+	+	+	+	+
K19	+	+	+	+	+
R2	+	+	+	NO	+
K65	+	+	NO	+	+
K22	+	+	+	+	+
K67	+	+	NO	+	+
K64	+	+	NO	+	+
K76	+	+	+	+	+
R7	+	+	+	+	+
K70	+	+	+	+	+
K81	+	+	+	NO	+
T3	+	+	+	+	+
K73	+	+	+	+	+
K6	+	+	+	+	+
R6	+	+	+	+	+
T1	+	+	+	+	+
K44	+	+	+	+	+
K57	+	+	+	+	+
K34	+	+	+	+	+
M36	+	+	+	NO	+
T19	+	+	+	+	+
T9	+	+	+	NO	+
K92	+	+	+	NO	+
T7	+	+	+	NO	+
R3	+	+	+	+	+
R4	+	+	+	+	+
T10	+	+	+	+	+
K87	+	+	+	+	+
K93	+	+	+	NO	+

Figure 2: Phylogenetic relationship among isolates of extended-spectrum beta-lactamases-producing strains of *Klebsiella pneumoniae* using pulsed-field gel electrophoresis data. The strains were clustered using UPGMA. CTX-M: bla_{CTX-M} gene, MDR: Multidrug Resistant, intI: Integron class 1, intII: Integron class 2

cephalosporins.[22] In Asia, the prevalence of ESBL-positive *K. pneumoniae* isolates varies within different regions. The ESBL frequency in neighboring countries of Iran varied from 31% to 85%.[23] Although in most of the studies, the ESBL prevalence was higher than our study.[23-26]

bla_{CTX-M} has been found to be widely disseminated among clinical Enterobacteriaceae such as *Escherichia coli* and *K. pneumoniae*.[27] The prevalence of CTX-M type producing *K. pneumoniae* in Asia is variable among countries.[28] The higher prevalence of bla_{CTX-M} among our isolates indicates the more dissemination of this class of ESBL in our region. We observed the production of ESBLs in about 71.4% of our MDR isolates and all isolates contained bla_{CTX-M} were MDR which indicate the cluster of resistant genes. The rate of class I and II integrons in our isolates was consistent with the results of previous studies.[29,30]

Molecular typing indicated two distinct features among genotypic patterns of *K. pneumoniae* strains; first, the strains with genotypic diversity and the second strains with similar genotypes. The genotypic polymorphism can reflect the genetic diversity of isolates or the various origins of them. The presence of strains with a similar genotypic pattern in our isolates may show the dissemination of strains among hospital patients, in particular in intensive care and burn wards. The hospitalized patients such as burned patients are at increased risk of infection with various nosocomial pathogens[31] due to the destruction of the skin barrier, suppression of immune system, and invasive procedures.[32] Strains with similar genotypic patterns from different hospitals may suggest the bacterial spread of patients transferring to different hospitals.

The calculated values for Simpson's diversity indicated the diversity of our strains. The D value is between 0 and 1, in which the 1 represents infinite diversity and 0 no diversity.[33]

The genetic diversity of our isolates showed that most strains were genetically different, indicating the dissemination of resistant strains of *K. pneumoniae*. In the same way, several studies between 2008 and 2012 on *K. pneumoniae* isolates

in Iran indicated the genotype diversity among isolates of this bacterium.[23,28,34-37] However, there are studies that have reported less diversity and more genotype similarity among isolates.[38-41] One explanation could be the fact that the most isolates in the above study were from limited sources in one hospital. The epidemiology of ESBL-producing *K. pneumoniae* is complex, and the genetic agents encoding the varied ESBL behave in different ways.[42]

No distinct association was found between the resistant phenotypes and pulsotypes by cluster analysis of the 35 *K. pneumoniae* strains. As expected, there was no significant (P = 0.29) relation among strains in terms of association between genotypes with ESBL production and antibiotic resistance phenotypes. This issue can be explained by the fact that the most ESBL and antibiotic-resistant genes are carried on plasmids that are not mostly large enough to make a difference in PFGE patterns (40–50 kb).[43]

Conclusions

The clonal diversity of isolates carrying resistance genes suggests the strain transmission may be responsible for the recent spread of *K. pneumoniae* infection in hospitals. The results of our study suggest dissemination of *K. pneumoniae* MDR strains with $bla_{\text{CTX-M}}$ and class I and II integrons in Kermanshah hospitals. Given the ability of integrons for spreading and expressing of newly acquired genes in bacteria, the high frequency of these gens in *K. pneumoniae* in our region is alarming. Therefore, it is crucial the spread of resistant genes, in particular, integrons, in *K. pneumoniae* regularly test and report in hospitals.

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Conflicts of interest

There are no conflicts of interest.

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