

Molecular epidemiology of ESBL-producing *Klebsiella pneumoniae* isolates in intensive care units of a tertiary care hospital, North of Iran

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Abstract: The emergence of extended-spectrum beta-lactamases (ESBL) producing strains become a great concern, because few antimicrobial agents remain active against them. Due to the lack of data on the genotyping characteristics and antibiotics resistance of clinical isolates of *Klebsiella pneumoniae* in the north of Iran, this study aimed to determine the occurrence of ESBL-producing isolates and their molecular characteristics in order to analyses their epidemiological relationships. This cross-sectional study performed on 60 *K. pneumoniae* isolates which were recovered from different clinical specimens within May and November 2016. Isolates were identified by standard microbiologic tests and confirmed by API 20E strip. Antimicrobial susceptibility testing was carried out by disk diffusion method. The genetic relatedness among the isolates was assessed by RAPD-PCR. Totally, the lowest level of susceptibility was toward amoxicillin/clavulanat, and nalidixic acid. On the other hand, the highest level of susceptibility was toward imipenem (86.7%). The rate of ESBL-producing isolates was 45% (27/60). There was a significant association between production of ESBLs and higher antibiotic resistance in tested isolates. The RAPD-PCR dendrogram revealed 5 major clusters with a similarity of 80% which indicates the high relatedness of the studied isolates. Twenty-one isolates out of the 27 ESBL-producing isolates were clustered in cluster A. In summary, results showed the high prevalence of multiple-drug resistant and ESBL-producing *K. pneumoniae* isolates in our ICUs. Also, results revealed a significant similarity between ESBL-producing isolates that necessitate restricted infection control policies and rational prescription and use of antibiotics.

Key words: *Klebsiella pneumoniae*; Antibiotic resistance; ESBL; MDR; RAPD typing.

Introduction

Klebsiella pneumoniae is a facultative anaerobic Gram-negative rod bacterium, and membrane of the *Enterobacteriaceae* family (1). *K. pneumoniae* is an opportunistic pathogen that accounts for a significant proportion of nosocomial infections including urinary tract infections (UTIs), pneumonia, septicemia, and soft tissue infections (2, 3). Recently, due to widespread and indiscriminate use of antibiotics drug-resistant strains has been increasing dramatically, which often lead to clinical failure, prolonged hospitalization, and increased risk of morbidity and mortality (3, 4).

Extended-spectrum β -lactam antibiotics have been widely used for the treatment of serious Gram-negative infections, particularly *K. pneumoniae* (5). However, the emergence of extended-spectrum beta-lactamases (ESBL) producing strains become a great concern, because few antimicrobial agents remain active against them (6-8). In this regard, carbapenem possesses a broad spectrum of activity and remain an important therapeutic option against drug-resistant strains (9, 10). The specific ESBL-producing strains have different genetic characteristics which possible their identification at the molecular level (5). This genetic diversity in the different ESBL-producing bacteria may cause to specific characteristics associated with pathogenesis, antibiotic resistance pattern, response to therapy and infection

control (5, 9).

Typing methods for discriminating the different type of bacteria even in the same species are useful epidemiological tools in infection prevention and control (11). The use of genotype markers, including plasmid analysis and PCR-based methods may provide suitable data for demonstrating differences between individual bacteria (12, 13). Among the PCR based methods, random amplified polymorphic DNA (RAPD) is a rapid, and low cost method which has proven as a valuable typing method for generating distinct DNA profiles (13).

The knowledge of the spread of ESBL-producing strains has epidemiological importance in order to monitor the broad spreading of both their pathogenicity and multi-resistance. Due to the lack of data on the genotyping characteristics and antibiotics resistance of clinical isolates of *K. pneumoniae* in the North of Iran, this study aimed to determine the occurrence of ESBL-producing isolates of *K. pneumoniae* and their epidemiological relationships.

Materials and Methods

Study design and bacterial isolates

In this cross-sectional study, 60 clinical isolates of *K. pneumoniae* were collected from hospitalized patients at a different part of Razi hospital, Rasht, Iran, and were recovered from various clinical sources between May

and November 2016. The study was approved by regional Ethics Committee and was in accordance with the declaration of Helsinki. The enrolled isolates were from urine (n = 40), blood (n = 7), sputum (n = 6), CSF (n = 4), and wounds (n = 3) specimens. The samples were cultured on MacConkey agar and Blood agar and recovered isolates identification was performed with standard microbiological tests and confirmed by API 20E strip (API-bioMérieux, France).

Antimicrobial susceptibility testing and ESBL detection

Susceptibility to 17 antimicrobial agents was determined using disk diffusion method on Muller Hinton agar (Merck, Germany) in accordance to the clinical and laboratory standards institute (CLSI) recommendation (14). The tested isolates were categorized as susceptible, intermediate-resistant or resistant based on CLSI criteria. Commercially available disk loaded with the following antibiotics were used: amoxicillin-clavulanat (20/10µg; AUG), cefepime (30 µg; CPM), cefotaxime (30 µg; CTX), ceftriaxon (30 µg; CRO), ceftazidime (30 µg; CAZ), ceftazidime (30 µg; CAZ), cefixime (5 µg; CFM), aztreonam (30 µg; AZM), imipenem (10 µg; IMI), tetracycline (30 µg; T), ciprofloxacin (5 µg; CIP), nalidixic acid (30 µg; NA), ofloxacin (5 µg; OFX), nitrofurantoin (30 µg; NI), cephalothin (30 µg; KF), co-trimoxazole (25 µg; TS), gentamicin (10 µg; GM). Multiple-drug resistant (MDR) isolates were estimated according to previously described definitions (15).

All isolates were tested for ESBL production using the double-disk synergy test using ceftazidime (30 µg) and cefotaxime (30 µg) alone and in combination with clavulanic acid (10 µg) disks as described by CLSI guidelines (14). *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive control strains, respectively.

DNA extraction and molecular typing

Genomic DNA was extracted from overnight grown bacteria using high pure PCR template prep kit for genomic DNA extraction (Roche Diagnostics, Germany). The extracted DNA in elution buffer was stored

at -20 °C for further use. To determine Bacterial clonal relatedness and genetic diversity, RAPD was performed for *K. pneumoniae* using the primer RAPD-7 (5' GTGGATGCGA 3') and analyzed to generate suitable RAPD banding profiles as previously reported (16). PCR reactions were prepared in a total volume of 25 µl per tube containing 1 µl of chromosomal DNA, 12/5 µl Taq DNA polymerase 2x Master Mix Red, 1 µl primer(10pmol) and 9.5 µl ddH₂O. Amplification was performed using the thermal cycler 5530 (Eppendorf Master, Germany) program consisting the initial denaturation at 94°C for 4 minutes and 30 cycles of denaturing at 94°C for 1 minutes, annealing at 31°C for 1 minutes and extension at 72°C for 1 minutes, followed by a final extension at 72°C for 9 minutes. Amplified PCR products were separated by electrophoresis on 1.5% agarose gel in TBE buffer at 100 volts for 2 hours and the DNA bands were visualized by DNA safe staining. The banding patterns were analyzed by the NTSYS software (version.2.0) and cluster analysis was performed to generate dendrogram by unweighted pair group method with arithmetic averages (UPGMA).

Statistical analysis

The analysis was performed by using SPSS™ software, version 21.0 (IBM Corp., USA). The results are presented as descriptive statistics in terms of relative frequency. Values were expressed as percentages of the group (categorical variables). Chi-square or Fisher's exact tests were used to estimate any statistical association. Statistical significance was regarded as P values < 0.05.

Results

Of a total of 60 *K. pneumoniae* isolates included in our study, 28 were obtained from females and 32 males with a mean age of 43.26 ± 40.5 years. The recovered isolates were from different wards including intensive care unit (ICU) (n=53, 88.3%), internal wards (n=4, 6.6%), and emergency (n=3, 5%).

Analysis of antibiotic susceptibility results showed the lowest level of susceptibility to amoxicillin/clavu-

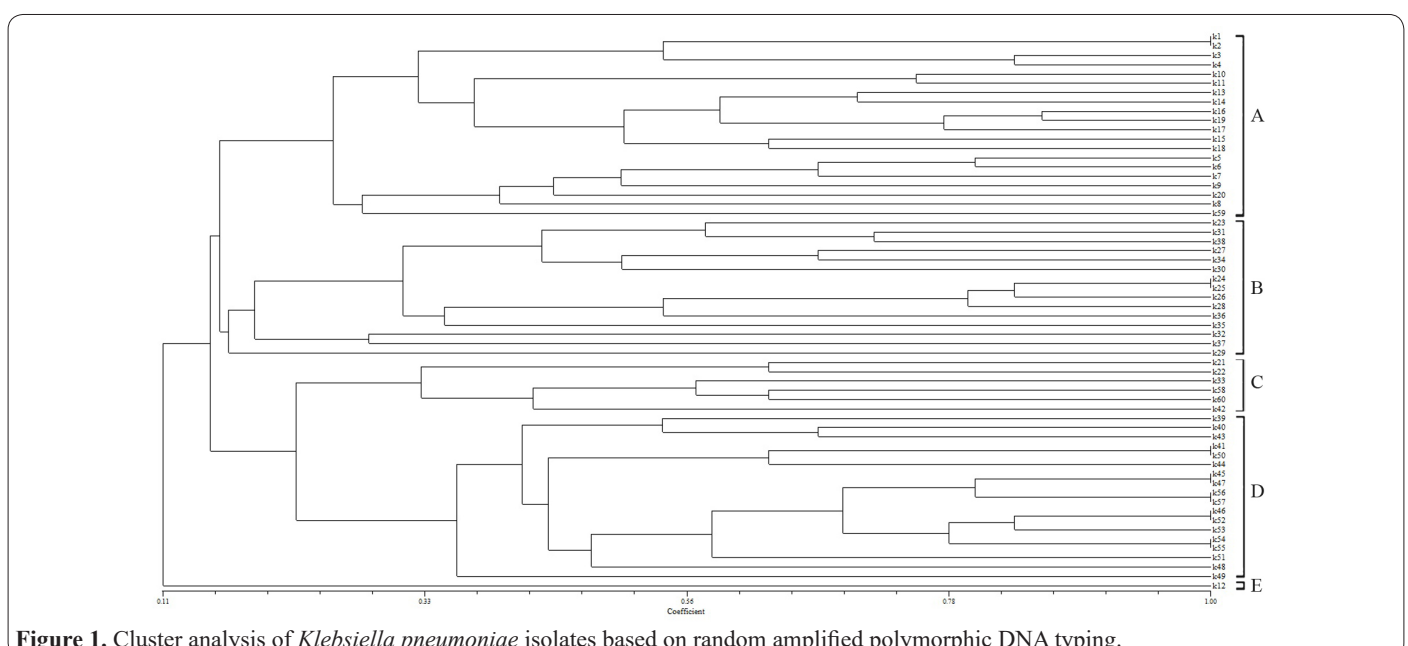


Figure 1. Cluster analysis of *Klebsiella pneumoniae* isolates based on random amplified polymorphic DNA typing.

Table 1. The results of antibiotic susceptibility pattern of *K. pneumoniae* isolates.

Class	Antibiotics	Total <i>K. pneumoniae</i> N = 60		Non-ESBL-producing <i>K. pneumoniae</i> N = 33		ESBL-producing <i>K. pneumoniae</i> N = 27		P value
		No.	%	No.	%	No.	%	
β-lactam/β-lactamase inhibitor	Amoxicillin/clavulanat	15	25	14	42.4	1	3.7	0.001
	Monobactams	Aztreonam	19	31.7	19	57.6	0	0
Cephalosporins I	Cephalothin	19	31.7	19	57.6	0	0	<0.001
Cephalosporins II	Cefoxitin	42	70	22	66.7	20	74.1	0.58
Cephalosporins III	Cefotaxime	19	31.7	19	57.6	0	0	<0.001
Cephalosporins III	Cefixime	19	31.7	19	57.6	0	0	<0.001
Cephalosporins III	Ceftazidime	17	28.3	17	51.5	0	0	0.001
Cephalosporins III	Ceftriaxone	20	33.3	20	60.6	0	0	0.001
Cephalosporins IV	Cefepime	24	40	20	60.6	4	14.8	<0.001
Tetracyclines	Tetracycline	34	56.7	24	72.7	10	37	0.01
Aminoglycosides	Gentamicin	38	63.3	22	66.7	16	59.3	0.6
Quinolones	Nalidixic acid	15	25	12	36.4	3	11.1	0.04
Quinolones	Ciprofloxacin	31	51.7	20	60.6	11	40.7	0.19
Quinolones	Ofloxacin	38	63.3	21	63.6	17	63	0.96
Carbapenem	Imipenem	52	86.7	26	78.8	26	96.3	0.06
Sulfonamides	Co-trimoxazole	25	41.7	20	60.6	5	18.5	0.001
Nitrofurans	Nitrofurantoin	20	33.3	11	33.3	9	33.3	1.0

lanat, and nalidixic acid. On the other hand, the highest level of susceptibility was toward imipenem (86.7%) followed by cefoxitin (70%). The full results of antibiotic susceptibility pattern for *K. pneumoniae* isolates are shown in Table 1. Further analysis revealed that the rate of ESBL-producing isolates was 45% (27/60). There was a significant association between production of ESBLs and higher antibiotic resistance in tested isolates (Table 1). Meanwhile, the most effective *in vitro* agents against ESBL-producing isolates were imipenem with 96.3% susceptibility. All of the ESBL-producing isolates (100%) and 60.6% of non-ESBL-producing isolates were MDR ($P < 0.001$).

All the isolates were typeable by RAPD-PCR. DNA profiles generated using primer RAPD-7 showed 5-10 bands in the range of 450-3000 bp in size. The RAPD-PCR dendrogram of *K. pneumoniae* isolates illustrated in Figure 1. The dendrogram revealed 5 major clusters on similarity level of 70% and 28 different groups on similarity level of 85% (Figure 1). From 60 *K. pneumoniae* strains, 20 strains were found in cluster A that shared $\geq 80\%$ similarity. This cluster contained the majority of ESBLs producing isolates (74/7%) obtained from ICUs. Fifteen strains related to cluster B and contained both ESBLs and non-ESBLs isolates. Of 6 isolates which were in cluster C, 2 isolates were ESBL-producing and obtained from blood samples in ICU, 1 ESBL-producing isolate that obtained from wound sample in ICU and 3 non-ESBL producing isolates that obtained from CSF, urine and sputum samples in ICU. Cluster D consists of 18 non-ESBL producing isolates, and cluster E contained one isolate having a unique RAPD pattern type obtained from a urine sample. Overall, RAPD showed satisfactory discriminatory power ($DI=0.8$) which indicated a heterogeneity among the *K. pneumoniae* isolates.

Discussion

K. pneumoniae is responsible for nosocomial outbreaks worldwide and because of spread rapidly in the hospital, associated with high morbidity and mortality (17, 18). The important concern that must be considered in the management of nosocomial infections caused by *K. pneumoniae* are periodic surveillance to identify the ESBL-producing strains for optimizing available infection control policies and treatment options in different parts of the hospital (9, 17, 19).

Here, we reported the antibiotic resistance and the prevalence of ESBL producing *K. pneumoniae* strains isolated from hospitalized patients. In our results, the prevalence of ESBL producing *K. pneumoniae* isolates was 45%, which in spite of the great discrepancy is consistent with the median values (range 19.6% to 75%) reported in previous Iranian studies (2, 20-23). Prevalence of ESBL producing *K. pneumoniae* varies around the world and has been reported around 12.9 to 26.8% in European countries, 7.5% in North America, 22.4% in Asia Pacific Rim, 44% in Latin America, 48.5% in Turkey, 51% in China, 71.4% in Mexico and 72% in India (24, 25).

ESBLs are plasmid-mediated beta-lactamases that are capable of hydrolyzing beta-lactams except for carbapenems and cephamycins (5). Consequently, most ESBL isolates can be resistant to other classes of antibiotics rather than β-lactams (5, 9). In the present study, all of *K. pneumoniae* isolates were fully resistant to penicillins, and also showed high rates of resistant to monobactams and cephalosporins. As we expected, in our results ESBL producing isolates showed a significant higher resistant rate to the most of tested agents. The only remarkable exception was resistance rates to cefoxitin. Such an observation may be because of this fact that cefoxitin resistance in Enterobacteriaceae is

mainly due to AmpC β -lactamases production (26). In agreement with previous studies, the high rates of imipenem susceptibility in our findings suggest that carbapenem can be the effective last-line treatment option for infections caused by MDR Gram-negative bacteria (2, 22, 23, 25, 27, 28). Despite the several studies which showed comparable results to our findings, antibiotic susceptibility patterns can be varied based on geographical distribution or source of infections (2, 21, 23, 25, 27).

Our understanding of the source of bacteria is very important to monitoring and preventing acquired infections in the hospital (11, 12). RAPD is a cost-beneficial and useful technique in our understanding of the distribution and genetic diversity of nosocomial pathogens at a regional scale (11). RAPD can detect strains that are epidemiologically dependent and thus identify the source of contamination and infection (16). A dendrogram analysis showed 5 clusters with a similarity of 80% which indicates the high relatedness of the studied isolates in our hospital. Twenty-one isolates out of the 27 ESBL-producing isolates were clustered in cluster A, indicating the high similarity of these isolates and possibility of the common origin of spreading in our ICUs. Given that, the most of the ESBL-producing isolates in our hospital were in a same genetic group and all of them originated from UTIs. In contrary to our findings, Fereshteh Eftekhari *et al.* in a hospital survey from Tehran (North of Iran) showed high rates of heterogeneity among tested ESBL and non-ESBL-producing isolates (29). Since our ESBL-producing isolates showed a high genetic similarity, it can be concluded that these isolates were originated from cross-contamination in ICUs, and not from other wards or outside of the hospital. In this regard, natural selection due to higher rates of antibiotic resistance in ESBL-producing isolates is the main factor contributing to successful colonization of this isolates in hospital environments (30). On the other hand, non-ESBL producing strains show a high genetic diversity and are classified into three different clusters. These isolates caused different infections in different parts of the hospital, including Emergency wards, ICUs, and Internal wards, which indicates the out or in-hospital source of infections.

There were some limitations related to the present study. First, the lack of investigation for probable mechanisms of ESBLs production in our isolates. Moreover, use of RAPD typing instead of technique with higher discriminatory power, because of budget restriction.

In summary, beside the limitations results showed the high prevalence of MDR and ESBL-producing *K. pneumoniae* isolates in our ICUs. Also, results revealed a significant similarity between ESBL-producing isolates that necessitate restricted infection control policies and rational prescription and use of antibiotics. Hopefully, carbapenem antibiotics still have promising effects against MDR isolates in our region. These findings provide experimental evidence for safe and effective managements of ESBL-producing *K. pneumoniae* infections.

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Conflict of Interest

None to be declared

Author's contribution

Study concept and design: A. Mojtahedi; acquisition of data and sampling: F Ghaffarian; analysis and interpretation of data: F Ghaffarian, Z Atrkar Roushan; drafting of the manuscript: F Ghaffarian, M Hedayati, H Sedigh Ebrahim-Saraie; critical revision of the manuscript for important intellectual content: A. Mojtahedi; study supervision: A. Mojtahedi, M Hedayati.

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