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Molecular Typing of *Klebsiella pneumoniae* isolates using Repetitive Extragenic Palindromic Sequence-Based PCR in a Hospital in Tehran, Iran

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Abstract

Background: The presence of extended-spectrum β-lactamases (ESBLs) is increasing worldwide and bla_{CTX-M1} is the predominant β-lactamase.

Objective: This study was conducted to determine the ESBL production and prevalence of bla_{CTX-M1}, bla_{SHV} and bla_{TEM} and AmpC genes and repetitive extragenic palindromic polymerase chain reaction (rep-PCR) pattern among *Klebsiella pneumoniae* isolates in Tehran from 2014 to 2016.

Materials and Methods: One hundred eleven isolates were collected during the study period. The PCR was employed to detect the bla_{CTX-M1}, bla_{SHV}, bla_{TEM} and AmpC genes. The genetic relation of isolates was performed using rep-PCR typing method.

Results: Eighty-three and 86 isolates showed Minimum inhibitory concentration (MIC) ≥2 against ceftazidime and cefotaxime, respectively and 80 (72%) isolates exhibited ESBL production. The prevalence of bla_{CTX-M1}, bla_{SHV}, bla_{TEM} and AmpC genes among ESBL producers was 92.5% (n=74), 66.2% (n=53), 56.2% (n=45) and 2.5% (n=2), respectively. The rep-PCR typing pattern of isolates showed a wide diversity, indicating the polyclonal spread of CTX-M type producing isolates.

Conclusion: The findings of this study highlighted the emergence and spread of *K. pneumoniae* isolates producing CTX-M and other ESBL enzymes with diverse genetic backgrounds in a hospital in Tehran.

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Background

During recent years, the development of *Klebsiella pneumoniae* with resistance to multiple antibiotics has become a great concern. Extended-spectrum β–lactamases are growing among enterobacteriacea.[1] The genetic loci encoding extended-spectrum β-lactamases (ESBLs) include mobile elements and chromosome.[2] Recent data have shown that bla_{CTX-M1} clones are mostly widespread at an endemic level worldwide and in Iran.[3-5] The ESBLs are increasing everywhere. These ESBLs are inhibited by clavulanic acid, sulbactam, and tazobactam applied for detection of them.[6] On the other hand, resistance due to ESBLs is often accompanied by resistance to other antibiotics including fluoroquinolones, aminoglycosides and sulfamethoxazole/trimethoprim.[7] Nosocomial infections caused by multidrug-resistant *K. pneumoniae* isolates producing CTX-M-15 (CTX-M-15-KP) have dramatically increased in recent years. CTX-M-type ESBLs are a complex and heterogeneous family which may be subdivided into 5 major groups (CTX-M-1, 2, 8, 9 and CTX-M-25).[8,9] The bla_{CTX-M} and bla_{TEM} ESBLs can hydrolyze third and fourth generation cephalosporins.[10] Several studies have demonstrated a relationship between ESBL enzymes and MIC of the third and fourth generation cephalosporins, including ceftazidime, cefepime and cefotaxime.[11] In the Ambler classification, AmpC β-lactamases are an important group of class C β-lactamases with the ability to hydrolyze penicillins, oxyimino-cephalosporins, cephamycins and aztreonam. Whereas they cannot be inhibited by clavulanate, sulbactam and tazobactam, they are inhibited by cloxacillin and phenylboronic acid.[12,13] The repetitive extragenic palindromic polymerase chain reaction (rep-PCR) have been applied for typing gram-negative species.[14,15] Several studies have shown that rep-PCR has the ability to fingerprint strains of *Escherichia coli* and *K.*

pneumoniae and other gram-negative species.[16-19] There has been an excellent correlation between multilocus sequence typing (MLST) and automated rep-PCR in *K. pneumoniae* fingerprinting.[20] The amplification of neighboring repetitive elements is implemented by common primers for differentiating bacterial strains.

Objectives

The aim of this study was to demonstrate ESBL positive *K. pneumoniae* strains and prevalence of bla_{CTX-M1}, bla_{SHV}, bla_{TEM} and AmpC types and rep-PCR typing of ESBL positive *K. pneumoniae* in Tehran.

Materials and Methods

Clinical Isolates

One hundred eleven non-duplicated *K. pneumoniae* isolates were collected from patients including 28 males (25.23%, mean age of 34.21) and 83 females (74.77%, mean age of 46.63), with age ranging from 2 to 73 years old in Loghman hospital, Tehran from 2014 to 2016. The isolates were identified by the conventional biochemical tests and preserved at -20°C in trypticase soy broth containing 30% glycerol.

Susceptibility and ESBL Production

Susceptibility testing was performed by the disk diffusion method following the guidelines of Clinical and Laboratory Standards Institute (CLSI) version 2016. Seventeen antimicrobial disks were used as indicated in Table 1. Ceftazidime-resistant isolates were adopted for ESBL production.

Escherichia coli ATCC 25922 (present as a stock in AJA University of Medical Sciences) was used for the quality control of susceptibility testing. The ESBL phenotype was detected by combined disk method using cefotaxime and ceftazidime with and without clavulanic acid (10 µg) and cefoxitin/boronic acid (400 µg) as described everywhere.

Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of ceftazidime and cefotaxime with a range of 0.25 to 128 µg/mL (CLSI 2014) was measured by microbroth dilution. Each isolate with MIC ≥2 was further tested for ESBL production.

PCR Amplification of AmpC and ESBL Genes

The CTX-M1, SHV, AmpC and TEM type ESBLs genes were amplified with specific primers as previously described.

The Repetitive Extragenic Palindromic Polymerase Chain Reaction Typing

The rep-PCR typing method was performed to determine genetic relatedness among strains. Genomic DNA was extracted using Cinagen kit. Briefly, one colony was dissolved in 500λ TE buffer (150 µg/ml) and centrifuged at 7500 g for 10 minutes. After discarding the supernatant, 100λ protease buffer (100 µg/ml) was added and kept at 95°C for 10 minutes. The lysis solution was added and homogenized by vortex. Then 300λ precipitation solution was added and kept at -20°C for 10 minutes. At the next stage, the tube was centrifuged at 12000 g for 10 minutes and the supernatant was discarded. The tube was dried and 1 mL of wash buffer was added. Then 50λ solvent buffer was added and shacked for 5 minutes at 65°C, and centrifuged for 30 seconds. The supernatant was transferred to a new tube and used as DNA template for rep-PCR. The electrophoresis (40-45 V) of rep-PCR products was conducted by preparing 2% agarose gel in 1X TAE buffer.

Statistical Analysis of the Data

Comparisons of relations were tested using the *t* test. A value of $P < 0.05$ was considered statistically significant.

Results

The Susceptibility Profile and ESBL Production

The antibiotic susceptibility testing of ESBL positive and negative *K. pneumoniae* have been demonstrated in Table 1.

In the microbroth dilution method, 92.7% of the isolates showed ceftazidime MIC ≥2, and in the combined disk method, 89 (80.1% of all) isolates were ESBL producers. Coexistence of resistance (ESBL + AmpC β-lactamases) was determined among 4 isolates. As shown, 83 (74.77%) and 86 (77.47%) isolates exhibited MIC ≥2 for ceftazidime and cefotaxime, respectively. Thirty isolates were ESBL-positive and high MIC_{CAZ} was associated with

Table 1. The Results of the Disk Diffusion Test for ESBL Negative and Positive Isolates

Antibiotic	ESBL-Positive (%)	ESBL-Negative (%)	*P* Value
Aztreonam	97.3	31.2	0.0014
Piperacillin	6.31	4.44	0.311
Augmentin	23.52	87.31	0.003
Cefotaxime	94.6	18.7	0.002
Meropenem	13.3	12.4	0.455
Piperacillin-tazobactam	5.5	4.6	0.777
Ceftazidime	92.4	23.6	0.002
Ofloxacin	87.2	31.1	0.014
Ciprofloxacin	77.7	33.4	0.023
Levofloxacin	73.3	32.4	0.034
Amikacin	23.3	5.3	0.025
Tobramycin	19.5	4.43	0.015
Gentamicin	30.3	11	0.031

the presence of the bla_{CTX-M} gene. In addition, DHA and CITM genes were detected in 5 isolates exhibiting MIC_{CAZ} ranging from 32 to 64 µg/mL for each.

Molecular Detection of ESBLs

The prevalence of bla_{CTX-M1}, bla_{SHV}, bla_{TEM} and *AmpC* (CITM and DHA) genes among *K. pneumoniae* ESBL producers was 92.5% (n=74), 66.2% (n=53), 56.2% (n=45) and 2.5% (n=2), respectively. Thirty isolates contained bla_{CTXM1}, bla_{SHV} and bla_{TEM} genes, two of which were positive for DHA and CITM as well. The rep-PCR typing pattern of isolates showed a wide diversity, indicating no genetic relation.

Rep-PCR Typing

The rep-PCR typing pattern of isolates showed a wide diversity, indicating no genetic relation. In the electrophoretic analysis of patterns by Complete Linkage software, each isolate exhibited a specific pattern. The isolates had been collected from different hospital wards. In fact, analysis by computer software revealed that isolates showed various fingerprints with no similarity and genetic relatedness.

It was demonstrated that TEM-positive isolates were grouped into 6 clusters (A-F) and SHV and CTXM1 were grouped into 5 clusters (A-E), while AmpC positive (n=5) isolates showed 1 cluster with 40 to 80% homology.

Discussion

In this study, 74 (92.5%) out of 80 ESBL-producing *K. pneumoniae* isolates in the study period (2014-2016) carried bla_{CTXM1}. This finding confirms the dominance of CTX-M enzymes in ESBL-producing *K. pneumoniae* isolates in Tehran.[21-23] CTX-M types have different geographical spread; for example, CTX-M15 belonging to CTX-M1 is the predominant allele distributed worldwide.[24-28] The results of this study demonstrated no clonal spread of CTX-M1 producing *K. pneumoniae* by rep-PCR. We investigated the genetic relatedness of CTX-M1 producing isolates by the rep-PCR method. Of 74 isolates examined by rep-PCR, 6 different genotype clusters (A-F) were determined. The isolates mostly showed 40 to 50% similarity, while 6 isolates showed 70% and more similarity. Our data in combination with other findings suggest that *K. pneumoniae* isolates producing CTX-M-type enzymes are genetically heterogeneous.[29] The emergence and polyclonal spread of CTX-M–producing *K. pneumoniae* isolates likely occurred among the strains with diverse genetic backgrounds. This hypothesis is in contrast with previous data which have shown the clonal spread of KPC or ESBL producing *K. pneumoniae*. We also observed a diverse genetic background among SHV and TEM type producing isolates. Among 5 AmpC producing *K. pneumoniae*, 1 cluster was drawn, in which isolates indicated 40 to 80% similarity.

Conclusion

In this study, 30 ESBL producers out of 89 contained CTX-M1, SHV and TEM type enzymes (MIC range: 8 to 128 µg/mL). The AmpC type enzyme was determined in 2 isolates producing TEM and SHV types (CITM/DHA+TEM+SHV, MIC=16 and 32). A shortcoming of this study is that only one hospital has been considered, and thus it is suggested that more hospitals should be investigated in various regions in the future. The findings of this study highlighted the emergence and spread of *K. pneumoniae* isolates producing CTX-M and other ESBL enzymes with diverse genetic backgrounds in Tehran. There are some limitations in the current study including patients' history and data from other hospitals of Tehran.

Authors' Contributions

ME, AG and MS designed and performed the work. FN, MV and HRV helped during process, advisory and data analysis.

Ethical Approval

This study was ethically approved by Shahrekord University of Medical Sciences, Shahrekord, Iran.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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