

Genetic characterization of ESBL-producing strains of *Klebsiella pneumoniae* from Tehran hospitals

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Abstract

Introduction: This study was conducted to determine the genetic characterization of extended-spectrum beta-lactamase (ESBL) producing strains of *Klebsiella pneumoniae* isolated from Iranian patients in hospitals in Tehran.

Methodology: Antibiotic susceptibility of 104 isolates was determined using the disk diffusion test. The Minimum Inhibitory Concentrations (MICs) of imipenem and meropenem were determined for isolates showing reduced susceptibility to carbapenems. The phenotypic confirmatory test (PCT) was used to screen the isolates for ESBL production. PCR was used to detect *bla*_{SHV}, *bla*_{TEM} and *bla*_{CTX-M} and the amplicons from selected clones were sequenced. Isolates producing ESBLs were analyzed by pulsed-field gel electrophoresis (PFGE).

Results: One isolate showed resistance to imipenem (MIC = 16 µg/ml). Resistance to amikacin and ciprofloxacin was 44.2% and 25.0%, respectively. ESBL production was detected in 72.1% (n = 75) of isolates. The prevalence of *bla*_{SHV}, *bla*_{TEM} and *bla*_{CTX-M} genes among the isolates was 55.7% (n = 58), 30.7% (n = 32) and 45.2% (n = 47), respectively. The sequencing revealed the amplicons corresponding to *bla*_(TEM-1, TEM-79, SHV-1, SHV-12, SHV-31, CTX-M-15) genes. While the *bla*_{CTX-M-15} is the dominant gene among the Iranian isolates, we detected the *bla*_{SHV-31} and *bla*_{TEM-79} genes for the first time in the country. PFGE differentiated the 71 ESBL-producing isolates into 62 different genotypes. Clonal dissemination of ESBLs was found in the neonatal intensive care unit and intensive care unit of one hospital.

Conclusion: The findings are evidence of the spread of multi-resistant clones of ESBL producers in Tehran hospitals.

Key words: *Klebsiella pneumoniae*; ESBL, PCR, PFGE, FIGE, Iran

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Introduction

With the widespread use of broad-spectrum antibacterial agents, problems in treatment of infections with drug resistant organisms, in particular those acquiring resistance to beta-lactams, including broad-spectrum cephalosporins, has increased. Resistance to the new cephalosporins is mediated by production of extended-spectrum beta-lactamases (ESBLs). The ESBL-producing isolates of *Klebsiella pneumoniae* have been associated with numerous outbreaks of nosocomial infections throughout Europe, the United States of America [1] and the Far East [2], though such outbreaks have recently been reported in Asia [3,4] including Iran [5-7]. Strains of *K. pneumoniae* producing ESBLs are widespread in Tehran hospitals but relatively little is known about

the epidemiology and genetic background of the involved organism. Dissemination of ESBL-producing *K. pneumoniae* in a hospital may be a complex event involving several modes of epidemic spread. Molecular typing techniques, particularly macrorestriction patterns of total genomic DNA, obtained by pulsed-field gel electrophoresis (PFGE), are now widely accepted as powerful epidemiological tools and have been applied for the molecular characterization in outbreaks [8,9].

The main aims of this study were to assess the susceptibility of *K. pneumoniae* strains isolated from the Iranian patients to different antimicrobial agents and to characterize the genes encoding the ESBL phenotypes. Since little data was available on the genetic relationships among the Iranian isolates of *K.*

pneumoniae, we also applied PFGE to investigate the possibility of clonal outbreaks or heterogeneity of ESBL-producing strains isolated from different wards of the study hospitals.

Methodology

Bacterial isolates and phenotypic characterization

Collectively, a total of 104 consecutive nonduplicate isolates of *K. pneumoniae* were collected from September 2006 to June 2007 from four university hospitals in Tehran. Two general hospitals located in the city center (Hospital A with 1,230 beds and Hospital D with 338 beds), 2 children's hospitals located in the city center (Hospital B with 350 beds), and one hospital in the north of the capital (Hospital C with 210 beds) were included in the study.

Bacterial isolates were identified using the standard procedures [10]. They were tested against the following antibiotics using the Clinical and Laboratory Standards Institute (CLSI) protocol [11]: imipenem, meropenem, amikacin, ciprofloxacin, levofloxacin, piperacillin–tazobactam, amoxicillin-clavulanic acid, ampicillin-sulbactam, aztreonam, cefotaxime, ceftazidime, ceftriaxone and cefepime. *K. pneumoniae* ATCC 700603 was used as the control in all experiments. The Minimum Inhibitory Concentrations (MICs) of imipenem (MAST, Merseyside, UK) against isolates showing reduced susceptibility to this carbapenem was determined using micro-broth dilution assay.

Isolates showing resistance to at least one of the third generation cephalosporins (3GCs) or aztreonam were tested for ESBL production using the phenotypic confirmatory test (PCT) as instructed by CLSI [11]. *K. pneumoniae* ATCC 700603 was used as the positive control and *Escherichia coli* ATCC 25922 was used as the negative control.

PCR amplification of beta-lactamase genes

DNA of all isolates was extracted by the boiling method. The *bla_{SHV}* and *bla_{TEM}* were amplified as described previously [5]. Plasmid DNA was extracted by miniprep kit (Metabion, Martinsried, Germany) and targeted for the *bla_{CTX-M}* gene using the primers described by Lee *et al.* [12] under the following conditions: 94°C for 3 minutes, and 35 cycles of 94°C for 30 seconds, 63°C for 1 minute, 72°C for 1 minute and a final extension in 72°C for 10 minutes. *K. pneumoniae* ATCC 700603, *E. coli* ATCC 35218 and *K. pneumoniae* 7881 were used as positive controls for *bla_{SHV}*, *bla_{TEM}* and *bla_{CTX-M}*, respectively.

The latter isolates were kindly provided by Professor Patrice Nordmann of Institut National de la Santé et de la Recherche Médicale, Paris, France.

Pulsed Field Gel Electrophoresis (PFGE)

The Genomic DNA was extracted using the protocol described by Bouallegue-Godet *et al.* [8] with some modifications. The embedded DNA in plugs was digested with XbaI (Fermentas, Vilnius, Lithuania) for 4 hours at 37°C. The digested DNAs were separated in 1% agarose gel by field inversion gel electrophoresis (FIGE), a prototype of PFGE (Faculty of Engineering, University of Tehran). Conditions were as follows: pulse time ramped linearly from 1 to 35 s, run time 20 hours, temperature 14°C, and constant voltage of 6 V/cm. The gels were stained with ethidium bromide and DNA patterns were photographed with UVP gel documentation (UVP, Cambridge, UK) (Figure 1). The DNA patterns were analysed with Zhen Negar software (Faculty of Mathematic and Computing Sciences, Sharif University, Tehran, Iran) and the strains were clustered by the unweighted pair group method with arithmetic averages strategy [13]. The DNA banding patterns were interpreted as instructed by Tenover *et al.* [14].

Sequencing of PCR products

Based on the results of PFGE analysis, the amplicons from isolates with identical DNA banding patterns were selected and sequenced. The sequences were analyzed using the BLAST program (www.ncbi.nlm.nih.gov=BLAST) and submitted to GenBank.

Results

Resistance of K. pneumoniae isolates

In total, 104 isolates of *K. pneumoniae* were isolated from urine (n = 33), respiratory tract (n = 14), blood (n = 18), wound exudates (n = 22) and other clinical specimens (n = 17). The majority of the strains (79.8%) were cultured from hospitalized patients and the remainder (20.2%) belonged to outpatients. Using the PCT method, ESBL production was found in 75 (72.1%) isolates.

All ESBL-positive isolates were susceptible to meropenem, but one isolate showed resistance to imipenem (MIC = 16 µg/mL). Following carbapenems, the lowest rates of resistance in ESBL-producing isolates were observed for amikacin (21.4%), piperacillin/tazobactam (21.3%), levofloxacin (25.3%) and ciprofloxacin (28.0%). The

highest rate of resistance belonged to aztreonam (92.0%), cefotaxime, ceftazidime (84.0%) and cefepime (76.0%). Piperacillin/tazobactam was more potent than ampicillin-sulbactam and amoxicillin/clavulanic acid; results showed that

69.3% of ESBL isolates were susceptible to this combination. The resistance rate for cefepime was 76%. In ESBL-positive isolates, 21 isolates (28%) had concomitant resistance to ciprofloxacin.

Table 1. Clones of *K. pneumoniae* detected by PFGE in regard to their ESBL phenotypes and the relevant wards

Clone	Isolates Number	Sample	Hospital wards	ESBL phenotype
A	305	Eye exudates	NICU	SHV-1, CTX-M-15
	308	Eye exudates	NICU	SHV-1, CTX-M-15
	309	Eye exudates	NICU	SHV-1, CTX-M-15
	359	Bronchial	NICU	SHV-1, CTX-M-15
	302	Wound	Surgery	SHV-1, CTX-M-15, TEM-1
	312	Bronchial	NICU	SHV-1, CTX-M-15
	313	Bronchial	NICU	SHV-1, CTX-M-15
B	344	Blood	ICU	CTX-M-15
	358	Abdominal exudates	ICU	SHV-1, CTX-M-15
C	329	Blood	ICU	TEM-79, CTX-M-15
	306	Blood	ICU	CTX-M-15, TEM-79, SHV-12
D	350	Eye exudates	NICU	SHV-31
	375	Umbilical exudates	NICU	SHV-31

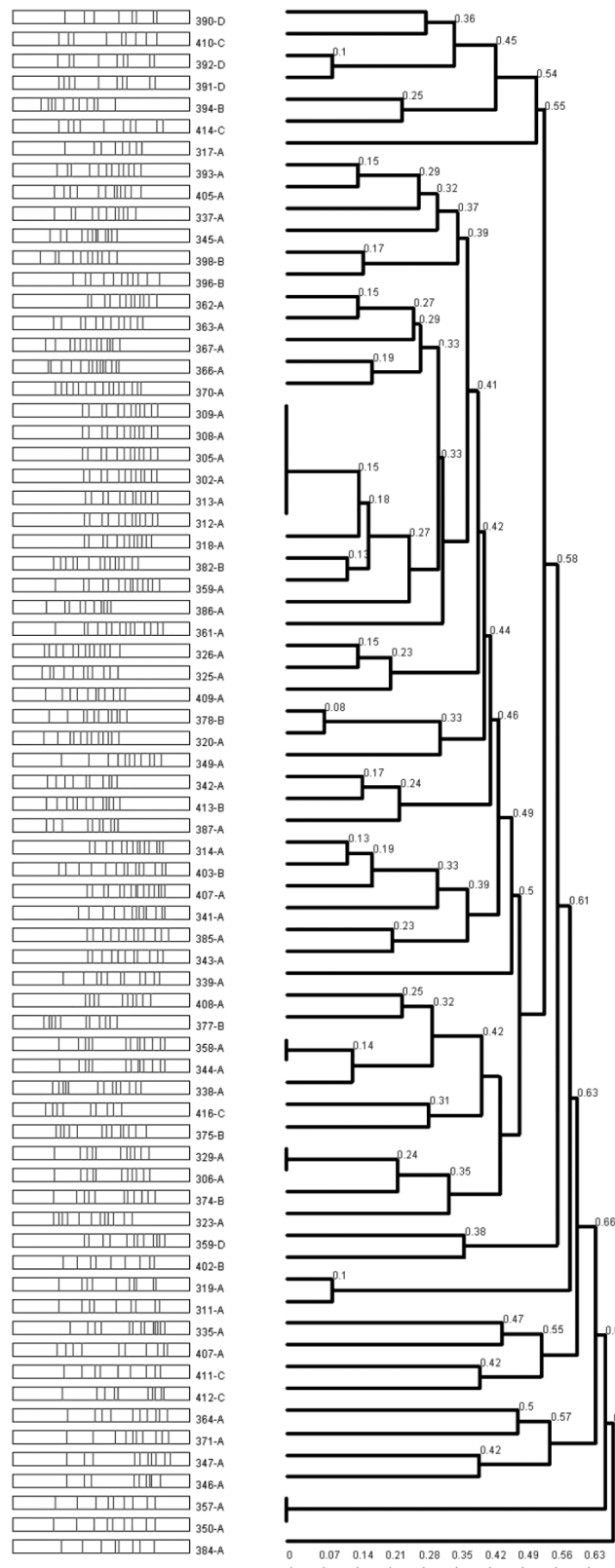
Prevalence of bla_{SHV}, bla_{TEM}, and bla_{CTX-M} genes and sequencing results

PCR showed that 55.7% (n = 58), 30.7% (n = 32) and 45.2% (n = 47) of isolates contained *bla_{SHV}*, *bla_{TEM}*, and *bla_{CTX-M}* genes, respectively. Coexistence of these genes was detected in 26 (34.7%) of isolates. Of 104 isolates tested for the above genes, only three isolates were not positive in PCT but produced

amplicons corresponding to *bla_{SHV}* and *bla_{TEM}*. They were negative for *bla_{CTX-M}*.

With the exception of two isolates, the results of sequencing showed that all *bla_{TEM}* amplicons belonged to *bla_{TEM-1}* (GenBank accession numbers GU734690-GU73469097). The excepted isolates cultured from blood specimens and possessed *bla_{TEM-79}* (Table 1). There was more diversity among sequences obtained for *bla_{SHV}*

Figure 1. Phlogenetic relationships among isolates of *K. pneumoniae* using data obtained by PFGE. The strains were clustered by the unweighted pair group method with arithmetic averages (UPGMA). The scale indicates the percentage of genetic distances. Hospitals: A; Emam Khomeini hospital, B; Markaz Tebbi Kudakan Hospital, C; Mofid Hospital, D; Sina hospital



genes and these belonged to *bla*_(SHV-1, SHV-12, SHV-31) (Table 1). The amplicons obtained for *bla*_{CTX-M} were characterized as *bla*_{CTX-M-15} after sequencing and the relevant sequences were submitted to GenBank under accession numbers HM002646-HM002660.

PFGE analysis

Analysis of ESBL-producing strains by PFGE showed that the majority of isolates were not related (Figure 1). Four isolates did not yield sufficient DNA. These isolates belonged to outpatients and were excluded from PFGE analysis.

Of 71 ESBL-producing strains analyzed by PFGE, isolates 51, 11, 5 and 4 belonged to hospitals A, B, C and D, respectively. The numbers of distinctive patterns identified in these hospitals were 39, 11, 5 and 4. Therefore, 13 strains from hospital A were clustered in 4 groups (Table 1). These isolates were cultured from patients in a neonatal intensive care unit (NICU) (n = 8), an intensive care unit (ICU) (n = 4), and a surgery unit (n = 1). One isolate collected from the surgery unit and 6 isolates from NICU were identical. However, genetically distinct strains were also detected in hospital A, which is the largest health care setting in Iran. The ICU isolates were collected from blood and abdominal exudates.

The remaining isolates (n = 58) were distributed through the dendrogram at different genetic distances (Figure 1).

Discussion

In Asia, the prevalence of ESBL-positive *K. pneumoniae* isolates varies within different regions. The ESBL frequency in neighboring countries varied from 85% in Russia, 66.7% in India [4], 54.7% and 61% in Turkey [3,15], 41% in United Arab Emirates [16] and 31.7% in Kuwait [17,] but it is lower in America and Europe [15,18]. In comparison with our previous studies [5, 7], in this study, we found ESBL production in 72.1% of isolates (n = 75) because most of these isolates were collected from hospitalized patients and ESBL production is more common among hospitalized patients (n = 66, 79.5%) than outpatients (n = 9, 42.9%) [18]. In agreement with recent trends [6], ESBL phenotypes showed remarkable increase in the CTX-M phenotype. Moreover, we found a new type of SHV genotype (*bla*_{SHV-31}) which had caused a large outbreak in the Netherlands [19]. This illustrates the necessity for continued phenotypic surveillance for ESBL-producing strains of *K. pneumoniae*. Three isolates were not positive in PCT but produced amplicons

corresponding to *bla*_{TEM} and *bla*_{SHV} genes, which shows that the sensitivity of the PCT assay is not 100% and that the PCR assay is more reliable [2].

The high rate of ESBL enzymes in Tehran hospitals probably is due to the frequent use of 3GCs or the spread of resistant plasmids among the bacterial isolates in the hospital environments as explained previously [20].

Cefepime is more stable to hydrolysis by ESBLs than the 3GCs [21]; however, the rate of resistance to this fourth-generation cephalosporin (76%) was considerably high in this study. This antibiotic has been administered in recent years and widely used against different infections in Iran. It seems that cefepime should not be used as the first choice of therapy against ESBL-producing organisms.

Similar to other studies, we found that ESBL-producing *K. pneumoniae* strains are associated with high aminoglycoside resistance [22]. Amikacin remained the most effective aminoglycoside against *K. pneumoniae*.

The spread of *K. pneumoniae* with resistance to both quinolones and broad-spectrum beta-lactams would severely limit treatment options. Levofloxacin, which is not being prescribed in Iran, was more effective than ciprofloxacin since it was active against 24 isolates with a ciprofloxacin resistance phenotype.

The high rate of resistance to amoxicillin/clavulanic acid among ESBL-producing isolates (n = 50, 66.7%) is probably due to the frequent use of this antibiotic to treat paediatric otitis media and gastritis caused by *Helicobacter pylori* [23]. Such rate of resistance was also reported from Malaysian hospitals [24].

The carbapenems, in combination with aminoglycosides, are the final therapeutic option against ESBL-producing isolates [25]. Unfortunately, we found the first imipenem resistant isolate recovered from the wound of a surgery patient. To our knowledge, such resistance has not been reported in Iran before. Similar cases of imipenem resistant *K. pneumoniae* were reported from Turkey in 2006 and 2007 [3,26]. In most European countries, Latin America, and East Asia, CTX-M variants have displaced TEM and SHV enzymes as the predominant beta-lactamases produced by Gram-negative bacteria such as *K. pneumoniae* [4,27] and our data confirms this document.

The high genetic diversity detected in the collection of isolates from hospital A can be attributed to the diversity of sources that is being

introduced to this hospital. It is the largest hospital in Iran and patients are referred to it from nearly all parts of the country. However, the PFGE profiles demonstrated a few clonal dissemination of ESBL-positive strains in hospital A, which shows that vertical spread of resistant isolates had occurred in this hospital. The isolates with identical patterns suggest that clonal spread also played a role in the dissemination of ESBL-producing isolates in the NICU and ICU. It appears that a multi-drug resistant *K. pneumoniae* clone detected in the NICU was related to a clone isolated from women's surgery, as both of them produced similar PFGE patterns.

In conclusion, carbapenems still show the highest potency against *K. pneumoniae* in Iran. However, emerging of resistance to this group of antibiotics, in particular resistance to imipenem, may limit their prescription in the future. The tremendous increase in prevalence of ESBLs (at least 26.7%) is the second concern since the majority of them were multi-resistant to other antibiotics. Importantly, inactivation of beta-lactamase inhibitors, in particular clavulanic acid, has reduced its efficacy in combination treatment with beta-lactams against ESBL-producing isolates.

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