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Antibiotic Resistance Patterns and Genetic Analysis of *Klebsiella Pneumoniae* Isolates from the Respiratory Tract

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ABSTRACT

Background: *Klebsiella pneumoniae is a pathogenic bacterium causing nosocomial infections in particular severe respiratory tract infections. Little information is available on the antibiotic susceptibility of pulmonary isolates of Klebsiella spp. The aims of this study were to determine the antibiotic resistance patterns and prevalence of extended-spectrum β-lactamase (ESBLs) producing Kleb. Among the respiratory isolates and to detect the possible clonal outbreaks associated with them.*

Materials and Methods: *The respiratory isolates of K. pneumoniae (n=33) received from two Tehran hospitals during 2002-2005 were evaluated. Disk diffusion was used to determine the susceptibility of isolates to 14 antibiotics. Phenotypic confirmatory and double disk synergy methods were used to detect extended spectrum β-lactamase producing isolates (ESBLs). Respiratory isolates were then analyzed by multilocus enzyme electrophoresis (MLEE).*

Results: *ESBL phenotype was detected in 75.75% of the isolates. The most effective antibiotic was imipenem followed by tazobactam/piperacillin. MLEE analysis revealed 13 electrophoretic types (ETs). The locus leucine-tyrosine peptidase showed the highest genetic diversity (0.733).*

Conclusion: *These 33 respiratory isolates consisted of 16.5% Klebsiella pneumoniae. This rate is lower than the neighboring country, Turkey (35%). However, ESBL-producing strains belonging to different genetic lineages are serious concerns at Tehran hospitals. Carbapenem is still considered one of the most effective antibiotics against multi-drug resistant isolates.* ***(Tanaffos 2007; 6(3): 20-25)***

Key words: *Klebsiella pneumoniae, Extended spectrum β-lactamase, Respiratory tract, Multilocus enzyme electrophoresis*

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INTRODUCTION

Klebsiella pneumoniae is a common hospital-acquired pathogen that causes severe respiratory infections such as pneumoniae. Other infections caused by this organism include urinary tract infections (UTIs), wound infections, abscesses and diarrhea (1). Treatment of Klebsiella infections is complicated in cases in which causative organisms produce extended spectrum beta-lactamases (ESBLs). ESBLs are enzymes which can hydrolyze penicillins as well as cephalosporins and monobactam. The majority of ESBL isolates are also resistant to other antibiotics such as fluroquinolones and aminoglycosides (2, 3).

Outbreaks of ESBL-producing *Klebsiella pneumoniae* infections have increased worldwide (4). The investigation of these strains requires suitable methods for recognition of ESBLs and strain dissemination. Thus, the use of chromosomal markers together with the evaluation of ESBLs are essential. Multilocus enzyme electrophoresis, which allows the characterization of closely related strains through the analysis of allelic variations at polymorphic enzyme loci, is well recognized as an efficient typing method for analyzing genetic relationships in bacterial populations in genetic and epidemiological studies (5).

To date, no information is available on the population genetic analysis of K. *pneumoniae* in Iran. The aim of this study was to determine the antibiotic resistance pattern of *Klebsiella pneumoniae* isolates causing respiratory tract infections at two Tehran hospitals. To characterize the genetic relationships between the isolate and outbreaks, the ESBL producing isolates were subsequently analyzed by Multilocus Enzyme Electrophoresis (MLEE).

MATERIALS AND METHODS

Screening of Bacterial isolates and Identification

Two-hundred isolates of *K. pneumoniae* were isolated from different clinical specimens at two Tehran hospitals during 2002-2005. They were identified by using conventional bacteriological methods(6).

Antimicrobial susceptibility testing

The method of disk diffusion was used to determine the susceptibility of isolates to imipenem (IMP), gentamicin (Gm), amikacin (Ak), ciprofloxacin (Cip), nalidixic acid (NA), nitrofurantoin (Nf), piperacillin /tazobactam (TZP), cephalexin (Cl), cefotaxime (Ce), ceftazidime (Ca), ceftriaxone (Ci), cefixime (Cfx), aztreonam (Ao) and amoxicillin-clavulanic acid (Ac) as recommended by NCCLS(7).

Detection of ESBL

The phenotypic confirmatory test was used to detect ESBL producing isolates. The resistance of isolates to ceftazidime and cefotaxime alone and in combination with clavulanic acid was determined. The results were positive when the zone diameter around ceftazidime- clavolanic acid was between 5 and 22 millimeters and for cefotaxime-clavulanic acid was between 3 and 27 millimeters(7,8).

The double-disk synergy test was used in parallel to detect ESBL strains(7,8). In brief, aztreonam, ceftazidime, ceftriaxone, and cefotaxime (30 µg) were placed at variable distances (20 to 30 mm from center to center) and around a disk containing amoxicillin (20 µg) plus clavulanic acid (10 µg). Enhancement of the inhibition zone toward the amoxicillin-plus-clavulanic acid disk was suggestive of ESBL production.

Multilocus Enzyme Electrophoresis

Preparation of bacterial extracts

Strains were grown in 20 ml of nutrient broth and after 6 hours were transferred to 300 ml of this media and kept for 18 hours at 37ºC with aeration. The cells were harvested by centrifugation (13,000×g for 15 minutes at 4ºC), washed by phosphate buffer saline (pH=7.2) and re-centrifuged with the same program. The sedimented cells were disrupted by sonication

and microfuged at 13,000 × g for 15 minutes at 4ºC. The supernatants were stored at -70ºC for electrophoresis(9).

Enzyme Electrophoresis

The lysate containing the enzymes was subjected to electrophoresis in horizontal starch gel. The preparation of starch gel and bufferic system was performed. The following 17 enzymes were determined by staining for specific enzyme activity: adenylate kinase (ADK), alpha-esterase (ESTα), phosphogluconate dehydrogenase (PGD), glutamate dehydrogenase (GDH 1,2), lysine dehydrogenase (LYD), hexikonase (HEK), beta-esterase (ESTβ), superoxide dismutase (SOD), phosphoglucomutase (PGM), peptidase (1,2,3), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), xanthine dehydrogenase (XDH) and glucose-6-phosphate dehydrogenase (G6PD).

Statistical analysis

Genetic diversity (h) for each enzyme locus (allele) examined in MLEE was calculated from the formula $h= (1-\Sigma P_i^2)[n/(n-1)]$ where P_i is the frequency of the *i*th allele and *n* is the number of electrophoretic types (ETs) in the sample(10). Genetic distances between ETs were calculated as the proportion of fixed loci at which dissimilar alleles occurred.

RESULTS

Of the 200 clinical isolates of *K. pneumoniae* received from the hospitals, 33 (16.5%) were isolated from respiratory tracts. The remaining isolates were from urine (n=134, 67%) or other sources (n=33, 16.5%). The antibiotic resistance patterns among respiratory isolates are shown in Table 1. Although 49% of all 200 isolates were found to be ESBL producers by double-disk synergy, the rate of ESBL producing strains among respiratory isolates was higher (75.75%). Imipenem was the most effective agent against respiratory isolates (100%), followed by piperacillin/tazobactam (85%).

Table 1. Rate of resistance to different antibiotics among the respiratory isolates of *K. pneumoniae* in relation to ESBL production

Antibiotic	Resistance N (%)	Production of ESBL N (%)
Ak	14 (42.42)	14 (56.00)
Gm	21(63.63)	19 (76.00)
Cip	15(45.45)	12 (88.00)
NA	22(66.66)	20 (80.00)
Nft	14 (42.42)	12 (88.00)
TZP	5 (15.15)	5 (20.00)
Cl	23(69.69)	19 (76.00)
Cfx	24(72.72)	20 (80.00)
Ca	24(72.72)	22 (88.00)
Ce	19(57.57)	17 (68.00)
Ci	19(57.57)	17 (68.00)
Ao	26(78.78)	22 (88.00)
Ac	21(81.81)	21(84.00)
Total	33 (16.5%)	25 (75.75%)

All respiratory strains showed the highest resistance to amoxicillin-clavulanic acid (81.81%), cefixime (72.72%), and ceftazidime (72.72%). Higher rates of resistance were found for cephalosporins including cephalexin (69.69%), cefixime (72.72%), ceftazidime (72.72%), cefotaxime (57.57%) and ceftriaxon (57.57%) among respiratory isolates.

Analysis of 26 respiratory isolates by MLEE produced 13 electrophretic types (ETs, Table 2). Three ETs contained isolates from urinary and respiratory tracts.

Of 17 enzyme loci analyzed, 6 loci including Le-Tyr, Le-Pro, Le-Gly peptidase, PGD, GDH (NADP), G6PD and AK were polymorphic. The loci HEK, LyD, PGM, IDH, MDH, XDH, EST (α,β), SOD and G6PD were monomorphic. The genetic diversity (GD) of the polymorphic loci ranged from 0.733 (LE-Tyr peptidase) to 0.387 (G6PD). The GD of other loci were as follows: PGD (0.614), Le-Gly peptidase (0.607), GDH and Le- Pro peptidase (0.424), Ak (0.420) and G6PD (0.387). The mean genetic diversity among all isolates was 0.515.

Table 2. Epidemiological and phenotypic characterization of 13 ETs among 26 respiratory strains of *K. pneumoniae*

Strain no.	Clinical specimen	Ward	Date of isolation (day.month.year)	ESBL *	ET**	Markers of resistance
110	CSF	ICU	03.07.04	+	1	Nf, Cip, NA, CL, Cfx, Ca, Ce, Ci, Ao
112	Blood	ICU	12.07.04	+	1	Cip, NA, CL, Cfx, Ca, Ce, Ci, Ao
114	Trachea	ICU	13.07.04	+	1	Gm, Cip, NA, CL, Cfx, Ca, Ce, Ao
116	Urine	OP	13.07.04	-	1	Cfx
111	Urine	OP	03.07.04	-	1	Cfx
101	Trachea	ICU	03.07.04	+	3	NA, CL, Cfx, Ca, Ce ,Ci, Ao
103	Trachea	ICU	15.07.04	+	3	NA, CL, Cfx, Ca ,Ce, Ci, Ao
104	Trachea	ICU	15.07.04	+	3	NA, CL, Cfx, Ca, Ce, Ci, Ao
142	Trachea	ICU	18.01.05	+	10	Ak, Cip, NA, TZP, CL, Cfx, Ca, Ce, Ci, Ao
139	Urine	OP	31.12.04	-	10	Cip, NA, Cl, Ao
113	Trachea	Women	12.07.04	-	12	Cfx
122	Trachea	ICU	22.09.04	+	14	Ak, Gm, Cip, NA, Cl, Cfx, Ca, Ce, Ci, Ao
74	Trachea	RCU	02.11.03	+	23	Ak, Gm, Cip, NA, Cl, Cfx, Ca, Ce, Ci
81	Trachea	RCU	17.11.03	+	24	Ak, Gm, NA, Nf, Cl, Cfx, Ca, Ce, Ci
94	Sputum	Women	27.11.03	+	24	Ak, Gm, Cip, NA, Nf, Cl, Cfx, Ce
132	Trachea	ICU	24.10.04	+	25	Ak, Gm, Cl, Cfx, Ca, Ce ,Ci, Ao
133	Urine	-	26.10.04	+	26	Ak, Gm, Cip, NA, Cl, Cfx, Ca, Ce, Ci, Ao
131	Trachea	-	26.10.04	+	26	Ak, Gm, Cl, Cfx, Ca, Ce, Ci, Ao
21	Trachea	ICU	29.09.03	+	30	Gm, NA, Cl, Ca
24	Trachea	ICU	29.09.03	+	30	Gm, NA, Cl, Ca
61	Sputum	RCU	27.10.03	+	33	Ak, Gm, Cip, NA, Nf, Cl, Cfx, Ca, Ce, Ci
102	Bronchi	ICU	18.11.04	+	38	Ak, Cip, NA, Nf, Cl, Cfx, Ca, Ce, Ci, Ao
166	Trachea	ICU	14.04.05	-	40	Gm ,NA, Cl, Cfx, Ca, Ce, Ci, Ao

* Extended spectrum beta-lactamase

** Electrophoretic type

DISCUSSION

The high rate of ESBLs among hospitalized patients is a global problem. It is generally thought that patients infected by an ESBL-producing organism are at an increased risk of treatment failure with an expanded-spectrum β-lactam. The prevalence of ESBL producing isolates of *K. pneumoniae* varies in different countries (11). In some parts of Asia, the percentage of ESBL-production in *E. coli* and *K. pneumoniae* varies from 4.8% in Korea to 8.5% in Taiwan and up to 12% in Hong Kong (11). Countries with a high rate of prevalence include Turkey (60%), Latin America (45.4%), Western Pacific (24.6%), and Europe (22.6%) (12).

This rate was 49% in our survey which is significantly higher than the western pacific, Europe and Asia but similar to Latin America.

The prevalence of respiratory isolates of *K. pneumoniae* with ESBL phenotype has been reported from less than 1% in Japan (3) to 83.3% in China (13). These rates also differ in Taiwan (8.5%), Hong Kong (13%) and Korea (28.4%) (3). Our study confirmed a rate of 75.75% in Iran which is the

second highest after China.

ESBL-producing strains were often resistant to other antibiotics such as aminoglycosides and fluroquinolones (2). In Chili, the rates of resistance in ESBL- producing strains to gentamicin and amikacin were 65% and 47% respectively (14). In Turkey, the rates of resistance to amikacin and ciprofloxacin among ESBL producing strains have been reported as 16.7% and 74.1% respectively (15). In our study, the rates of ESBL-producing strains showing resistance to amikacin (18%), gentamicin (16%) and ciprpfloxacin (18%) are lower than figures reported from Chili but close to those of Turkey.

Multilocus enzyme electrophoresis has been used as a standard method in population genetics and systematics of bacteria (9). This has proved to be a powerful means of characterizing the genetic diversity of *K. pneumoniae* (5). MLEE can differentiate the respiratory isolates (n=26) into 13 ETs. ET 1 contained 5 isolates that were cultured from patients over a period of 10 days. Isolates 110, 112 and 114 were isolated from patients in the ICU ward in one of the study hospitals. They belonged to ESBL phenotype and exhibited the same antibiotic resistance patterns (Cipr, NAr, Clr, Cfxr, Car, Cer, Cir, Aor, Acr) except for Gmr and Nfr (Table 2). This finding suggests the dissemination of a clone with ESBL phenotype in the ICU. Other isolates in this ET, 111 and 116, were cultured from urine samples of outpatients. Both of them showed similar resistance patterns (Cfxr) and were negative in ESBLs detection tests.

Strains 101, 103 and 104 belonged to ET3. They were isolated from patients in the ICU and showed ESBL phenotype. The results of MLEE analysis were consistent with drug susceptibility testing and ESBL detection test (Table. 2). Moreover, they had been cultured from the trachea of patients over a period of 12 days. This finding provides evidences for the persistence of infection with ESBL strains in the ICU of the under-study hospital.

Strains 142 and 139 belonged to the same ET. They were isolated during 18 days from different wards and with different clinical sources. In spite of different antibiotic resistance patterns, the isolates exist in the same ET. However, they have some common antibiotic resistance patterns (Cipr, NAr, Clr, Aor, Acr).

Strains 133 and 131 belonged to ET 26. Both of them produced ESBL and shared common resistance to AKr, Gmr, Clr, Cfx, Car, Cer, Cir, Aor, Acr. However, they were isolated from different clinical specimens. There were 2 respiratory isolates in ET 24 that showed resistance to amikacin and gentamicin from aminoglycoside family. These strains produced ESBL. ET 30 also had 2 strains with the same antibiotic resistance pattern. This data showed the close relationships among these isolates.

MLEE had been reported to be more discriminatory than ribotyping for typing of *K. pneumoniae* strains as it can detect polymorphic enzyme loci in chromosomal genes (5). This method was used here as an epidemiological marker. Some strains producing ESBL were differentiated by MLEE and those strains with the same ET in MLEE differed from each other in antibiotic resistance patterns. This work showed the importance of respiratory tract infections with *K. pneumoniae* among Iranian patients in terms of genetic diversity and high rate of resistance to third generation cephalosporins as well as other antibiotics.

In conclusion, spreading ESBL-producing strains is a concern, as it causes limitations to the antimicrobial agents for optimal treatment of patients. The most reliable and effective antimicrobial treatment for infections caused by this organism is imipenem (16). Analysis of strains with MLEE showed the existence of certain clones in the ICU wards and the persistence of infection with ESBLs producing isolates.

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