Phenotypic characterization and plasmid analysis of Klebsiella pneumoniae strains from Iranian patients

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Abstract

Local knowledge of antimicrobial susceptibilities of Klebsiella pneumoniae is important for implementation of effective hospitals anti-infective policies. One hundred isolates of K. pneumoniae collected from 3 different hospitals in Iran during 2004 were screened for their susceptibilities to thirteen different antibiotics using disk diffusion test and macro broth dilution assay. Isolates were then subjected to restriction endonuclease analysis of plasmid DNA. All isolates were susceptible to imipenem. The rates of resistance to other antibiotics were in the following order: amikacin (10%), piperacillin-tazobactam (%2), ciprofloxacin (20%), ceftizoxime (14%), cefexime (31%), ceftazidime (28%), cefotaxime (33%), nalidixic acid (32%), cephalexin (32%), gentamicin (30%), nitrofurantoin (31%) and piperacillin (66%). The production of extended spectrum betalactamase (ESBL) hydrolyzing ceftazidime and cefotaxime was detected in 54% of isolates. Of 100 isolates tested, 67 harbored plasmids and the remaining lacked any plasmid. Though the prevalence of ESBL phenotype in Iran is higher than western countries, it is close to figures reported from the region. Evidences for outbreaks with certain isolates of K. pneumoniae were found by restriction endonuclease analysis of plasmid DNA. This technique also showed the persistence of infections in the urinary tract of several patients.

Keywords*: Klebsiella pneumoniae - ESBL strains- Plasmid profiling- Tehran Hospitals; Nosocomial infections- Drug resistance.*

INTRODUCTION

Nosocomial infection with K. pneumoniae is a serious problem in Iran. The prevalence of infection with this organism has been reported as 70% in one of the hospitals in the north east of country [1]. The organism has also been isolated from 37.8% of cases with neonatal septicemia [2]. The extended spectrum ß-lactamase producing isolates of K. pneumoniae has created a major problem in antibiotic therapy due to lack of effective drugs against some multi resistant Isolates.[3,4]

ESBLs are typically encoded by plasmids that can be exchanged between bacterial species. In many cases, these plasmids also harbor other antimicrobial resistance genes. Therefore, it is common for organisms with ESBL phenotype to express multiple resistance to aminoglycosides, trimethoprim-sulfamethoxazole and tetracyclines.[5] Outbreaks due to dissemination of ESBL-producing strains of Klebsiella spp. vary geographically. The prevalence of ESBLs within isolates of K. pneumomiae in Asian Pacific area and China has been reported as 25.2 and 51% respectively. [6,7]

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The aim of this study was to investigate the susceptibility of K. pneumoniae isolates to different antibiotics and to determine the prevalence of ESBL producers in the population of this organism in 3 hospitals in Tehran. Plasmid profiling was used as a typing technique to determine the genetic relationships between the isolates and possible outbreaks between different wards of study hospitals.

MATERIALS AND METHODS

Bacterial culture and susceptibility testing

One hundred and fifteen isolates of Klebsiella spp were collected from clinical specimens at different hospitals between March to October 2004. These hospitals are located at different parts of the capital city. Conventional biochemical tests were used for identification of isolates to the species level [8]. Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 29213, Pseudomonas aeruginosa ATCC 27853, Klebsiella pneumoniae ATCC700603, and Enterococcus faecalis ATCC 29212 were used as control strain.

Susceptibility of isolates to antimicrobial agents was determined using commercially available disks impregnated with the following antibiotics: imipenem (10µg), amikacin (30µg), piperacillin-tazobactam (10/100µg), ciprofloxacin (5µg), ceftizoxime (30µg), cefexime (5µg), ceftazidime (30µg), cefotaxime (30µg), nalidixic acid (30µg), cephalexin (30µg), gentamicin (10µg), nitrofurantoin (300µg) and piperacillin (10µg) according to NCCLS guidelines.[9]

Isolates showing resistance or with decreased susceptibility to ceftazidime and cefutaxime were tested for ESBL production. Disks containing 10 µg of clavulanic acid plus 30 µg of ceftazidime were placed on the inoculated plates containing Muller Hinton agar. Similar assay was fulfilled with cefotaxime plus cefotaxime/clavulenic acid in parallel. A positive test result defined as a 5-mm increase in zone diameter comparison to a disk without clavulenic acid [10]. All disks were purchased from Oxoid (Oxoid, UK).

The macro-broth dilution assay [11] was used to determine the minimum inhibitory concentration of ceftazidime, gentamicin and ciprofloxacin (Exir, Borojerd, Iran) for 40 isolates and the results were compared with those obtained by disk diffusion tests.

Plasmid profile analysis

Plasmid DNA was extracted using alkaline lysis method [12]. Plasmids were digested by Hin dIII and Eco RI and separated by agarose gel lectrophoresis and visualized under ultraviolet light after staining of the gel with ethidium bromide. A supercoiled DNA ladder (2–16 kb) was used as the reference for small plasmids.

RESULTS

One hundred isolates were identified as K. pneumoniae and the remaining were K. oxytoca (n=15). These isolates were cultured from the clinical specimens including urine (n=78), trachea (n=9), blood (n=4), wounds (n=3), sputum (n=2) and others (n=4). The ratio of females to male patients infected with K. pneumoniae was 76 to 24.

The results of drug susceptibility testing obtained by disk diffusion method is shown in Table 1. None of the isolates was resistant to imipenem. With a resistant rate of 66%, piperacillin was the least effective antibiotic against isolates of K. pneumoniae in this study. Combination of piperacilin with tazabactam reduced the rate of resistance to 2%. The rates of resistance to nitruforantoin (31%), nalidixic acid (32%), cephalexin (32%), cefotaxime (33%), cefexime (31%), gentamicin (30%) and ceftazidime (28%) were close together. The resistance rates for amikacin, ciprofloxacin and ceftizoxime were 10%, 20%, and 14% respectively. Of 29 isolates showing resistance to ceftazidime, 25 (86%) were also resistant to gentamicin. Multi-resistance to ceftazidime, gentamicin and ciprofloxacin were obsereved in 8% of isolates.

The results of macro-broth dilution assay on 100 isolates were compared with those obtained by disk diffusion method. No significant differences were found between both methods.

Production of ESBL was detected in 54 isolates of which 22 and 41 used ceftazidime and cefotaxime as substrates respectively. 9 isolates hydrolyzed both antibiotics. Of 55 ESBL producing

Table 1 - Results of drug susceptibility testing on 100 isolates of *Klebsiella pneumoniae* cultured from patients at Tehran hospitals.

Antibiotics	sensitive	intermediate	resistance	total
Piperacillin	12	22	66	100
Nitrofurantoin	48	31	31	100
Nalidixic acid	67	1	32	100
Cephalexin	66	2	32	100
Cefexime	68	1	31	100
Gentamicin	69	1	30	100
Ceftazidime	68	4	28	100
Ceftriaxone	67	15	18	100
Cefotaxime	66	1	33	100
Ciprofloxacin	73	7	20	100
Ceftizoxime	71	15	14	100
Amikacin	80	10	10	100
Piperacillin-Tazobactam	74	24	2	100
Imipenem	100	0	0	100

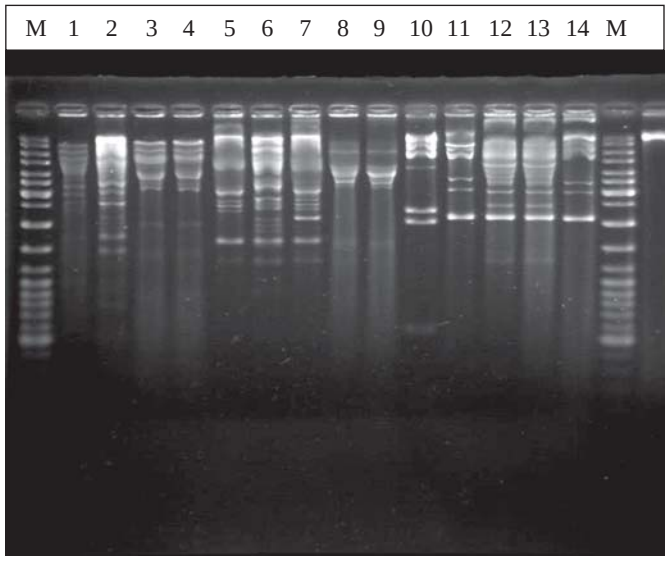

Figure 1 - Digestion of plasmid DNA with Eco RI.

Note: lanes M : Molecular weight marker (100bp10000bp); 3-4: isolates. T89 & T90 belongin to an outpatients; 8-9 isolates L107 & L108 recovered from two patients at different wards; 12-13 isolates L102 & L104 recovered from two patients at different wards.

Table 2 - The results of plasmid fingerprinting on selected isolates recovered from different specimens of patients stayed at hospital wards.

Isolates number	Patients	Plasmid fingerprints	ESBL	Specimen	Ward
F21	1F	A	-	Trachea	ICU
F24	1F	B	+	Trachea	ICU
L73	2F	C	+	Trachea	RCU
L74	2F	C	+	Urine	RCU
ch78	3M	D	+	Trachea	ICU
ch79	3M	D	+	Urine	
T89	4F	E	-	Urine	Out patient
T90	4F	E	-	Urine	
ch95	5M	F	+	Blood	ICU
ch96	5M	G	+	Trachea	
ch97	5M	F	+	Venal catheter	
L70	6F	H	+	Urine	Transplant
L75	6F	I	+	Urine	
L107	7F	J	-	Urine	Internal Urology
L 108	8F	J	-	Urine	
102	9M	K	+	Wound	Urology
04	10F	K	+	Urine	Urology

isolates, 21 (38.18%) were resistant to gentamicin and 6 (10.90%) were resistance to ciprofloxacin.

Plasmids were found in 67% of isolates. Isolates originated from different hospitals showed distinct plasmid profiles in terms of size and number of plasmids. Plasmid DNA from 30 isolates with similar profiles (n=30) were selected and subsequently digested with Eco RI and Hind III. The results showed that isolates cultured from different body organs might be distinct or identical (TABLE 2; FIGURE 1). For example two isolates recovered from patient 5M were identical but the third one produced different DNA fingerprints. Two isolates recovered from trachea of patient 1F yielded different plasmid fingerprinting by endonuclease Eco RI. Repetitive isolation of K. pneumoniae from certain patients may produce identical plasmid profiles (Patients 2F, 6 3M, 4F) or distinct DNA banding pattern (patients 1F, 5M and 6F) (FIGURE 1).

DISCUSSION

This study was conducted to determine the drug resistant patterns of K. pneumoniae at Tehran hospitals. K. pneumoniae has been the second gram negative bacteria involved in urinary tract infections (7%) in Iran [13]. Isolates of this organism had been involved in 35-37% cases of neonatal septicemia in the country with a mortality rate as high as 47%.[14]

There was agreements between the results obtained by disk diffusion method and those of macro-broth dilution assay. Piperacillin is inactivated by b-lactamase, but its bactericidal effect may return if it is used in combination with tazobactam. Therefore, the rate of resistance to this antibiotic was dropped from 66% to 2% when it was combined with tazobactam. However, up to 24% of isolates in this study showed intermediate level to this combination. Therefore, we suggest that prescription of piperacillin-tazobactam to be limited to the susceptible isolates only. Aminoglycosides, in particular amikacin, may be used in the treatment of infections with ESBL-producing organisms if the strain is susceptible. Of 30 isolates showing resistance to gentamicin, 14 (46%) were susceptible to amikacin and 8 (26%) showed resistance to both antibiotics. Therefore, the mechanism of resistance to amikacin appears to be independent from gentamicin. In the current study 48% of ESBL producing were resistant to amikacin. Therefore, like piperacillin/tazobactam, there is limitation in treatment of ESBL producing isolates with amikacin in Iran.

Finding resistance to various antibiotics among ESBL producing isolates is predictable since the genes encoding ESBL are typically located on self-transferable plasmids that often carry other determinants of antibiotic resistance.[15,16,17]

Of 55 isolates with ESBL phenotype, 9 (31%) were also resistant to ciprofloxacin. This rate is much higher than 18% that was reported in a multinational study.[18]

ESBLs are now a problem in hospitalized patients worldwide. It is generally thought that patients having infections caused by an ESBL-producing organism are at increased risk of treatment failure with an expanded-spectrum b-lactam. The prevalence of ESBLs found in this study is much higher than northern European countries (1-5%), but is close to the figure reported from Turkey (48.5%) [19]*. It appears that most of the Iranian strains with ESBL phenotype use cefotaxime as substrate rather than ceftazidime.*

Plasmid profiling technique has been used in parallel with other typing technique to study the epidemiology of infections with K. pneumoniae [20]*. This technique is relatively rapid, inexpensive, technically simple and provide readily interpretable results. In our study it could differentiate the isolates frequently recovered from patients (TABLE 2). We showed that certain plasmids circulate between different wards of study hospitals.*

Though we did not type the isolates by PFGE, differentiation of ESBL strains recovered from patients such as 5M and 6F was achieved by plasmid fingerprinting. The ESBL strains may co-exist with susceptible strains in the same or different organs as demonstrated in patient 24F. This finding shows the simplicity and usefulness of plasmid fingerprinting for differentiating of isolates frequently recovered from trachea of patients. Moreover, it was shown that isolates recovered from different specimens of patients staying at ICU and RCU can yield identical plasmid fingerprints. Transmission of strains with certain plasmid among different wards of hospitals i.e., isolates from patients 7F and 8F, is an evidence for common source of outbreak in the study hospitals and can be used for tracing the source (TABLE 2).

Caracterização fenotípica e análise plasmidial de cepas de *Klebsiella pneumoniae* em pacientes iranianos

Resumo

É importante os conhecimentos locais de susceptibilidade antimicrobiana para *Klebsiella pneumoniae* a fim de que haja uma implementação efetiva de política hospitalar em relação aos antibacterianos. Foram isoladas 100 culturas para *K. pneumoniae* coletadas a partir de 3 diferentes hospitais no Irã durante o ano de 2004; para a susceptibilidade foram selecionados treze antibióticos diferentes, utilizando o método de difusão em disco e ensaio em caldo de diluição. Os isolados foram então submetidos à análise de endonucleases restritas ao DNA plasmidial. Todos os isolados foram sensíveis ao imipenem. As taxas de resistência a outros antibióticos foram na seguinte ordem: amicacina

(10%), piperacilina-tazobactam (2%), ciprofloxacina (20%), ceftizoxima (14%), cefexime (31%), ceftazidima (28%) , cefotaxima (33%), ácido nalidíxico (32%), cefalexina (32%), gentamicina (30%), nitrofurantoína (31%) e piperacilina (66%). A produção de betalactamase de espectro estendido (ESBL) que hidrolisa a ceftazidima e a cefotaxima foi detectada em 54% dos isolados. Das 100 amostras testadas, 67 portavam plasmídeos e o restante faltava qualquer plasmidial. Embora a prevalência do fenótipo ESBL no Irã seja superior a países ocidentais, os números reportados são restritos de uma região. As evidências de focos com certos isolados de *K. pneumoniae* foram encontradas pela análise das endonucleases restritas de DNA plasmidial. Esta técnica também mostrou a persistência de infecções do trato urinário de vários pacientes.

Palavras-chave: *Klebsiella pneumoniae* - cepas (ESBL)- perfil plasmidial- Hospital de Tehran; Infecções nosocomiais- Resistência a drogas.

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