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Plasmid Profiling of *Klebsiella* sp. and its Relation with Antibiotic Resistance at two Hospitals of Urmia (Iran)

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Abstract: The aim of the present study was to compare the antibacterial susceptibility patterns with the presence of plasmids in *Klebsiella* isolates. Thirty nine isolates were collected from urine specimens submitted to two educational hospitals in Urmia/Iran. The susceptibility patterns were determined against antibiotics, Plasmids were extracted by alkaline lysis method, electrophoresed and investigated by a UV transilluminator. Single digestion of plasmids with *Eco*RI and *Hinc*II were performed and the restriction patterns were compared using a DNA ladder. The rates of resistance were determined to antibiotics as follows: gentamicin 46.1%, tobramycin 48.7%, ceftizoxime 41%, co-trimoxazole 46.1%, amikacin 33.3%, cephtazidime 51.3%, ciprofloxacin 30.8%, kanamycin 53.8%, nalidixic acid 30.8% ampicillin 79.5% and Nitrofurantoin 41%. 25.6% of isolates harbored plasmids. Restriction enzyme analysis of plasmids showed unique pattern for all of plasmid positive isolates. There was a meaningful correlation between the presence of plasmid in isolates and resistance to the tested antibiotics. The absence of plasmids from the majority of isolates showed low typeability power of this technique, so using of other molecular typing techniques in companion with plasmid profiling and restriction enzyme analysis suggested for further studies.

Key words: *Klebsiella*, plasmid profiling, restriction enzyme analysis

INTRODUCTION

Klebsiella sp. is a group of short, non-motile, gram negative rods and they are present in the respiratory tract and feces of about 5% of normal individuals. They can cause different kinds of infections, for example, pneumonia, urinary tract infection and bacteremia in immunocompromised hosts. *K. pneumoniae* and *K. oxytoca* cause hospital- acquired infections (Brooks *et al*., 2004).

Multi-drug resistant *Klebsiella* has been recognized as a cause of hospital acquired infections worldwide (Eisen *et al*., 1995; Keynan and Rubinstein, 2007). They are resistant to numerous antibiotics, including aminoglycosides, penicillins and extended spectrum cephalosporins. Their resistance to antibiotics restricts the choice of antibiotics for therapy (Hogg *et al*., 1993).

There are different mechanisms for development of resistance in *Klebsiella* sp. including the acquisition of plasmids which code for the production of extended spectrum beta-lactamases and aminoglycoside modifying enzymes. The genetic origin of drug resistance may be plasmid or chromosomal. Plasmids carry genes for resistance to often more than one antibiotics, these plasmids so-called R-plasmids. R-plasmids can easily transfer between different species and even genera of bacteria, so, determination of the genetic origin of resistance in clinical isolates of bacteria can predict the rate of transferring resistance between clinical isolates (Oktem *et al*., 2008).

Investigation of the plasmid profiles of nosocomial isolates and determination of the pattern of restriction enzyme analysis of plasmids are reliable methods for typing of hospital bacterial isolates, however, there is no data in the literature about plasmid profiles and pattern of restriction enzyme analysis of plasmids of *Klebsiella* sp. about clinical isolates in hospitals of Urmia, North-West of Iran.

In this study, we investigated antibiotic resistance pattern, plasmid profiles and patterns of restriction enzyme analysis of plasmids in clinical isolates of *Klebsiella* sp. As well as relation between plasmid pattern and resistance to different antibiotics were determined.

MATERIALS AND METHODS

Bacterial isolates: A total of 39 isolates were collected from urine specimens submitted to two educational hospital clinical laboratories in Urmia/Iran during a three months period from December 2006 until March 2007. The

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isolates were further processed by the standard methods to identify as the *Klebsiella* sp. In brief some of biochemical tests were used for identification of the isolated bacteria as *Klebsiella* sp. was indole production, methyl red, voges-proskauer, citrate, SH_2 production, urea hydrolysis, Phenylalanine deaminase, Lysine decarboxylase, Arginine dihydrolase, motility and several sugars fermentation (Baron and Finegold, 1990). Isolated bacteria were maintained for long storage on skimmed milk medium (BBL) by adding 10% glycerol in -60°C, cultures were maintained for daily use on Nutrient agar (BBL) slants on 4°C.

Susceptibility testing: The Muller Hinton Agar (MHA) and Muller Hinton Broth (MHB) media (Merck) were used for detection of antibiotic resistance of isolates. The susceptibility of isolates to different antibiotics was tested using agar disk diffusion method (Bauer *et al*., 1966; Jazani *et al*., 2007). To represents different classes of antibacterial agents commonly used for treatment of *Klebsiella* infections, gentamicin, tobramycin, ceftizoxime, co-trimoxazole, amikacin, cephtazidime, ciprofloxacin, kanamycin, nalidixic acid, ampicillin and nitrofurantoin were used in present study (Hi-media, Mombay, India).

Extraction, purification and electrophoresis of plasmids: Plasmids were extracted by alkaline lysis method with some modifications. In brief, isolates were cultured in LB medium and after centrifugation, cellular pellet were re-suspended in 100 μL of GTE buffer (2 mL EDTA 0.5 M, 10 mL Glucose 0.5 M and 2.5 mL Tris-base 1 M pH = 8). Two hundred microliter of lysis buffer (1 mL NaOH 1 M and 0.5 mL SDS 10%) were added and tubes were incubated in ice for 5 min. Then cold sodium acetate solution was added and tubes were incubated in ice for 10 min, after centrifugation.

Phenol/chloroform/isoamyl alcohol solution in a 1:24:25 ratio was added to supernatant and after another round of centrifugation, isopropanol was added to supernatant and samples were centrifuged for 15 min, resulting white pellet washed with cold ethanol and after centrifugation supernatant was discarded and micro tubes allowed to dry, pellet solved in 50 μL of buffer (Tris-HCl pH = 8 10 mM, EDTA 1 mM and RNase) (Sambrook and Russel, 2001).

To run electrophoresis 5 μL of loading buffer (Takara, japan) was added to 35 μL plasmid solution and then 20 μL of aliquots was loaded on 0.8% agarose gel(contained ethidium bromide). Plasmids were separated by agarose gel electrophoresis at 45 V for 4-5 h and visualized under UV illumination.

Restriction digestion of plasmid DNA: Single digestion of plasmid DNA extracts with *Eco*RI (Fermentase) and *Hinc*II (Cinnagen, Tehran, Iran) were performed as recommended by the manufacture. The cleavage products of single digestion were electrophoresed on 1% agarose gel. The restriction patterns of different plasmid DNA extracts were compared using DNA ladder (100-12000) as a molecular size marker.

Statistical analysis: Fisher's exact statistical test was used for analysis of qualitative data. p-value was considered significant if <0.05.

RESULTS AND DISCUSSION

Sensitivity of bacterial species to antibiotics: Based on the results of drug susceptibility testing, 16 different antibiotypes numbered from 1 to 16 were found. Antibiotype 1 consisted of 8 isolates that showed resistance to all investigated antibiotics. It was the most prevalent antibiotype among the isolates (20.5%). Four isolates were sensitive to all tested antibiotics (antibiotype 2). The remaining antibiotypes consisted of 27 isolates were resistant to different antibiotics. Ampicillin (79.5%) and kanamycin (53.8%) showed the highest rate of resistance and ciprofloxacin and nalidixic acid (30.8%) demonstrated the lowest (Table 1).

The list of 22 MDR phenotypes identified is shown in Table 2, with majority showing resistance to the 11 antimicrobials.

Plasmid profile analysis indicated that ten isolates (25.6%) harbored one plasmid with identical patterns in all isolates (Fig. 1). The restriction pattern of the ten isolates is identical after single digestion of plasmids with *Hinc*II

Table 1: The rates of resistance to different antibiotics for 39 isolates of *Klebsiella* from two educational hospitals in a six months period

	Antibiotics										
	Am	K	CAZ	Tob	Ct	SXT	Gm	AN	Cp	NA	FN
Resistance	79.5	53.8	51.3	48.7	41	46.1	46.1	33.3	30.8	30.8	41

Gentamicin (Gm), Tobramycin (Tob), Ceftizoxime (Ct), Co-trimoxazole (SXT), Amikacin (AN), Cephtazidime (CAZ), Ciprofloxacin (Cp), Kanamycin (K), Nalidixic Acid (NA), Ampicillin (Am) and Nitrofurantoin (FN)

Table 2: Antibiotic resistance phenotypes of 22 clinical MDR isolates

Resistance pattern	No. of isolates (%)
Am Cp NA	2 (5.1)
Am Caz K Sxt	2 (5.1)
Am Caz Gm K Tob Sxt	2 (5.1)
Am Ct Caz Gm K Tob Sxt	3 (7.6)
Am Ct Caz An Gm K Tob Sxt	2 (5.1)
Am Ct Caz An Gm K Tob Fn Sxt	1 (2.6)
Am Ct Caz Gm K Tob Cp NA Fn Sxt	2 (5.1)
Am Ct Caz An Gm K Tob Cp NA Fn Sxt	8 (20.5)

Fig. 1: Electrophoresis of plasmids extracted from *Klebsiella* isolates from two hospitals in Urmia/Iran. Lane 1 and 2 unique profile of plasmid band extracted from 25.6% of isolates

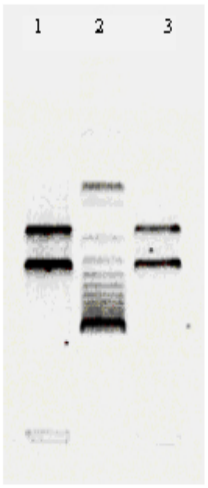

Fig. 2: Electrophoresis of plasmids digested by restriction enzymes. Lane 2: molecular size marker, Lane 1: Single digestion of plasmid band with *Hinc*II, Lane 3: Single digestion of plasmid band with EcoRI

or *Eco*RI. Two bands obtained after restriction enzyme analysis of plasmids. The sizes of these bands were found to be approximately 4 and 2.8 kbp (Fig. 2). The association between presence of plasmids and resistance to antibiotics was significant ($p<0.05$) (Table 3). All the plasmid containing isolates were resistant to at least ten different antibiotics.

Klebsiella is an opportunistic pathogen and is a causative agent of several kinds of infections in humans.

Table 3: Relation between presence or absence of plasmid and antibiotic resistance with Fisher's exact statistical test. There is meaningful relations between the presence of plasmid and resistant to all tested antibiotics with the exception of ampicillin

Resistance to different antibiotics	Plasmid positive resistant isolates/ plasmid positive isolates (%)	Plasmid negative resistant isolates/ plasmid negative isolates (%)	p-value
Ampicillin (Am)	100	72.4	0.086
Kanamycin (K)	100	34.4	0.010
Ceftazidime (CAZ)	100	34.4	0.010
Tobramycin (Tob)	100	31.0	0.000
Ceftizoxime (Ct)	100	20.6	0.000
Co-trimoxazole (SXT)	100	27.6	0.000
Amikacin (AN)	80	17.2	0.010
Nitrofurantoin (FN)	100	20.6	0.000
Ciprofloxacin (Cp)	100	7.0	0.000
Nalidixic acid (NA)	100	7.0	0.000
Gentamicin (Gm)	100	27.6	0.000

It is one of the major pathogens in nurseries, intensive care units and in hospital wards in spite of many effective antibiotics now available (Eisen *et al*., 1995).

The use of broad spectrum antibiotics in hospital environments exerts selective pressure on bacteria, results in promoting infections by multi-antibiotic resistant isolates. Present finding showed that the most useful antibiotics for infections caused by *Klebsiella* sp. were amikacin, nalidixic acid and ciprofloxacin. Resistance to some antibiotics such as ampicillin, gentamicin, kanamycin and nitrofurantoin showed increases in comparison with previous studies in different countries (Rasool *et al*., 2003; Talbot *et al*., 1980).

Isolates grouped in the main antibiotype (antibiotype 1) consisting of eight isolates were resistant to all tested antibiotics, two isolates grouped in antibiotype 15 were resistant to ten antibiotics and only showed intermediate sensitivity to amikacin.

Molecular methods such as plasmid profile analysis and restriction enzyme analysis of plasmids have recently been used for determining the bacterial strains which cause nosocomial outbreaks. Tayfour *et al*. (2005) used plasmid profiling, Restriction Endonuclease Analysis of Plasmids (REAP) and antibiotic sensitivity tests for detection the source of resistance in *Staphylococcus aureus* strains. Tolmasky *et al*. (1988) reported a multiresistant *Klebsiella pneumoniae* strain isolated from neonates in Argentina, harbored a 48 kbp plasmid, with genetic determinants for resistance to amikacin and also ampicillin, kanamycin, streptomycin and tobramycin. Gaynes *et al*. (1988) reported that clinical isolates of *Klebsiella* at a hospital that had used amikacin as its principal aminoglycoside demonstrated high-level resistance to amikacin, kanamycin, gentamicin, netilmicin and tobramycin. The resistant strains contained an identified 6.8 kbp plasmid. In the present study plasmid DNA was detected in 25.6% of isolates, however 80% of

these isolates were resistant to all tested antibiotics and 20% were resistant to 10 antibiotics and showed intermediate sensitivity to amikacin. The prevalence of plasmids in our isolates are relatively low, therefore it is postulated that most of the resistance genes in our isolates are chromosomal. However all the isolates containing plasmid were resistant to at least 10 antibiotics indicating the importance of presence of plasmid in Multi drug resistant isolates. The relationship between presence of certain plasmids and resistance to some antibiotics in *Klebsiella* sp. has been demonstrated previously (Tolmasky *et al*., 1988; Gaynes *et al*., 1988; Nikbin *et al*., 2007).

The absence of plasmids from the majority of isolates showed low typeability power of this technique, so, using of the other molecular typing techniques in companion with plasmid profiling and restriction enzyme analysis suggested for further studies.

CONCLUSION

In conclusion these results remind us again that physicians should be aware of current antimicrobial susceptibility patterns of pathogens in their communities and design strategies to diminish non specific use of antimicrobial drugs in the hospitals.

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