Correlation of Multi-drug Resistance, Integron and blaESBL Gene Carriage With Genetic Fingerprints of Extended-Spectrum β-Lactamase Producing Klebsiella pneumoniae

Mitra Ashayeri-Panah 1; Mohammad Mehdi Feizabadi 2; Fereshteh Eftekhar 1,7

1Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, General Campus, Tehran, IR Iran
2Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, IR Iran
*Corresponding author: Fereshteh Eftekhar, Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, General Campus, Tehran, IR Iran. Tel: +98-2129903208; Fax: +98-2122431664; Email: f-eftekhar@sbu.ac.ir

Background: Some genetic and phenotypic variables are associated among distinct microbial populations. Objectives: The associations between multi-drug resistance (MDR) phenotypes, prevalence of antibiotic resistance integrons (ARIs), blaSHV, blaTEM and blaCTX-M gene carriage and genetic fingerprints of random amplified polymorphic DNA (RAPD), confirmed by pulsed field gel electrophoresis (PFGE), were investigated among extended-spectrum β-lactamases (ESBL)-producing nosocomial isolates of Klebsiella pneumoniae.

Materials and Methods: Susceptibility of 35 ESBL-producing K. pneumoniae nosocomial isolates to 22 antimicrobial agents was determined. Integron carriage was detected using specific primers for intI1, intI2 and intI3 genes by PCR.

Results: All isolates were resistant to piperacillin and susceptible to imipenem. MDR phenotype was observed in 94.3% of the isolates. Class 1 integrons were detected in 21 (60%) and class 2 integrons in 3 (8.57%) of the isolates. Two of the isolates carried both classes and none harbored class 3 integrons. Significant correlations were observed between resistance to aminoglycosides, fluoroquinolones and sulfonamides, and between genotype groups with carriage of ARIs, MDR phenotype and blaSHV gene carriage. ARI carriage was also significantly associated with MDR phenotype.

Conclusions: Our findings suggest the possible co-carriage of some blaSHV genes and ARIs on the same plasmids harboring the MDR genes. Possible role of integrons in dissemination of ESBL-encoding blaSHV genes among ESBL-producing K. pneumoniae nosocomial isolates may be inferred.
prevalence of ARIs, \textit{bla}\textsubscript{ESBL} genes and RAPD profiles were investigated among ESBL-producing \textit{K. pneumoniae} nosocomial isolates.

3. Materials and Methods

3.1. Bacterial Strains

Thirty five ESBL-producing nosocomial isolates of \textit{K. pneumoniae} were randomly selected from a collection previously described (9). Bacteria were isolated from hospitalized patients at different wards of Labbafinejad teaching hospital, Tehran, Iran, during March 2008 – March 2009; subjects consisted of 23 (65.7%) male patients and 12 (34.3%) females. These isolates were recovered from urine (n = 23; 65.7%), trachea (n = 4; 11.4%), wounds (n = 4; 11.4%), blood (n = 2; 5.7%), sputum (n = 1; 2.9%) and unknown sources (n = 1; 2.9%). ESBL production was confirmed using the phenotypic confirmatory test and susceptibility of the isolates to 22 antimicrobial agents (Hi media, India) shown in Figure 1, was determined by the disc diffusion method according to the CLSI criteria (10).

3.2. Screening for Antibiotic Resistance Integrons

Genomic DNA was extracted from overnight grown bacteria using High Pure PCR template Prep kit for Genomic DNA extraction (Roche Diagnostics, Mannheim, Germany). PCR amplification of classes 1, 2 and 3 integrase genes was performed in 25 µL reaction mixtures containing 30 ng DNA template, 0.4 mM of each dNTP, 150 µM MgCl\textsubscript{2}, 0.2 U Super Taq DNA polymerase (CinnaGen, Tehran, Iran) and 1 pmol of each primer (FazaBiotech, Tehran, Iran) as follows: \textit{Int}1F; CCTCCCGCACGATGATC, \textit{Int}1R; TCCACG and \textit{Int}3R; TGTTCTTGTATCGGCAGGTG) (11). Amplifications were performed in a Bioer TC25/H Thermal Cycler (Bioer Technology Ltd, Hangzhou, China) using the following program: initial denaturation at 95°C for 5 minutes followed by 35 cycles of 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C with a final extension at 72°C for 10 minutes. The amplified PCR products were resolved by electrophoresis in 1% agarose gels and visualized after staining with ethidium bromide.

3.3. Genetic Fingerprinting and Characterization of \textit{bla}\textsubscript{ESBL} Genes

Genetic profiles of the isolates by RAPD, confirmed by PFGE have been reported in our previous article (7). Presence of \textit{bla}\textsubscript{ESBL} genes (\textit{bla}\textsubscript{SHV}, \textit{bla}\textsubscript{TEM} and \textit{bla}\textsubscript{CTX-M}) and the sequencing result for the isolates were also previously reported (9).

3.4. Statistical Analyses

To assess the strength and statistical significance of correlations between the studied variables including patient gender, type of specimen, antimicrobial susceptibility, MDR phenotypes (resistance to 6 or more antibiotics), carriage of ARIs, \textit{bla}\textsubscript{SHV}, \textit{bla}\textsubscript{TEM} and \textit{bla}\textsubscript{CTX-M} genes and genotype grouping, and also measure the association between resistance to each of the aminoglycoside, quinolone and sulfonamide antibiotics, separate bivariate analyses were performed by use of the non-parametric Spearman’s rank correlation test. To confirm the association between each pair of significantly correlated variables after factoring out the effect of other effective variables, partial correlation analyses were used. To interpret the results of correlation analyses, we considered correlation coefficients (r values) as well as the levels of significance (P values).

4. Results

The antibiotic susceptibility results are shown in Figure 1. As observed, all isolates were resistant to piperacillin followed by 97.1% resistance to co-amoxiclav, 94.3% to aztreonam, 88.6% to kanamycin and cefotaxime, 85.7% to cefpodoxime, 82.9% to tobramycin and ceftazidime, 74.3% to ceftiraxone and ampicillin/sublactam, 71.4% to spectinomycin and cotrimoxazole, 68.6% to cefepime, 60% to norfloxacin, 48.6% to gentamicin, 45.7% to ciprofloxacin, 28.6% to amikacin, 25.7% to piperacillin/tazobactam and 11.4% to nitrofurantoin and colistin. All isolates were susceptible to imipenem. Streptomycin resistance was not observed but 31.4% of the isolates showed intermediate resistance. The most active antibiotic was imipenem followed by streptomycin, colistin and nitrofurantoin. Significant associations were observed between resistance to kanamycin, tobramycin, gentamicin, amikacin, norfloxacin, ciprofloxacin and cotrimoxazole (Table 1). Class 1 integrons were detected in 21 isolates (60%) and class 2 integrons in 3 isolates (8.5%). Two of the isolates carried both classes and none harbored class 3 integrons.
Genetic profiles of the isolates by RAPD (Figure 2) which were confirmed by PFGE, showed six major clusters (a-f) on a similarity level of 70%, and 21 different groups on a similarity level of 85% (7). Characterization of \( \text{bla}_{\text{ESBL}} \) genes from our previous work showed that 27 isolates (77.1%) harbored \( \text{bla}_{\text{SHV2}} \), \( \text{bla}_{\text{SHV5}} \) and \( \text{bla}_{\text{SHV11}} \) 17 (48.6%) carried \( \text{bla}_{\text{TEM}} \) genes characterized as \( \text{bla}_{\text{TEM4}} \) by sequencing, 16 (45.7%) carried \( \text{bla}_{\text{CTX-M-1}} \) which belonged to \( \text{bla}_{\text{CTX-M-15}} \) and 10 (28.57%) contained \( \text{bla}_{\text{CTX-M-III}} \) characterized as \( \text{bla}_{\text{CTX-M-8}} \) (9).

### Table 1. Statistical Analyses Regarding the Associations Between Resistance to Aminoglycosides, Quinolones and Sulfonamides Among the ESBL-Producing Nosocomial Isolates of *K. pneumoniae*

| Antibiotic classes | Aminoglycosides | Quinolones | Sulfonamides |
|--------------------|----------------|------------|--------------|
|                     | KM \(^a\)     | TN \(^a\)  | GM \(^a\)   | AK \(^a\)   | SM \(^a\)   | NOR \(^a\)   | CIP \(^a\)   | TS \(^a\)   |
|                    | r \(^b\)    | p \(^c\)  | r  | p  | r  | p  | r  | p  | r  |
| Aminoglycosides    | KM             | TN 0.789 0.1% | 0.447 1% | 0.460 1% | 0.463 5% | NS | NS | 0.456 1% | NS | NS |
|                    | TN             | GM 0.412 5% | 0.447 1% | - | - | 0.436 1% | NS | NS | 0.421 5% | NS | NS |
|                    | AK             | GM 0.363 5% | 0.460 1% | 0.436 1% | - | - | NS | NS | NS | 0.470 1% | NS | NS |
|                    | GM             | AK 0.363 5% | 0.460 1% | 0.436 1% | - | - | NS | NS | NS | 0.470 1% | NS | NS |
|                    | SM             | GM 0.363 5% | 0.460 1% | 0.436 1% | - | - | NS | NS | NS | 0.470 1% | NS | NS |
| Quinolones         | NOR 0.456 1% | 0.421 5% | NS | NS | NS | NS | NS | NS | 0.504 1% | NS | NS |
|                    | CIP 0.456 1% | NS | NS | NS | NS | 0.470 1% | NS | NS | NS | 0.504 1% | NS | NS |
| Sulfonamides       | TS 0.410 5% | NS | NS | NS | NS | NS | NS | NS | 0.410 5% | NS | NS |

\(^a\) Abbreviation: KM, kanamycin; TN, tobramycin; GM, gentamicin; AK, amikacin; SM, Streptomycin; NOR, norfloxacin; CIP, ciprofloxacin; TS, co-trimoxazole; NS, non-significant.

\(^b\) Correlation coefficients range between -1 (perfect negative relationship) and +1 (perfect positive relationship). A value of 0 indicates absence of any linear relationship.

\(^c\) Level of significance.

\(^d\) not available.

### Figure 2. Cluster Analysis of the ESBL-Producing *K. pneumoniae* Nosocomial Isolates Based on RAPD Typing, Using the Dice Similarity Coefficient

Isolate numbers are presented on the vertical axis.
Genotyping results were significantly correlated with carriage of ARIs (r = 0.700, P < 0.001; Spearman rank correlation test), blaSHV (r = 0.742, P < 0.001) and MDR phenotype (r = 0.560, P < 0.001). Significant association was also found between ARI carriage and MDR phenotype (r = 0.398, P < 0.05). Although at the 95% confidence level, no significant association was observed between ARIs with blaSHV, blaTEM and blaCTXM among the isolates. A positive association was found between class 1 integrons with blaSHV, blaSHV-5 and blaSHV-12 at a lower confidence level (r = 0.298, P < 0.1) (Table 2). Results of partial correlation analyses were also confirmatory (data not shown). No correlation was observed between the patient gender or specimen source with any of the genetic variables.

5. Discussion

Infections due to ESBL-producing strains, have been most commonly reported regarding K. pneumoniae (3). ESBL encoding genes are usually located on plasmids which may also carry other antibiotic resistance determinants. Reports have suggested a close association between ESBL production and ciprofloxacin resistance in K. pneumoniae (8). Co-resistance with other classes of antibiotics such as fluoroquinolones, aminoglycosides, tetracyclines, chloramphenicol and sulfonamides are also widespread among ESBL producing strains (12). This may explain the significant associations found between resistance to aminoglycosides (kanamycin, tobramycin, gentamicin and amikacin) in this study. The same trend was observed for the association of resistance between norfloxacin with kanamycin, tobramycin, ciprofloxacin and cotrimoxazole. Similarly, resistance to ciprofloxacin and gentamicin were associated showing a relationship as a sign of co-carriage.

Bivariate correlation analyses followed by partial correlation analyses in order to distinguish between direct and indirect interactions, confirmed the results. Despite high heterogeneity observed among the isolates of this study, genotyping results were strongly correlated with carriage of ARIs and blaSHV genes. Although almost all K. pneumoniae isolates carry chromosomal non-ESBL blaSHV, nearly all ESBL encoding blaSHV genes found in K. pneumoniae are plasmid borne (13, 14). In this study, RAPD profiles were strongly correlated with the presence of blaSHV genes suggesting that plasmid mediated blaSHV-5 and blaSHV-12 (the two prevalent ESBL encoding blaSHV genes among our isolates) had some influence on RAPD patterns. Possible contribution of plasmid DNA to RAPD patterns was suggested in K. pneumoniae (15). However, Elaichouni et al. found no influence of plasmid DNA on the RAPD profiles in Escherichia coli and claimed that the amount of chromosomal DNA per cell in natural conditions inhibits observable plasmid amplification (16). The association of blaESBL genes with ARIs occurs when both form parts of complex integrons or are located on the same plasmid (4, 17).

We found a positive association between class 1 integrons and blaSHV, blaSHV-5 and blaSHV-12 at the confidence level of 90% (P < 0.1). Since genotyping results were highly correlated with the carriage of both ARIs and blaSHV, it could be concluded that ARIs and blaSHV genes are carried on the same plasmids, or blaSHV genes are located within ARIs at least among some of our isolates. Association between ARIs and blaSHV-5 as well as co-location of blaSHV-12 and a class 1 integron on the same plasmid have been reported (17, 18). However, other investigators have found a low rate of association between integrons and ESBL genes with the exception of blaCTXM-5 (19).

Presence of plasmids that carry ESBL encoding genes as well as integron mediated antibiotic resistance has been reported among nosocomial isolates of K. pneumoniae (17, 19, 20). In most of these studies, ESBL encoding genes

Table 2. Statistical Associations Between Genotypes, Carriage of Antibiotic Resistance Integrons (ARIs), blaSHV Genes, and Multi-Drug Resistance (MDR) Phenotypes Among the ESBL-Producing Nosocomial Isolates of K. pneumoniae

| First Variable | Second Variable | r a,b     | P Value |
|---------------|----------------|-----------|---------|
| Genotype      | ARL5          | 0.700     | 0.1%    |
|               | blaSHV        | 0.742     | 0.1%    |
|               | MDR           | 0.560     | 0.1%    |
| Antibiotic susceptibility | -     | NS a    |         |
| ARIs          | blaSHV        | 0.298     | 0.097 (NS) |
|               | MDR           | 0.398     | 5%      |
| Antibiotic susceptibility | -     | NS       |         |
| blaSHV        | MDR           | -         | NS      |
| Antibiotic susceptibility | -     | NS       |         |

a Abbreviations: r, correlation coefficient; NS, non-significant.
b coefficients range between -1 (perfect negative relationship) and +1 (perfect positive relationship). A value of 0 indicates absence of any linear relationship.
were located on plasmids but not within the integrons. Although most of the findings so far suggest contribution of integrons in the acquisition and transmission of resistance genes among bacteria, further investigations are needed to evaluate the involvement of other factors in transmission of linked resistance genes.

Acknowledgements
There were no acknowledgements.

Authors’ Contribution
Study concept and design: Eftekhar, Feizabadi. Analysis and interpretation of data: Eftekhar, Feizabadi, Ashayeri-Panah. Drafting of the manuscript: Eftekhar, Ashayeri-Panah. Critical revision of the manuscript for important intellectual content: Eftekhar, Feizabadi. Statistical analysis: Ashayeri-Panah. Study supervision: Eftekhar, Feizabadi.

Financial Disclosure
There is no financial interest to disclose.

Funding/Support
The financial support of this research was provided by the Shahid Beheshti University Research Council.

References
1. Podschun R, Ullmann U. Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev. 1998;11(4):589–603.
2. Bruisse S, Grimon F, Grimon PAD. The Genus Klebsiella. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E editors. The Prokaryotes: a handbook on the biology of bacteria. Berlin: Springer-Verlag; 2006. p. 159–196.
3. Paterson DL, Bonomo RA. Extended-spectrum beta-lactamases: a clinical update. Clin Microbiol Rev. 2005;18(4):657–86.
4. Cantor R, Coque TM, Baquero F. Multiresistant Gram-negative bacilli: from epidemics to endemics. Curr Opin Infect Dis. 2003;16(4):315–25.
5. Cambray G, Guerout AM, Mazel D. Integrons. Annu Rev Genet. 2010;44:341–65.
6. Li W, Raoult D, Fournier PE. Bacterial strain typing in the genomic era. FEMS Microbiol Rev. 2009;33(3):892–916.
7. Ashayeri-Panah M, Eftekhar F, Feizabadi MM. Development of an optimized random amplified polymorphic DNA protocol for fingerprinting of Klebsiella pneumoniae. Lett Appl Microbiol. 2012;54(4):272–9.
8. Paterson DL, Mulazimoglu L, Casellas JM, Ko WC, Goossens H, Von Gottberg A, et al. Epidemiology of ciprofloxacin resistance and its relationship to extended-spectrum beta-lactamase production in Klebsiella pneumoniae isolates causing bacteremia. Clin Infect Dis. 2000;30(3):471–8.
9. Feizabadi MM, Delfani S, Raji N, Majnooni A, Aligholi M, Shahcheraghi F, et al. Distribution of bla(TEM), bla(SHV), bla(CTX-M) genes among clinical isolates of Klebsiella pneumoniae at Labafinejad Hospital, Tehran, Iran. Microb Drug Resist. 2010;16(1):349–53.
10. 21st informational supplement. Performance standards for antimicrobial susceptibility testing. Wayne, PA: Clinical and Laboratory Standards Institute; 2011.
11. Goldstein C, Lee MD, Sanchez S, Hudson C, Phillips B, Register B, et al. Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotic. Antimicrob Agents Chemother. 2000;45(3):723–6.
12. Nathisuwan S, Burgess DS, Lewis JS, 2nd. Extended-spectrum beta-lactamases: epidemiology, detection, and treatment. Pharmacotherapy. 2001;21(8):920–8.
13. Babini GS, Livermore DM. Are SHV beta-lactamases universal in Klebsiella pneumoniae? Antimicrob Agents Chemother. 2000;44(8):2220.
14. Turner MS, Anderson P, Bell JM, Turnidge JD, Harris T, Giffard PM. Plasmid-borne blaSHV genes in Klebsiella pneumoniae are associated with strong promoters. J Antimicrob Chemother. 2009;64(5):960–4.
15. Gori A, Espinasse F, Deplano A, Nonhoff C, Nicolas MH, Stuelens MJ. Comparison of pulsed-field gel electrophoresis and randomly amplified DNA polymorphism analysis for typing extended-spectrum-beta-lactamase-producing Klebsiella pneumoniae. J Clin Microbiol. 1996;34(10):2448–53.
16. Elai'houchi A, van Emmelo J, Claey's G, Verschraegen G, Verhelst R, Vaneechoutte M. Study of the influence of plasmids on the arbitrary primer polymerase chain reaction fingerprint of Escherichia coli strains. FEMS Microbiol Lett. 1994;115(2-3):335–9.
17. Jones LA, McEvoy CJ, Kim MJ, Rawlinson WD, White PA. The aadB gene cassette is associated with blaSHV genes in Klebsiella species producing extended-spectrum-beta-lactamases. Antimicrob Agents Chemother. 2005;49(2):754–7.
18. Gruteke P, Goessens W, Van Gils J, Peerbooms P, Lemmens-Den Toom N, Van Santen-Verheuvel M, et al. Patterns of resistance associated with integrons, the extended-spectrum-beta-lactamase-producing SHV-5 gene, and a multidrug efflux pump of Klebsiella pneumoniae associated with whole genome. J Antimicrob Chemother. 2005;56(11):2190–9.
19. Machado E, Ferreira J, Novais A, Peixe L, Canton R, Baquero F, et al. Preservation of integron types among Enterobacteriaceae producing extended-spectrum-beta-lactamases in a Spanish hospital over a 15-year period (1988 to 2003). Antimicrob Agents Chemother. 2007;51(6):2201–4.
20. Bhattacharjee A, Sen MR, Prakash P, Gaur A, Anupurba S, Nath G. Observation on integron carriage among clinical isolates of Klebsiella pneumoniae producing extended-spectrum-beta-lactamases. Indian J Med Microbiol. 2010;28(3):207–10.