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Original Article

Microbiology Section

Correlation of RAPD-PCR Profiles with ESBL Production in Clinical Isolates of *Klebsiella pneumoniae* in Tehran

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ABSTRACT

Background: Multidrug resistant *K. pneumoniae*, particularly the extended-spectrum β-lactamase (ESBL) producing strains, are often responsible for the failure of antibiotic treatment in nosocomial infections. Employing molecular methods to distinguish between ESBL and non-ESBL producing isolates can help quick identification of these multidrug resistant pathogens and thereby initiating appropriate antibiotic therapy. The aim of this study was to employ RAPD-PCR to distinguish the genetic fingerprints of ESBL producing clinical isolates of *K. pneumoniae* from ESBL negative strains.

Materials and Methods: Antibacterial susceptibility of 104 *K. pneumoniae* clinical isolates was determined to 13 antibacterial agents by disc diffusion. ESBL production was measured by the double disc synergy test followed by phenotypic confirmatory tests. Genetic fingerprinting was carried out by RAPD-PCR.

Results: All isolates were susceptible to imipenem. Antibiotic resistance rates were: piperacillin (100%), ceftazidime (62.5%), cefotaxime (57.6%), aztreonam (52.8%), cefepime (51.9%), kanamycin (50.9%), gentamicin (41.3%), ciprofloxacin (37.5%), nitrofurantoin (30.6%), nalidixic acid (22.1%), piperacillin/tazobactam (21.1%) and amikacin (9.6%). ESBL production was observed in 14 isolates (13.4%). Genetic fingerprinting performed on 43 isolates (14 ESBL positive and 29 ESBL negative) by RAPD-PCR, showed that 46.5% of the isolates belonged to a single profile (genotype 1), of which, the majority (62.1%) were non-ESBL producers.

Conclusion: RAPD-PCR results showed heterogeneity among the isolates. There was no association between ESBL production with any specific genetic fingerprint.

Keywords: ESBL, Genetic fingerprinting, *Klebsiella pneumoniae*, Multidrug resistance, Nosocomial isolates, RAPD-PCR

INTRODUCTION

Klebsiella pneumoniae is a frequent cause of nosocomial infections including urinary, respiratory and blood infections in immune-compromised patients [1]. Extended-spectrum ß-lactam antibiotics have been widely used for treatment of serious Gram-negative infections since the 1980s. However, bacterial resistance has emerged mainly due to the production of extended-spectrum ß-lactamases (ESBL) which are capable of hydrolyzing extended spectrum β-lactam antibiotics such as ceftazidime, ceftriaxone, cefotaxime and aztreonam [2,3]. Hospital outbreaks due to multidrug resistant *K. pneumoniae,* specifically the ESBL producing strains, have been increasing worldwide [3,4]. Identification of multidrug resistant, ESBL producing isolates by quick molecular techniques could aid in the choice of suitable antibiotic therapy. DNA fingerprinting using pulsed field gel electrophoresis (PFGE) and PCR-based methods has been employed to show the genetic diversity among *K. pneumoniae* isolates [5,6]. Among the PCR based methods, random amplified polymorphic DNA (RAPD) is a fast method for generating DNA profiles comparable to PFGE which is considered the gold standard in genetic fingerprinting. We recently developed an optimized RAPD protocol which could reliably differentiate between *K. pneumoniae* clinical isolates for epidemiological purposes [5]. Due to the role of ESBLs in multidrug resistance, the aim of this research was to employ the modified RAPD-PCR to distinguish ESBL producing clinical isolates of *K. pneumoniae* from non-ESBL isolates collected during a 4 month period from Imam Hossein hospital in Tehran.

MATERIALS AND METHODS

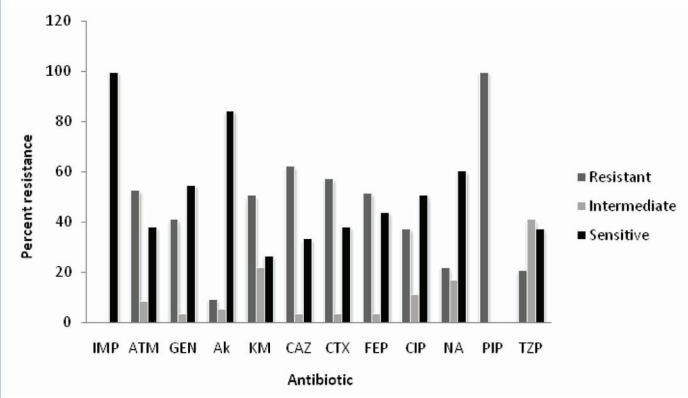
Bacterial strains: One hundred and four non-replicate nosocomial isolates of *K. pneumoniae* were collected from Imam Hussein hospital in Tehran between July and October 2010. The majority of the isolates were from urine (n=49; 47.1%) followed by sputum (n=21; 20.2%), catheters and wounds each (n=9; 8.65%), blood (n=6; 5.8%), exudates (n=3; 2.9%), eye infection (n= 3; 2.9%) and other unknown sources (n=4; 3.8%). The bacterial isolates were stored at -20°C in nutrient broth containing 1% dimethyl sulfoxide until use.

Antimicrobial susceptibility: Antimicrobial susceptibility was determined by the disc diffusion method according to the CLSI criteria [7]. The antibiotic discs were obtained from Himedia (India) and were: amikacin (30μg), gentamicin (10μg), kanamycin (30μg), imipenem (10μg), aztreonam (30μg), ceftazidime (30μg), cefotaxime (30μg), cefepime (30μg), piperacillin (100μg), piperacillin/tazobactam (100/10μg), ciprofloxacin (5μg), nalidixic acid (30μg) and nitrofurantoin (300μg). *K. pneumoniae* (ATCC 700603) and *E. coli* (ATCC 25922) were used as susceptible controls.

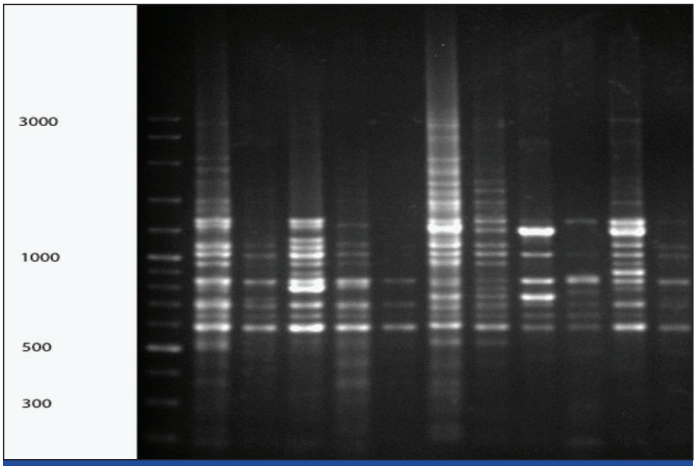
Detection of ESBL production: The isolates were initially screened for ESBL production by the double disc synergy test (DDST) using cefotaxime (30 μg), ceftazidime (30 μg) and cefepime (30 μg), placed 20 mm (center to center) from an amoxicillin/clavulanic acid disc (20+10 μg) (Himedia, India). ESBL production was detected when synergy was observed between the inhibition zones of cephalosporins and amoxicillin/clavulanic acid, and was further confirmed by the phenotypic confirmatory test (PCT) using ceftazidime and cefoaxime alone or in combination with clavulanic acid [8,9].

DNA extraction: DNA was extracted from overnight grown bacteria using a rapid phenol-chloroform method [10]. The extracted DNA in TE buffer (10 mm Tris-HCl, 1 mM EDTA, pH, 8.0) was stored at -20°C until use.

Generation of DNA fingerprints by RAPD-PCR: Primer 640 (5'-CGTGGGGCCT-3') (FazaBiotech, Tehran, Iran) was used to generate suitable RAPD banding profiles as previously reported [5]. Reaction mixtures (25 μl) contained: 4 mM $MgCl_2$, 0.4 mM of each



[Table/Fig-1]: Antibiotic susceptibility of 104 clinical isolates of *Klebsiella pneumoniae*. IMP, imipenem; ATM, aztreonam; GEN, gentamicin; AK, amikacin; KM, kanamycin; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime, CIP, ciprofloxacin; NA, nalidixic acid; PIP, piperacillin; TZP, piperacillin/tazobactam



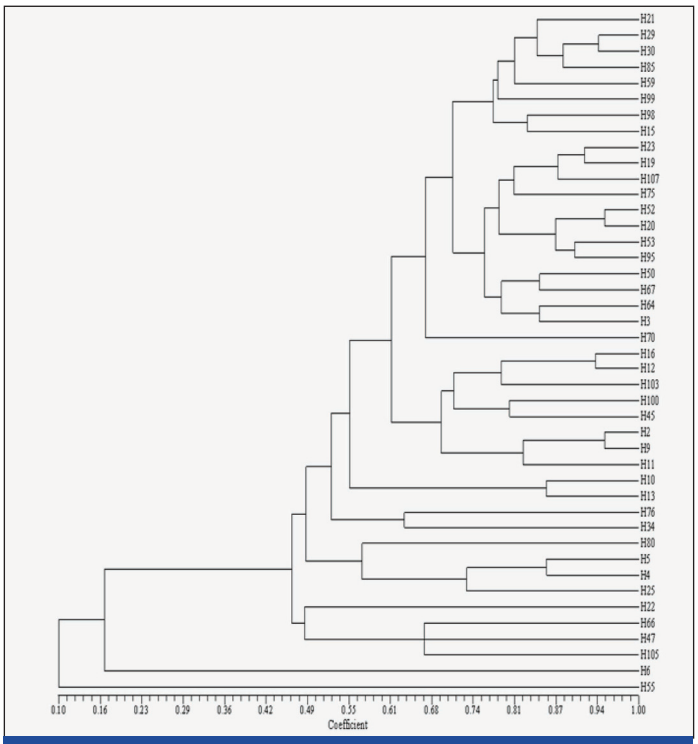
[Table/Fig-2]: RAPD profile of a number of *K. pneumoniae* isolates amplified by primer 640. From left to right: 100 Kb DNA ladder, isolates number H15, H16, H19, H103, H47, H85, H99, H100, H105, H107 and H5

dNTP, 0.9 µM of primer, 1.5 U of Taq DNA polymerase and 3 µl of DNA template. RAPD-PCR was performed in a thermal cycler (Bioer TC25/H Thermal Cycler, Bioer Technology, China) using the following program: 5 min at 95°C followed by 50 cycles of 1 min at 95°C, 1 min at 34°C and 2 min at 72°C followed by a final extension at 72°C for 10 min [5]. PCR products were electrophoresed on 1.2% agarose gels and the DNA bands were visualized by ethidium bromide staining. RAPD fingerprints were analyzed optically and binary scoring was carried out. SAHN clustering in the unweighted pair group method with arithmetic averages (UPGMA) was applied to generate dendrograms (NTSYS-pc, version 2.0).

RESULTS

The antibiotic susceptibility profile of the 104 *K. pneumoniae* isolates is shown in [Table/Fig-1]. As observed, all isolates were resistant to piperacillin and susceptible to imipenem. The DDST and PCT results confirmed that 14 isolates (13.5%) were ESBL producers, all of which were urinary isolates. However, comparison of resistance rates to piperacillin and piperacillin/tazobactam suggests that the majority of the isolates were β-lactamase producers. There was no association between antibiotic susceptibility profiles and ESBL phenotype.

For generation of DNA fingerprints using RAPD-PCR, the 14 ESBL producers and 29 non-ESBL strains were employed. DNA profiles generated using primer 640, showed 29 bands in the range of 300-3000 bps [Table/Fig-2]. [Table/Fig-3] presents the dendrogram obtained for the 43 clinical isolates of *K. pneumoniae* and [Table/Fig-4] shows RAPD fingerprints in ESBL positive and negative isolates. As observed, fifteen clusters were found on a similarity level of 70% and 32 groups were obtained on a similarity level of 85%,



[Table/Fig-3]: Cluster analyses of *K. pneumoniae* strains based on RAPD typing using primer 640. Isolate numbers are shown on the right

reflecting the heterogeneity of the isolates [5]. However, despite the heterogeneity observed, the majority of non-ESBL isolates (n=18, 62.1%) belonged to a single cluster (cluster 1). On the other hand, the ESBL positive isolates were more heterogeneous and their RAPD fingerprints were spread among 8 clusters [Table/Fig-4]. Overall, the results show that all isolates in cluster 1 (regardless of ESBL production) were more closely related than others [Table/Fig-3].

DISCUSSION

The widespread use of antibiotics coupled with transmissibility of resistance determinants mediated by plasmids, transposons and integrons continue to increase the rate of antibiotic resistance in bacterial pathogens. Emergence and spread of multidrug resistant, ESBL producing strains of *K. pneumoniae* have aroused concerns about antibiotic treatment failure, especially in hospital settings.

Prevalence of ESBL producing *K. pneumoniae* varies around the world and has been reported around 12.9 to 26.8% in European countries, 7.5% in North America, 22.4% in Asia Pacific Rim, 44% in Latin America, 48.5% in Turkey, 51% in China, 71.4% in Mexico and 72% in India [11-13]. The prevalence of ESBL producing *K. pneumoniae* in Iran is reported to range from 19.6 to 75% [14-17]. ESBL production in our urinary isolates was lower than the other reports from Iran.

Molecular typing methods such as RAPD-PCR and PFGE have been mostly used as epidemiological tools to discriminate between *K. pneumoniae* isolates and have shown heterogeneity among these organisms [18-20]. In our previous studies, using an optimized RAPD protocol in parallel with PFGE, we have shown the heterogeneity of nosocomial *K. pneumoniae* isolates [5,21]. In the present research, we employed our optimized RAPD-PCR protocol in an attempt to discriminate between ESBL positive and negative *K. pneumoniae* isolates. Overall, we found heterogeneity among the 49 test isolates regardless of their potential to produce ESBL and no correlation between the antibiotic resistance phenotype with RAPD fingerprints. However, despite the low number of ESBL producing isolates tested, our results showed a distinct heterogeneity among them compared to the non-ESBL strains where the majority shared one RAPD profile. As in the present study, Aladag et al., found no association between the antibiotic resistance phenotype with specific RAPD

ESBL positive RAPD profile	No. isolates	ESBL negative RAPD profile	No. isolates
4	3	1	18
1	2	3	3
3	2	2, 6, 8-13	4 (1 each)
5	2		
9	2		
7,14,15	3 (1 each)		

[Table/Fig-4]: RAPD-PCR profiles of 14 ESBL positive and 29 ESBL negative *K. pneumoniae* isolates

fingerprints in ESBL producing *K. pneumoniae* isolates [22]. Lim et al., showed a high degree of heterogeneity among 51 Malaysian *K. pneumoniae* isolates and found no correlation between DNA profiles with antibiotic susceptibility. In addition, they observed that a number of ESBL genes (blaSHV in particular) were integron-mediated and were carried on transmissible plasmids [23]. Bhattacharjee et al., found associations between integron carriage with antibiotic resistance and ESBL gene carriage (SHV and CTX-M) among nosocomial isolates of *K. pneumoniae*. In addition, they showed that integrons were carried by conjugative plasmids and suggested their role in horizontal transfer of ESBL-mediated resistance genes [24]. We have previously shown correlations between integron carriage and antibiotic resistance as well as significant associations between RAPD and PFGE fingerprints with the presence of blaSHV type ESBL genes and in *K. pneumoniae* isolates [25]. Considering that ESBL encoding genes are mostly located on integrons and carried by plasmids, presence of these mobile elements can affect the bacterial genetic profiles generated by molecular methods, and contribute to a higher degree of heterogeneity in ESBL producing isolates. Agreement between plasmid profiles and RAPD patterns of ESBL-producing *K. pneumoniae* has been shown by some Investigators [18,26]. However, further studies are needed to test the influence of mobile genetic elements on DNA profiles and therefore, heterogeneity among these organisms.

CONCLUSION

There was no association between antibiotic resistance profiles, ESBL phenotype and genetic fingerprints in our *K. pneumoniae* clinical isolates. In fact, contrary to our expectation, ESBL producing isolates were more heterogeneous than the non-ESBL strains.

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