

Research Paper

Genetic profiling of *Klebsiella pneumoniae*: comparison of pulsed field gel electrophoresis and random amplified polymorphic DNA

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Abstract

In this study, the discriminatory power of pulsed field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) methods for subtyping of 54 clinical isolates of *Klebsiella pneumoniae* were compared. All isolates were typeable by RAPD, while 3.6% of them were not typeable by PFGE. The repeatability of both typing methods were 100% with satisfying reproducibility ($\geq 95\%$). Although the discriminatory power of PFGE was greater than RAPD, both methods showed sufficient discriminatory power ($DI > 0.95$) which reflects the heterogeneity among the *K. pneumoniae* isolates. An optimized RAPD protocol is less technically demanding and time consuming that makes it a reliable typing method and competitive with PFGE.

Key words: *Klebsiella pneumoniae*, pulsed field gel electrophoresis, random amplified polymorphic DNA, genotyping.

Introduction

Klebsiella pneumoniae has been reported as one of the leading causes of nosocomial infections including urinary, respiratory and blood infections (Podschun and Ullmann, 1998). Endemic and epidemic infections caused by multidrug-resistant strains of *K. pneumoniae*, have become a major concern in hospital settings (Paterson and Bonomo, 2005). Understanding the route and dynamics of dissemination in outbreaks of infections with this organism relies on using accurate typing methods. Compared to phenotyping, genotyping has become widely used for bacterial strain typing due to its higher resolution. Among different genotyping methods, time and cost effectivity of DNA banding pattern based typing methods, have made these methods matters of interest (Li *et al.*, 2009). Among various DNA banding pattern based typing methods,

pulsed field gel electrophoresis (PFGE) has been considered as the gold standard for typing of *K. pneumoniae* (Arlet *et al.*, 1994; Li *et al.*, 2009). Since PFGE is technically demanding, time consuming and expensive, interests have been raised in easier, faster and more economical typing methods such as random amplified polymorphic DNA (RAPD) (Gori *et al.*, 1996). Despite the economical and practical merits offered by RAPD, the reproducibility of this method remains a challenge (Atienzar and Jha, 2006). After systematic optimization, RAPD has been suggested as a reliable, sensitive and reproducible assay for molecular typing of bacteria (Atienzar and Jha, 2006; Blixt *et al.*, 2003). In this study, 54 nosocomial isolates of *K. pneumoniae* were fingerprinted using an optimized RAPD protocol and a PFGE method. Typeability, repeatability, reproducibility and discriminatory power of the optimized RAPD protocol were compared with the PFGE method.

Materials and Methods

Bacterial strains and growth

Fifty four nosocomial isolates of *K. pneumoniae* were randomly selected from a collection previously described (Feizabadi *et al.*, 2010a). Bacteria were isolated from patients at different wards of Labbafinejad Hospital, Tehran, Iran, consisted of 26 (48.1%) males and 27 (50%) females and one (1.9%) of unknown gender. The majority of the isolates were from urine (n = 38; 70.4%) followed by trachea (n = 4; 7.4%), wounds (n = 4; 7.4%), blood (n = 2; 3.7%), sputum (n = 2; 3.7%) and others (n = 4; 7.4%). Susceptibility of the isolates to 17 antimicrobial agents was determined and none of the isolates were resistant to imipenem. Thirty six isolates (66.7%) were confirmed as ESBL-producers using phenotypic confirmatory test (Feizabadi *et al.*, 2010a).

PFGE

Genomic DNA extraction was carried out from overnight grown cultures on Mueller-Hinton agar (Merck, Darmstadt, Germany) at 37 °C as reported previously with minor modification (Feizabadi *et al.*, 2010b). Genomic DNA was digested with *Xba*I (Fermentas, Vilnius, Lithuania) for 4 h at 37 °C. The digested DNAs were then separated in 1% agarose gel using a counter-clamped homogenous electric field mapper II system, a prototype of PFGE (Faculty of Engineering, University of Tehran, Iran) with a linear pulse time ramped from 2 to 70 s for 24 h at 14 °C and constant voltage of 6 V/cm. A *Xba*I chromosomal digest of *Salmonella* serotype Braenderup strain H9812 was used as a molecular size marker. The DNA patterns were stained with ethidium bromide, visualized and photographed by UVP gel documentation (UVP, Cambridge, UK).

RAPD

Briefly, bacteria were grown overnight in Mueller-Hinton broth (Merck, Darmstadt, Germany) at 37 °C and genomic DNA was extracted using High Pure PCR template Prep kit for Genomic DNA extraction (Roche Diagnostics, Mannheim, Germany). The PCR reaction mixture contained 1 µM of primer AP4 (5'- TCACGATGCA-3', FazaBiotech, Tehran, Iran) (Gori *et al.*, 1996), 2.5 U of Taq DNA polymerase, 4 mM MgCl₂, 0.4 mM for each dNTP (CinnaGen, Tehran, Iran) and 90 ng DNA template in a final volume of 25 µL. The following cycling program was performed using a Bioer TC25/HH Thermal Cycler (Bioer Technology, China): 4 min at 94 °C, 50 cycles of denaturing at 94 °C for 1 min, annealing at 34 °C for 2 min, extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis in 1.2% agarose gels and were visualized after staining with ethidium bromide. The typeability, repeatability, reproducibility and discriminatory power of the

RAPD protocol were calculated and compared with those of PFGE. To estimate the possibility of plasmid DNA contribution in RAPD patterns, dendrograms of ESBL and non-ESBL producers were compared. For both PFGE and RAPD, DNA patterns were analyzed optically and binary scoring was carried out. The band matching coefficient of Dice and the unweighted pair group method with arithmetic averages (UPGMA) were applied for clustering (NTSYS-*pc*, version 2.0) (Rohlf 1992; Sneath and Sokal, 1973). Discriminatory power of the PFGE and the RAPD protocols were calculated using Simpson's diversity index (DI) (Hunter and Gaston, 1988). To evaluate the repeatability of each method, the procedures were repeated twice at four weeks intervals on 10 randomly selected isolates and similarity between fingerprints was calculated using Pearson's similarity coefficient. To assess reproducibility of the PFGE, the experiments were repeated using different subcultures of the same isolates. Similarly, the RAPD protocol was re-checked using two other brands of thermal cyclers (Techne TC-312, UK and Corbett GP-001, Corbett Research, Australia).

Results

All the isolates were typeable by RAPD but 3.6% of them (two out of 56) were not typeable by PFGE. The PFGE and RAPD profiles of some of the isolates are shown in Figures 1 and 2, respectively. PFGE generated 30 bands within the range of approximately 15-700 kb and the optimized RAPD protocol generated 32 bands within the range of 200-5000 bp. On a similarity level of 70%, PFGE generated 22 major clusters (A-W) and for RAPD seven major clusters (a-g) were observed among the *K. pneumoniae* isolates. On a similarity level of 85%, PFGE showed 42 differ-

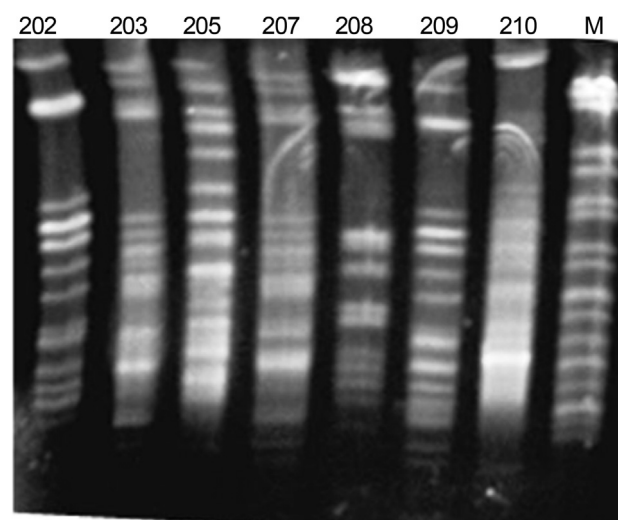


Figure 1 - Genomic DNA macrorestriction profiles of *Klebsiella pneumoniae* produced by PFGE after *Xba*I digestion. From left to right, respectively *K. pneumoniae* isolates number 202, 203, 205, 207, 208, 209 and 210. M: molecular weight marker (*Xba*I chromosomal digest of *Salmonella* serotype Braenderup strain H9812).

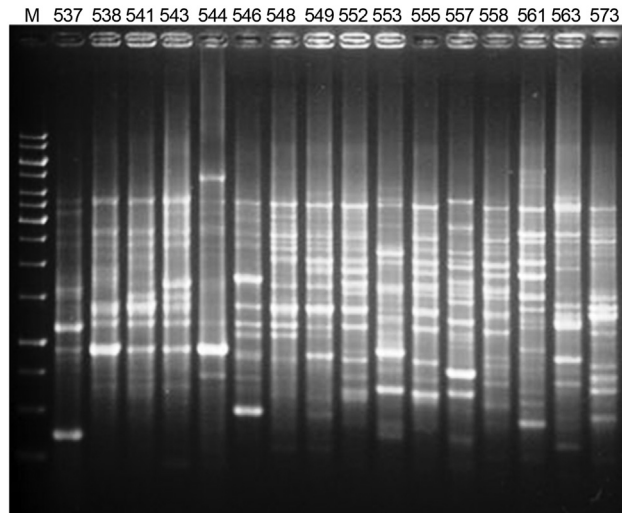


Figure 2 - RAPD profiles of *Klebsiella pneumoniae* isolates amplified by primer AP4. From left to right, respectively *K. pneumoniae* isolates number 537, 538, 541, 543, 544, 546, 548, 549, 552, 553, 555, 557, 558, 561, 563 and 573. M: DNA molecular weight marker (1Kb DNA ladder; Fermentase, Germany).

ent groups (Figure 3) and RAPD generated 32 different groups. The overall similarity of band patterns in the RAPD dendrogram of ESBL producers was 61%, compared to 64% in that of non-ESBL producer strains (Figure 4). Although both RAPD and PFGE profiles were highly consistent with respect to the size and number of the bands, there

were variations in the intensity of minor bands in RAPD but not in PFGE profiles. Overall, both PFGE and RAPD showed satisfactory discriminatory power (DI for PFGE = 0.989 and DI for RAPD = 0.982), which demonstrated heterogeneity among the *K. pneumoniae* isolates. Both protocols were highly repeatable (similarity coefficient, ~100%). Reproducibility of PFGE and RAPD were 100% and 95%, respectively.

Discussion

To evaluate the efficiency of a typing method, criteria such as typeability, reproducibility and discriminatory power should be taken into consideration (Hunter and Gaston, 1988). To choose a reliable typing method for a laboratory setting, other parameters such as complexity of performance and interpretation, time and expenses should also be considered (Krawczyk *et al.*, 2005; Tenover *et al.*, 1997).

Among various DNA banding pattern based typing methods, PFGE is accepted as the most efficacious one for typing many bacteria (Li *et al.*, 2009; Pingault *et al.*, 2007). However, as PFGE is technically demanding and time consuming, alternative methods such as RAPD have received attention (Gori *et al.*, 1996). It has been suggested that optimization of RAPD conditions, selection of optimally discriminating RAPD primers and parallel use of several primers could contribute to generate RAPD protocols with discriminatory ability equal, or even superior, to that of

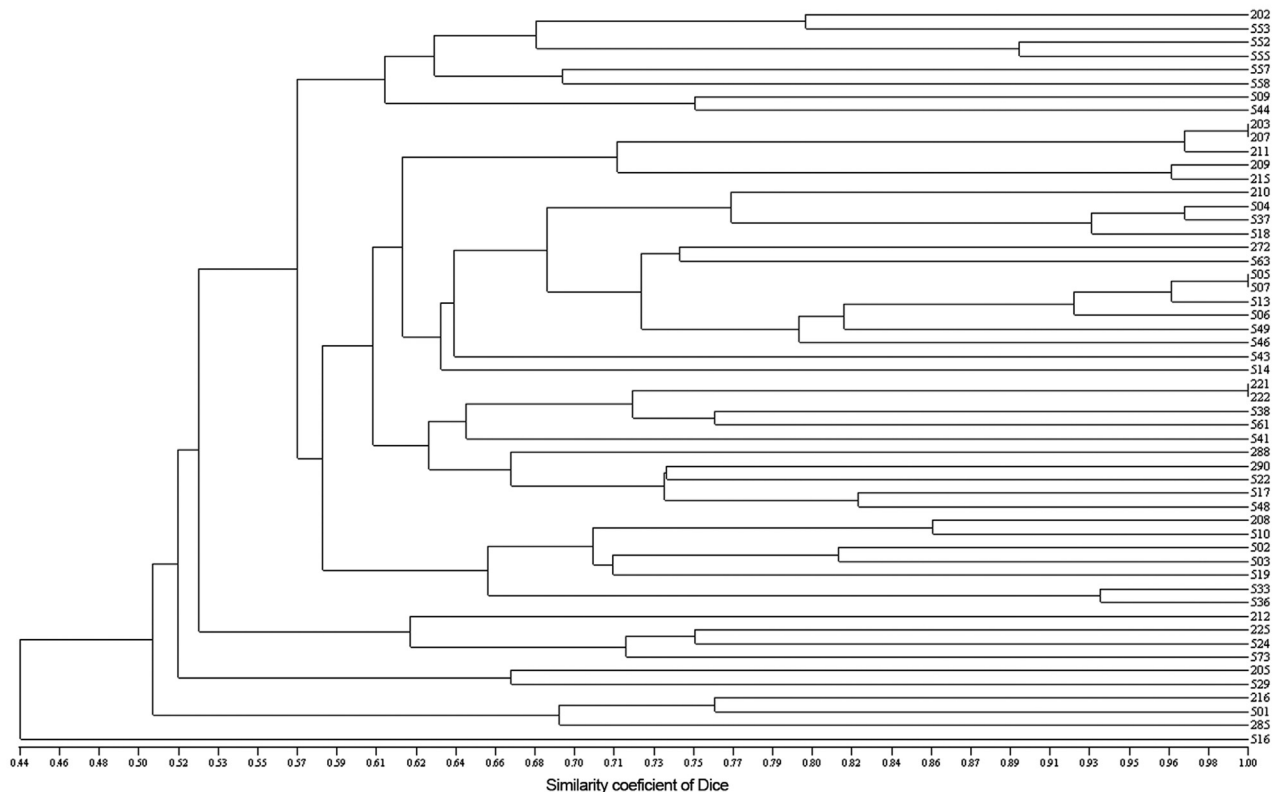


Figure 3 - Dendrogram of PFGE obtained by UPGMA group analysis method using similarity coefficient of Dice.

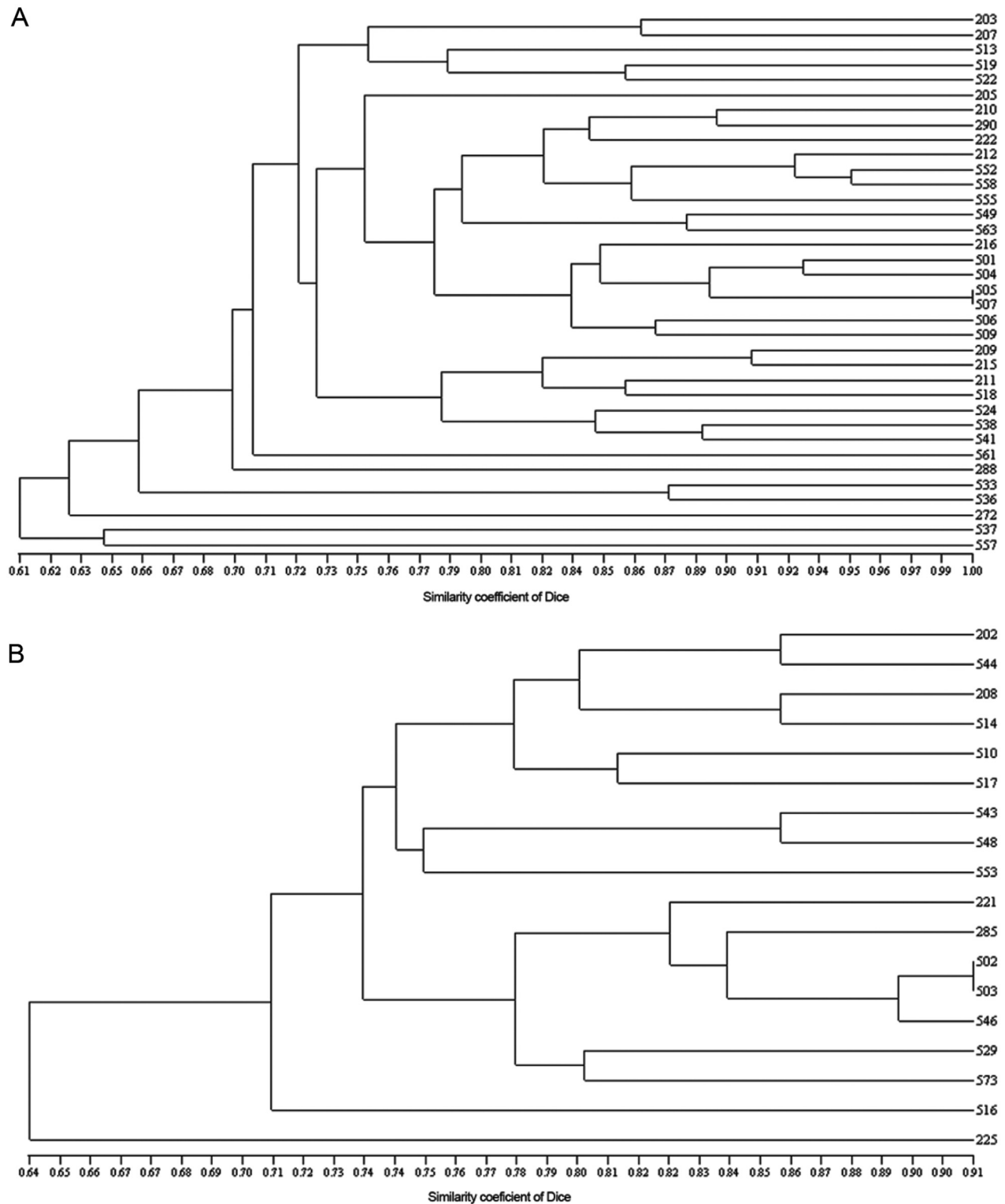


Figure 4 - Cluster analysis of *Klebsiella pneumoniae* isolates based on RAPD typing with primers AP4, using similarity coefficient of Dice. A – ESBL-producing *K. pneumoniae*. B – Non ESBL-producing *K. pneumoniae*.

PFGE (Larrasa *et al.*, 2004; Marois *et al.*, 2001). In this study, the optimized RAPD protocol was put to the test in comparison with a PFGE method for ESBL and non-ESBL producing nosocomial isolates of *K. pneumoniae*. In a

study on *Dermatophilus congolensis*, standardization of DNA extraction method, RAPD reagents, amplification conditions and primer selection showed great reproducibility of RAPD banding. Although Larrasa *et al.* (2004)

highlighted the use of at least two independent primers in order to improve the discriminatory power of RAPD, regarding our observation, meticulous optimization of RAPD protocol could eliminate the need for using several primers.

Comparison between PFGE and RAPD was previously reported for typing of ESBL-producing *K. pneumoniae* (Gori *et al.*, 1996). Although PFGE with *Xba*I showed more discrimination than combined RAPD with three primers, reliability of RAPD was validated with good but not calculated discriminatory power and reproducibility (Gori *et al.*, 1996). In comparison, we applied systematic optimization to accomplish a more reliable RAPD protocol competitive to PFGE and evaluated each method based on the precise calculation of the determinative criteria. In a more recent study, three PCR-based fingerprinting techniques were compared with PFGE for typing of *K. pneumoniae* and the usefulness of RAPD was concluded. However, lower discriminatory index and reproducibility were reported in comparison to our results, perhaps due to use of an optimized RAPD protocol in our study (Cartelle *et al.*, 2004). In a study on *Mycoplasma synoviae*, the reproducibility of both PFGE and RAPD typing techniques were evaluated 100% (Marois *et al.*, 2001). Lower typeability for PFGE in contrast to RAPD in present study could be a result of DNA degradation by bacterial DNase during long procedure of PFGE. In our study, the major drawback of PFGE was the relatively high percentage of non-typeable isolates as observed by other investigators (Marois *et al.*, 2001). Gori *et al.* (1996) suggested a possible involvement of plasmid DNA in RAPD patterns in *K. pneumoniae*. However, Elaichouni *et al.* (1994) found no influence of plasmid DNA on the RAPD results in *E. coli*. Since ESBL production is mostly mediated by plasmids, it is possible that presence of the plasmids harboring ESBL genes could affect the RAPD banding (Gori *et al.*, 1996). As in this study, overall similarity level of RAPD patterns between non-ESBL producer strains was higher than that of ESBL producer strains, our observation confirmed contribution of ESBL plasmids to the RAPD patterns. However, little accordance was observed between RAPD types and ESBL production, as well as PFGE. As van Belkum *et al.* (1995) suggested, excessively high discriminatory power of RAPD could be a sign of deterioration in the relationship between genotyping data and phenotypic characteristics in epidemiological studies. A more accurate comparison could be made if RAPD was performed on genomic DNA obtained by melting the PFGE agarose blocks (Marois *et al.*, 2001). Overall, both RAPD and PFGE results suggested that there was no outbreak with a particular *K. pneumoniae* strain in the different wards of Labbafinejad Hospital.

In conclusion, the optimized RAPD showed greater typeability but negligibly lower discrimination and reproducibility compared to PFGE. On the other hand, PFGE was more discriminatory and reproducible but with lower

typeability. Overall, our results showed that PFGE and optimized RAPD are comparable and equally valuable for typing of *K. pneumoniae* nosocomial isolates. As RAPD is less technically demanding and time consuming, we recommend RAPD typing as the preliminary method for fast and inexpensive investigation and PFGE typing as a confirmatory method if needed.

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