대한임상검사학회지 : 37권 제3호, 149-154, 2005

Random Amplified Polymorphic DNA Analysis for Typing Extended-Spectrum-β-Lactamase of *Klebsiella pneumoniae*

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Fifty-one extended-spectrum-β-lactamase(ESBL) producing *Klebsiella pneumoniae* strains were isolated from national university hospitals. All *K. pneumoniae* strains showed resistance to broad-spectrum antibiotic and most of them presented resistance to amikacin, gentamicin and ciprofloxacin. The results of amplified polymorphic DNA (RAPD) pattern for randomly isolated fifty-one strains were as follows; both twenty-one strains from Chungnam National University hospital and ten strains from Chungbuk National University hospital showed RAPD type Ia and Ib. However, twenty strains isolated from Gyeongsang National University hospital belonged to RAPD type IIa and IIb. All isolates were divided into four molecular types and showed high level of genetic diversity. These results suggested that RAPD analysis provided a rapid and simple method for analysing genotypes of ESBL.

Key Words : *Klebsiella pneumoniae*, Extended-spectrum-β-lactamase, Randomly amplified polymorphic DNA

I. INTRODUCTION

Klebsiella pneumoniae has been increasingly recognized as a cause of hospital-acquired infections internationally. These organisms are resistant to a number of antibiotics, including extended-spectrum cephalosporins and aminoglycosides, because of the acquisition of plasmids which code for the production of extended-spectrum-β-lactamases (ESBL) and aminoglycoside-modifying enzymes (Jarlier et al, 1988; Hogg et al, 1993; Meyer et al, 1993). Traditional epidemiologic tools, including biotyping and serotyping, are not useful in distinguishing between strains of *K. pneumoniae*. Molecular techniques, including plasmid analysis and ribotyping, have suggested very useful techniques in distinguishing between strains of *K. pneumoniae*(Brousseau et al, 1933). Distinctive polymorphisms generated by the random amplified polymorphic DNA analysis(RAPD) are now being utilized for differentiating strains. The aim of this study is to suggests the use of RAPD analysis to a cluster of ESBL-producing *K. pneumoniae* infections detected by routine infection control surveillance at the hospitals (Ellsworth et al, 1993).

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II. MATERIALS AND METHODS

1. Bacterial strains

Between February 2005 and May 2005, 51 *K. pneumoniae* strains were isolated from the university hospitals(Table 1). They consisted of five consecutive clinical isolates(24 from sputum, 10 from urine, 3 from blood, 2 from mucosal swabs, and 12 from others).

2. Phenotyping

Routine identification and antibiotic susceptibility tests of the isolates were performed with the automated Vitek system. Susceptibility to antimicrobial agents was tested

Table 1. RAPD genotyping of ESBL K. pneumoniae from various isolates

Strains	Source	RAPD type	Strains	Source	RAPD type
CUH1	sputum(GS)	Ib	CBH27	sputum(GS)	Ib
CUH2	urine(GS)	Ia	CBH28	sputum(ICU)	IIa
CUH3	urine(CS)	Ia	CBH29	urine(GS)	Ia
CUH4	mucosal(GS)	Ib	CBH30	sputum(GS)	Ib
CUH5	other(CS)	Ib	CBH31	blood(ICU)	Ib
CUH6	sputum(CS)	Ib	GUH32	sputum(GS)	IIa
CUH7	sputum(CS)	Ia	GUH33	other(IM)	Ib
CUH8	sputum(CS)	Ia	GUH34	mucosal(CS)	IIa
CUH9	urine(NS)	Ia	GUH35	otherGS)	IIb
CUH10	sputum(NS)	Ia	GUH36	sputum(CS)	Ib
CUH11	mucosal(GS)	Ib	GUH37	sputum(CS)	IIa
CUH12	other(GS)	Ia	GUH38	sputum(CS)	IIb
CUH13	blood(CS)	Ia	GUH39	urine(Ped)	IIb
CUH14	sputum(ICU)	Ia	GUH40	sputum(Ped)	IIa
CUH15	sputum(Ped)	Ia	GUH41	urine(CS)	IIb
CUH16	sputum(Ped)	Ia	GUH42	sputum(GS)	IIb
CUH17	urine(IM)	Ib	GUH43	urine(CS)	IIb
CUH18	blood(IM)	Ib	GUH44	urine(CS)	IIa
CUH19	urine(GS)	Ia	GUH45	sputum(CS)	IIb
CUH20	blood(CS)	Ib	GUH46	sputum(CS)	IIa
CUH21	other(GS)	Ia	GUH47	sputum(IM)	IIb
CBH22	sputum(NS)	Ib	GUH48	urine(CS)	IIa
CBH23	sputum(GS)	Ia	GUH49	other(GS)	IIa
CBH24	other(NS)	Ib	GUH50	other(ICU)	IIa
CBH25	sputum(NS)	Ia	GUH51	sputum(ICU)	IIb

CUH, Chungnam University Hospital; CBH, Chungbuk University Hospital; GUH, Gyoungsang University Hospital; GS, general surgery; ICU, intensive cure unit; CS, chest surgery; Ped, pediatrist; NS, neurosurgery; IM, internal medicine.

by the disk diffusion method on muller-hinton agar. The production of clavulanic acid-susceptible ESBL was detected by using the double-disk synergy test. Antibiotic susceptibility disks containing amoxicillin + clavulanate (AMC) were placed on the center of Petri dishes. Cefotaxime (CTX) and ceftazidine (CAZ) disks were placed 25～30 mm apart circularly around the co-amoxiclav disk. The agar plates were incubated for 24 hours at 35℃. When the disk containing co-amoxiclav extended to any of the other antibiotic disk inhibition zones, ESBL production was inferred. Additionally, inhibition zone diameters of various β-lactams, including aztreonam and imipenem, aminoglycosides, chloramphenicol, and other antibiotics, were measured.

3. RAPD analysis

Bacteria were grown overnight on trypticase soy broth. The broth was suspended of lysis buffer (50 mM Tris-Cl [pH 8], 50 mM EDTA, 1% sodium dodecyl sulfate, 30 ㎍ RNase per mL). The bacteria were then lysed, and RNA was digested by incubation of the lysate at 37℃ for 1h. After incubation, the lysate was cleared by brief centrifugation and 0.5 mL was removed to a fresh tube. One third volume of saturated ammonium acetate was added, to the content of the mix. DNA pellet was collected from the cleared lysate by ethanol precipitation. The DNA pellet was dissolved in 100 μL on TE buffer (Tris-EDTA). The following five primers were used for

PCR : 208, 228, 241, 270 and 272 (Table 2). The reaction mixture contained Tris-HCl, $MgCl_2$, each primer, dNTP, Taq polymerase, and 4 μL of DNA extract in a final volume of 20 μL. Amplification was performed in a GeneAmp PCR 9600 thermal cycler with 45 consecutive cycles of 15s at 94℃, 15s at 36℃, and 70s at 72℃, with a single final extension step of 5 min at 72℃. PCR products were separated by electrophoresis in a 1.5% agarose gel with 1×TBE running buffer at 90V for 1hr and stained with ethidium bromide and photographed under UV light.

Table 2. Oligonucleotide primers for RAPD for ESBL K. penumoniae

Primer	Sequence (5' to 3')
208	ACGGCCGACC
228	GCTGGCCGAC
241	GCCCGAGCGG
270	TGCGCGCGGG
272	AGCGGGCCAA

4. Statistical analysis

Polaroid photographs of the gels were scanned and saved. The images were normalized, a similarity matrix was produced by using the multivariate statistical package (MVSP), and dendrogram was constructed from the resulting data by the unweighted pair group method.

Ⅲ. RESULTS

1. Antimicrobial susceptibility test

Fifty-one strains of *K. pneumoniae* were isolated from three university hospitals. All strains exhibited a β-lactam susceptibility profile consistent with the production of ESBL: 1) decreased susceptibility or resistance to amoxicillin, piperacillin, cephalothin, cefamandole, extended-spectrum cephalosporins and aztreonam 2) full susceptibility to imipenem, temocillin and cephamycins 3) a positive disk synergy test with extended-spectrum cephalosporins(Fig. 1).

Fig. 1. Detection of ESBL production of CUH1 strain in double disc synergy tests.
(A) discs : left, ceftazidine; centre, amoxicillin+ clavulanate; right, cefotaxime.
(B) discs : left, ceftazidine; centre, ticarcillin+ clavulanate; right, cefotaxime.

2. RAPD analysis

For each of the primers used, RAPD analysis yielded four groups of closely related fingerprints (major types Ia through IIb), each exhibited by CUH1, CBH22, GUH32, GUH33 and GUH50 strains respectively. For group I, two subtypes obtained with primer 208 and three subtypes obtained with primer 228 were distinguished by single-band variations of the core pattern. Primer 241 produced for clearly different groups of fingerprints based on bands with high intensity. Besides several common bands, one subtype (Ia) showed an additional fragment of high intensity (approximate molecular size of 0.7 kb) and the disappearance of a low-intensity fragment with a size<0.7 kb. For another subtype (Ib), the modification consisted of an additional fragment of low intensity with a molecular size of approximately 0.6 kb. The resulted of cluster analysis of the RAPD results with MVSP software. 21 strains from Chungnam National University Hospital

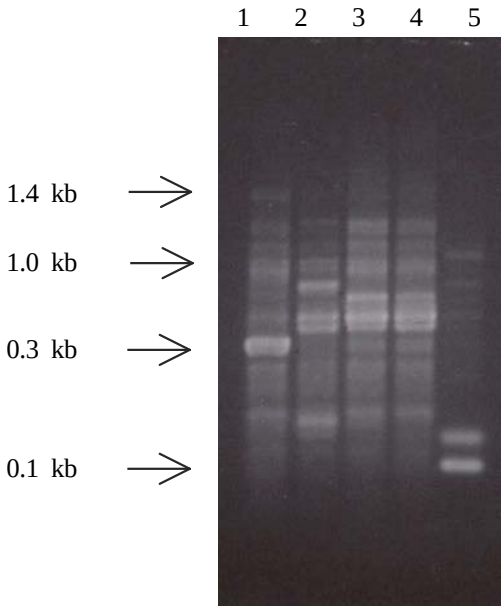

Fig. 2. RAPD patterns of *K. pneumoniae* isolates produced by using primer 241.
Lanes: 1, CUH1; 2, CBH22; 3, GUH32 4, GUH33; 5, GUH50.

and 10 strains from Chungbuk National University Hospital including the type strain were represented to Ia and Ib pattern but 20 strains from Gyeongsang National University Hospital belonged to IIa, IIb pattern (Fig. 2, 3).

Ⅳ. DISCUSSION

Nosocomial outbreaks caused by ESBL-producing *K. pneumoniae* have been reported in Europe and in the United States. Infection caused by these strains, particularly in adult patients admitted to ICUs as observed in this study, have been described elsewhere (Baraniak et al, 2002; Bingen et al, 1993). In the past two decades, a significant number of nosocomial outbreaks of infection by *K. pneumoniae* have been reported, causing increasing concern in hospitals (Asensio et al, 2000). In this study, the RAPD patterns classified two major groups Ia, Ib and IIa, IIb. The first pattern limited in Chungnam, Chungbuk National University Hospital and type strain, but the second pattern only presented in Gyeongsang national university hospital. It appears from this study, the RAPD patterns were more identical in particular area not specimens or patients from isolated strains. As shown in Fig. 3 the test primers (208, 228, 270 and 272) were not clearly different groups of fingerprints (data not shown). But, in the case of primer 241, the RAPD pattern is very good to fingerprints (Fig. 3). So, I think that this primer is very useful in distinguish among ESBL-producing *K. pneumoniae* in our country for ribotyping.

RAPD is a new tool that is being used in such studies. The simplicity and wide applicability of the method are dependent on the use of short nucleotide primers which are not related to known DNA sequences of the target organism. Genetic mapping and determination of the degree of relatedness between strains have been performed, with validation by ribotyping (Welsh et al, 1990; Williams et al, 1990). The banding pattern derived

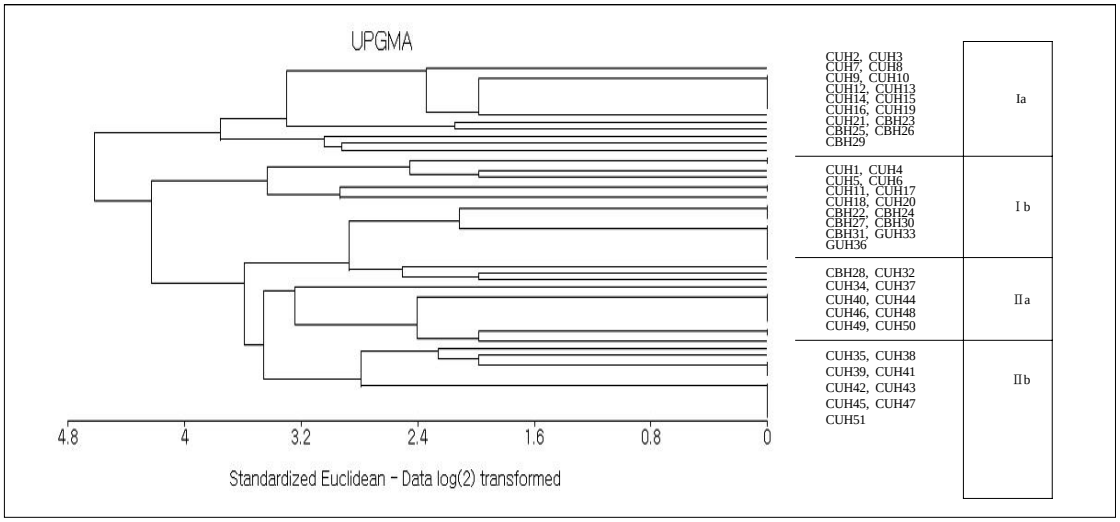

Fig. 3. Dendrogram showing the results of cluster analysis for 51 *K. pneumoniae* strains by using RAPD with primer 241.

from this process allows the identification of similar strains by a method significantly less complicated and time-consuming than ribotyping. Analysis of an accurate antibiogram did not always reliably differentiate between strains.

In this study, it is suggested that RAPD analysis provides rapid and simple typing method of *K. pneumoniae* strains for epidemiological studies and genotyping.

ACKNOWLEDGMENTS

This study was supported by a grant from Jinju Health College.

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=국문초록=

대학병원의 임상검체에서 extended-spectrum-β-lactamase (ESBL)생성 *Klebsiella pneumoniae* 51균주를 분리하였다. ESBL생성 *K. pneumoniae* 51균주는 광범위한 항생제에 내성을 보였고 대부분의 균주는 amikacin, gentamycin과 ciprofloxacin항생제에 내성을 나타내었다. 대학병원에서 분리한 51균주를 randomly amplified polymorphic DNA (RAPD)로 분석한 결과 충남대학병원 21균주와 충북대학병원에서 분리한 10균주는 Ⅰa 와 Ⅰb에 속하였고 경상대학 병원에서 분리한 20균주는 Ⅱa, Ⅱb에 속하였다. ESBL 생성 *K. pneumoniae* 51균주는 RAPD 분석으로 4가지의 유전형으로 구분 할 수 있었고 유전적으로 다양하였다. 이상의 결과로 RAPD 분석은 유전형분석에 빠르고 단순하고 경제적인 방법임을 알 수 있었다.