Preliminary Study of Randomly-Amplified Polymorphic DNA Analysis for Typing Extended-Spectrum Beta-Lactamase (ESBL)-Producing *Klebsiella pneumoniae*

Chanwit Tribuddharat MD, PhD*,
Somporn Srifuengfung PhD*, Wararat Chiangjong MSc*

** Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok*

Objective: *Extended-spectrum beta-lactamases (ESBLs) are most prevalent in Klebsiella pneumoniae. This organism is frequently isolated from clinical specimens and can cause septicemia, pneumonia or urinary tract infection. There were occasionally suspicious outbreaks of ESBL-producing K. pneumoniae in patients' wards. The objective is to determine whether the randomly amplified polymorphic DNA (RAPD), which is a polymerase chain reaction (PCR)-based typing technique, can be used as a typing method for studying the molecular epidemiology of ESBL-producing K. pneumoniae.*

Material and Method: *The present study was carried out by using 30 ESBL-producing K. pneumoniae isolates obtained from different patients admitted to Siriraj Hospital between January and February 2004. RAPD was evaluated for three primers. All isolates were re-examined by using Southern blot hybridization.*

Results: *It was found that 29 DNA band patterns were generated individually by either AP4 or HLWL74 and R108 primers (30 patterns) for RAPD analysis and 30 patterns for Southern blot hybridization with class 1 integron (intI1) probe. Different patterns indicated that these 30 isolates could not be the cause of an outbreak in Siriraj Hospital.*

Conclusion: *The RAPD typing is good and can be used as a screening, rapid and inexpensive test for ESBL-producing K. pneumoniae during investigation of outbreaks.*

Keywords: *Klebsiella pneumoniae, Extended-spectrum beta-lactamase, ESBL, Randomly amplified polymorphic DNA, RAPD*

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In recent years, bacterial resistance to beta-lactam antibiotics has risen dramatically[1,2]. In gram-negative bacteria, beta-lactamase production remains the most important contributing factor to beta-lactam resistance[3,4]. Extended-spectrum beta-lactamases (ESBLs) constitute a growing class of beta-lactamases that have been found in the *Enterobacteriaceae* family[5,6].

Correspondence to: Srifuengfung S, Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, 2 Prannok Rd, Bangkoknoi, Bangkok 10700, Thailand. Phone: 0-2419-9684, Fax: 0- 2411-3106, E-mail: sissf@mahidol.ac.th

There were occasionally suspicious outbreaks in patients' wards by ESBL-producing *K. pneumoniae* in Siriraj Hospital, which is a tertiary university hospital in Bangkok and the largest hospital in Thailand (2,400 beds). Initially, *K. pneumoniae* isolates were characterized by capsular serotyping and plasmid analysis. Subsequently, pulsed-field gel electrophoresis (PFGE) analysis of genome macro-restriction fragments was shown to be a more discriminating typing technique[7-9]. However, PFGE is a technically demanding, expensive and time-consuming technique which requires specific equipment. Polymerase chain reaction (PCR)-based typing technique, such as randomly amplified

polymorphic DNA (RAPD), is fast and easy to perform[10]. Interestingly, ESBL-producing *K. pneumoniae* is an important hospital-acquired bacterial pathogen in many countries[11]. Mostly, ESBL-encoding genes are located within transposons or integrons, which strongly facilitates antibiotic-resistant gene transfer between bacterial species resulting in cross-transmission, thereby spreading resistance among related and unrelated gram-negative bacteria[12].

The purpose of the present study was to study epidemiological typing of 30 ESBL-producing *K. pneumoniae* isolates collected in January-February 2004 from patients admitted to Siriraj Hospital. This was done by using RAPD and re-examination of these isolates using Southern blot hybridization with a class 1 integron (*intI1*) probe.

Material and Method

Clinical isolates

Thirty isolates of ESBL-producing *K. pneumoniae* from clinical specimens (blood, urine, sputum, pus) were studied. They were obtained from different patients admitted to Siriraj Hospital during the 2 months period in January-February 2004. The confirmation of ESBL-producing *K. pneumoniae* was tested by the standard disk diffusion method as described by CLSI using third generation cephalosporin i.e., cefotaxime with and without clavulanate disks, and ceftazidime with and without clavulanate disks[13]. The positive control ESBL-producing *K. pneumoniae* ATCC 700603 and the negative control *E. coli* ATCC 25922 were used.

RAPD analysis

All isolates of ESBL-producing *K. pneumoniae* were extracted for DNA by a Puregene DNA Purification kit (Gentra Systems, USA). The following three primers were used: i.e., AP4 (5'-TCA CGA TGC A-3')[14], HLWL74 (5'-ACG TAT CTG C- 3')[15] and R108 (5'-GTA TTG CCC T-3')[16]. The reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 4 µM each primer, 400 µM each deoxynucleoside triphosphate, 2.5 U of *Taq* DNA polymerase (DyNAzyme™ II DNA Polymerase, USA) and 100-500 µg of genomic DNA in a final volume of 25 µl. Amplification was performed in a PCR Sprint ThermoHybaid (Hausen Berstein, Germany), for 5 minutes at 94ºC and 30 consecutive cycles of 1 minute at 94ºC, 1 minute at 36ºC, and 2 minutes at 72ºC, with a single final extension step of 10 minutes at 72ºC. The amplified products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide. Patterns were visually interpreted by two independent observers blinded to the origin of the isolates. The latter showing patterns, which differed by 1 to 2 and more than 2 bands, were considered minor and major variants of a given type, respectively.

Southern blot analysis

All isolates of ESBL-producing *K. pneumoniae* DNA were digested with *Bam*HI. The positive and negative controls for the *intI1* probe were pCTF202 plasmid and DH5□chromosomal DNA, respectively[17]. The DNA patterns of all ESBL-producing *K. pneumoniae* were detected by a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany). Patterns were visually interpreted by independent, blinded observers. Isolates showing patterns which differed by a single band were considered a given major type.

Results

All *K. pneumoniae* isolates in the present study were confirmed to be ESBL producers by antimicrobial susceptibility tests. The ESBL-producing *K. pneumoniae* isolates were typed by RAPD analysis and Southern blot hybridization with *intI1* probe. For RAPD analysis, the numbers of band patterns generated individually by AP4, HLWL74 and R108 primers were 29, 29 and 30 types as shown in Fig. 1-3, respectively. In 29 different patterns, AP4 primer gave one similar pattern (pattern A23), whereas HLWL74 and R108 primers gave different patterns in these 2 isolates. In 29 different patterns, HLWL74 primer gave one similar pattern (pattern H3), whereas AP4 and R108 primers gave different patterns in these 2 isolates. For Southern blot analysis, the number of band patterns which used an *intI1* probe numbered 30 for *Bam*HI restricted fragments (data not shown). The comparison between RAPD analysis and Southern blot hybridization with an *intI1* probe is shown in Table 1.

Discussion

ESBL-producing *K. pneumoniae* has been, and still is, mainly a nosocomial pathogen [11]. Nosocomial outbreaks caused by ESBL-producing *K. pneumoniae* have been reported in Europe and the USA[4,18-20]. A major risk factor for colonization or infection with ESBL-producing bacteria is long term antibiotic exposure[19]. In Siriraj Hospital, ESBL-producing *K. pneumoniae* may be the cause of outbreaks in the future. The AP4, HLWL74 and R108 primers were used in RAPD analysis in a preliminary study. Amplification of each primer

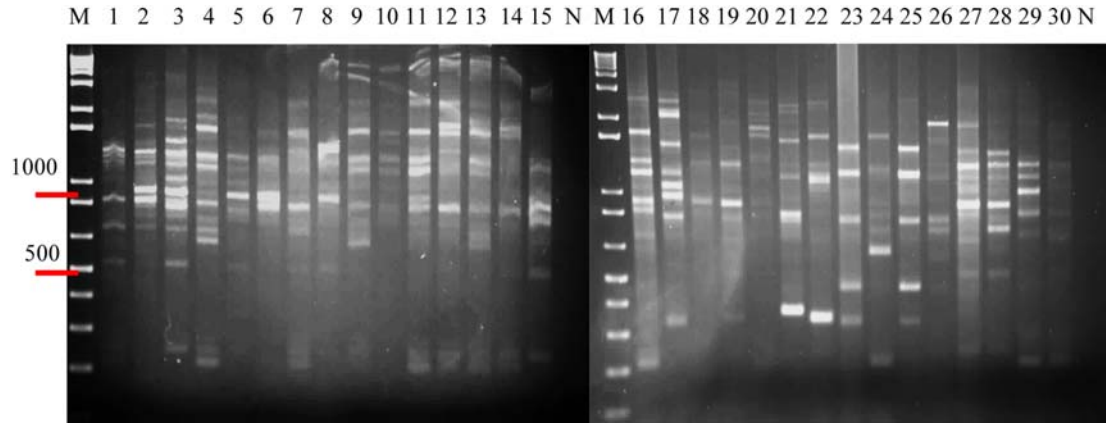

Fig. 1 Lanes 1-30 show band patterns of 30 ESBL-producing *K. pneumoniae* isolates, which DNA were amplified by using AP4 primer, lane M = the standard 100 bp DNA size marker and lane N = negative control

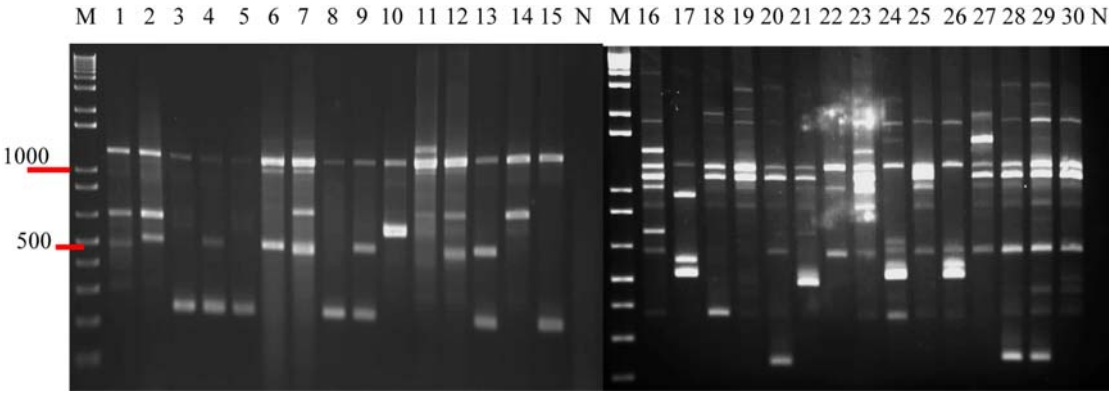

Fig. 2 Lanes 1-30 show band patterns of 30 ESBL-producing *K. pneumoniae* isolates, which DNA were amplified by using HLWL74 primer, lane M = the standard 100 bp DNA size marker and lane N = negative control

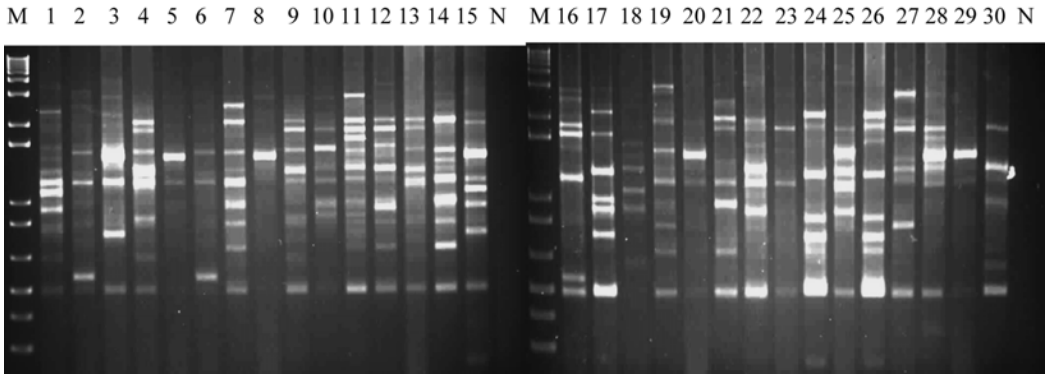

Fig. 3 Lanes 1-30 show band patterns of 30 ESBL-producing *K. pneumoniae* isolates, which DNA were amplified by using R108 primer, lane M = the standard 100 bp DNA size marker and lane N = negative control

could give 2-15 bands so they could be performed efficiently for the 30 ESBL-producing *K. pneumoniae* typing. For interpretation in the present study, one to two bands difference was considered a minor variant of a given major type, because triplicates of RAPD in an isolate could be given 1-2 bands difference. Differences of more than 2 bands were considered a given major type.

A previous report[10] used AP4, HLWL74 and R108 primers in RAPD analysis for their ESBL-producing *K. pneumoniae* from Erasme Hospital, Belgium and found that HLWL74 primer had more discriminating power than AP4 and R108 primers. However, in the present study, AP4 and HLWL74 primers gave 29 different patterns, whereas R108 primer gave 30 different patterns in these 30 isolates. Therefore, these primers had similar discriminating power. It may be possible that their isolates from Belgium have a DNA sequence different from the isolates in Siriraj Hospital. In Table 1, one similar pattern (A23 pattern) in 2 isolates (isolation numbers 23 and 25), which were amplified by AP4 primer gave different patterns in these isolates when they were amplified by using HLWL74 and R108 primers. In the same way, one similar pattern (H3 pattern) in 2 isolates (isolation numbers 3 and 5), which were amplified by HLWL74 primer gave different patterns in these isolates when they were amplified by using AP4 and R108 primers. Thus, the best RAPD analysis in the present study should use either a combination of AP4 plus HLWL74 primers or AP4 plus R108 primers (each primer in each reaction) so that the combination could give 30 major patterns in these 30 isolates.

The main cause of the problem is the result of drug resistant genes migrating among bacterial species, carried by plasmids and transposons[21]. Class 1 integrons, located on plasmids and transposons, make up the majority of the integrons found in clinical isolates and are associated with the multidrug resistance seen in the hospital environment[22-24].

In the re-examination, Southern blot analysis with *intI1* probe was used for detection. This technique was used because it was easier and less expensive than using PFGE for the purpose of re-examination. Southern blot analysis had more discriminating power than RAPD analysis because it gave 30 patterns in the 30 isolates. Surprisingly, all isolates, which were ESBL producers, had class 1 integron elements. In addition, integron was important, because it was a mobile genetic element and it was able to integrate drug-resistant gene cassettes[20]. Interestingly, various *intI1* patterns indicated dissemination of class 1 integron in Siriraj Hospital. However, the discrimination power of RAPD was good in the present study, since all epidemiologically unrelated isolates that were found differently by Southern blot analysis with *intI1* probe also exhibited distinct RAPD patterns. Reproducible patterns were obtained in independent experiments (data not shown).

Table 1. The comparison between RAPD typing and Southern blot hybridization with an *intI1* probe of ESBL-producing *K. pneumoniae* band patterns

Isolation number	RAPD typing[a]			Southern blot hybridization with an *intI1* probe[b]
	AP4 primer	HLWL74 primer	R108 primer	
1	A1	H1	R1	B1
2	A2	H2	R2	B2
3	A3	H3	R3	B3
4	A4	H4	R4	B4
5	A5	H3	R5	B5
6	A6	H6	R6	B6
7	A7	H7	R7	B7
8	A8	H3a	R8	B8
9	A9	H4a	R9	B9
10	A10	H10	R10	B10
11	A11	H11	R11	B11
12	A12	H12	R12	B12
13	A13	H13	R13	B13
14	A14	H14	R14	B14
15	A15	H15	R15	B15
16	A16	H16	R16	B16
17	A17	H17	R17	B17
18	A18	H18	R18	B18
19	A19	H19	R19	B19
20	A20	H20	R20	B20
21	A21	H21	R21	B21
22	A22	H22	R22	B22
23	A23	H23	R23	B23
24	A24	H24	R24	B24
25	A23	H25	R25	B25
26	A26	H24b	R24a	B26
27	A27	H27	R27	B27
28	A28	H28	R28	B28
29	A29	H28b	R29	B29
30	A30	H30	R30	B30

[a] RAPD typing patterns, which differed by one band, were considered a minor variant of a given major type, by two bands were considered major variants of a given major type, and by more than 2 bands were considered a given major type

[b] Southern blot hybridization with an *intI1* probe, showing patterns differed by a single band, was considered a given major type

Compared with Southern blot analysis with *intI1* probe in the present study and PFGE in general, RAPD typing is not expensive, significantly simpler to perform and produces results more rapidly. Therefore, RAPD typing is more suitable for first-pass screening of clinical isolates during investigation of outbreaks. Nevertheless, inter-assay reproducibility may be a problem with RAPD typing because of minor variation in PCR reagents or cycling parameters. This was not a problem in the present study, in which all isolates could be processed within single assay.

In conclusion RAPD typing can be used as a screening test for ESBL-producing *K. pneumoniae* during the investigation of outbreaks. Different patterns indicated that these isolates could not be the cause of an outbreak in Siriraj Hospital. This was relevant, because these isolates were collected from patients in different wards and the time of study expanded a 2 months period.

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การศึกษาเบื้องต้นของการใช้ randomly-amplified polymorphic DNA (RAPD) ในการแยกสายพันธุ์ของเชื้อ Klebsiella pneumoniae ที่ผลิตเอนไซม์อีเอสบีแอล

ชาญวิทย์ ตรีพุทธรัตน์, สมพร ศรีเฟื่องฟุ้ง, วรารัตน์ เชียงจง

วัตถุประสงค์: *ศึกษาเชื้อ Klebsiella pneumoniae ที่ผลิตเอนไซม์อีเอสบีแอลจำนวน 30 สายพันธุ์ ซึ่งแยกได้จากผู้ป่วย 30 รายที่เข้ารับการรักษาในโรงพยาบาลศิริราชระหว่างเดือนมกราคมถึงกุมภาพันธ์ พ.ศ. 2547 เพื่อวิเคราะห์ว่าจะสามารถนำวิธี randomly-amplified polymorphic DNA (RAPD) มาใช้ในการศึกษาเบื้องต้นว่าเกิดการระบาดของเชื้อ K. pneumoniae ในหอผู้ป่วยได้หรือไม่ เนื่องจากวิธี RAPD ให้ผลรวดเร็วกว่า ทำได้ง่ายและค่าใช้จ่ายถูกกว่าวิธีอื่นมากเพราะใช้หลักการของเทคนิคพอลิเมอเรสเชน รีเอกชัน*

วัสดุและวิธีการ: *การศึกษานี้ใช้ดีเอนเอ-ไพรเมอร์ 3 เส้น คือ AP4, HLWL74 และ R108 นอกจากนี้ได้ทำการทดสอบเชื้อทั้งหมดซ้ำโดยใช้วิธีทางระดับโมเลกุลชนิดอื่น คือ Southern blot hybridization ซึ่งดีเอนเอ-ติดตามของวิธีหลังนี้คือ class 1 integron (intI1 probe)*

ผลการศึกษา: *พบว่าวิธี RAPD สามารถตรวจแยกสายพันธุ์โดยการใช้ไพรเมอร์ AP4 หรือ HLWL74 ให้แบบแผนดีเอนเอ จำนวน 29 แบบ และไพรเมอร์ R108 ให้แบบแผนดีเอนเอ จำนวน 30 แบบ ซึ่งผลการศึกษานี้เหมือนกับการใช้วิธีศึกษา Southern blot hybridization ซึ่งให้แบบแผนดีเอนเอจำนวน 30 แบบ*

สรุป: *เทคนิคการตรวจแยกสายพันธุ์โดยวิธี RAPD สามารถนำมาใช้ในการแยกสายพันธุ์ของเชื้อ K. pneumoniae ได้ และเชื้อที่นำมาศึกษาเป็นเชื้อคนละสายพันธุ์กันไม่ได้เป็นสาเหตุการระบาดของโรคติดเชื้อ*