# Randomly amplified polymorphic DNA typing: a useful tool for rapid epidemiological typing of *Klebsiella pneumoniae*

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## SUMMARY

Discriminatory typing methods are invaluable in the investigation of outbreaks of infectious diseases. Single primers were used to generate randomly amplified polymorphic DNA (RAPD) profiles from *Klebsiella pneumoniae* isolates of various serotype and *K. pneumoniae* isolates from cases of sepsis at a Malaysian hospital and two English hospitals. RAPD profiles of acceptable reproducibility, a maximum of three minor band variations, were produced using a rapid DNA extraction method. RAPD typing of *K. pneumoniae* was shown to be as discriminatory as restriction fragment length polymorphism analysis using pulsed field gel electrophoresis yet quicker and less costly. The findings suggest that RAPD typing may be a useful tool for the epidemiological typing of *K. pneumoniae*.

## INTRODUCTION

*Klebsiella pneumoniae* continues to be a common and serious cause of nosocomial infection [1]. Being able to differentiate between strains of *K. pneumoniae* is therefore important for infection control and epidemiological surveillance in hospitals [2]. Currently available methods of typing *K. pneumoniae* include antibiogram typing, biotyping, capsular serotyping, bacteriophage typing and bacteriocin typing [2, 3]. Biotyping and antibiogram typing are too limited in their ability to discriminate between strains to be used for the epidemiological typing of *K. pneumoniae* [2, 4]. Whereas capsular serotyping, phage typing and bacteriocin typing have more discriminatory power, all three are time consuming and expensive methods and not all strains of *K. pneumoniae* are successfully typed by these methods [2, 4–6]. At present the most discriminatory method of typing *K. pneumoniae* involves the use of restriction fragment length polymorphism (RFLP) techniques [6–8]. However, these techniques are costly, labour-intensive and have lengthy processing times [9–11]. An alternative molecular typing method makes use of randomly amplified polymorphic DNA (RAPD) [12, 13]. RAPD is produced by amplifying segments of genomic DNA through a polymerase chain reaction (PCR) using primers of arbitrary sequence [12, 13]. RAPD methods are quick, relatively inexpensive and have already been successfully used to type several microbial species including *Escherichia coli* [9], *Aspergillus fumigatus* [14],

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*Proteus mirabilis* [15], *Legionella pneumophila* [16] and *Borrelia burgdorferi* [17]. The following study was therefore undertaken to develop and assess the use of RAPD methods for the typing of *K. pneumoniae*.

## METHODS

### Capsular serotyping and restriction fragment length polymorphism analysis

Klebsiella capsular serotyping and RFLP typing with contour clamped homogeneous electric field electrophoresis (CHEF) was performed at the Gram Negative Unit at Central Public Health Laboratory (CPHL), London. For CHEF analysis, chromosomal DNA was incorporated into agarose blocks using a previously described method [18] and digested with the restriction endonuclease *Xba* I as follows. Small portions (2·5 mm × 5 mm × 1 mm) of each block were equilibrated for 1 h in 100 $\mu$l of the buffer recommended by the manufacturer of the enzyme. Following the removal of the equilibration buffer, the blocks were incubated overnight in a 37 °C water bath in 100 $\mu$l fresh buffer containing 20 units of enzyme. DNA fragments were separated in 1·2 % agarose in 0·5 × TBE (44·5 mM Tris, 44·5 mM Boric acid, 1 mM EDTA) using the Biorad CHEF DRII system. Samples were electrophoresed for 24 h at 200 V with pulse times ramped from 1–35 sec. Gels were stained in 1 $\mu$g/ml ethidium bromide for 1 h, then destained for 1 h in distilled water before visualisation under UV transillumination (T. L. Pitt, personal communication).

The klebsiella isolates described below produced CHEF patterns of 13–20 bands. Isolates were considered to be indistinguishable if their CHEF patterns showed less than a three band variation.

### Organisms

Four groups of klebsiella isolates were used. The first group comprised seven *K. pneumoniae* isolates (C1–7) of different serotypes obtained from CPHL. The second group consisted of eight *K. pneumoniae* isolates (M1–8) from cases of neonatal sepsis detected over a 7-week period at a Malaysian hospital nursery. At this nursery, multiresistant *K. pneumoniae* has been the predominant cause of neonatal sepsis for at least the past 2 years and there was no change in the incidence of *K. pneumoniae* neonatal sepsis during the 7-week period. All eight isolates had identical antibiotic sensitivity patterns and API (bioMerieux, Lyon) profiles and none of the eight isolates were typable with the set of antisera used at CPHL. By grouping isolates whose CHEF patterns showed less than three band variations, the Malaysian isolates were divided into four subgroups: M2, M5; M1, M3, M4 and M6; M7 and M8. The third group comprised 13 *Klebsiella* isolates (A1–13) from cases of sepsis on the oncology ward of an English hospital (Hospital A). These cases were clustered over a 18-week period. Isolate A5 was a *Klebsiella oxytoca* strain and the remaining 12 isolates were *K. pneumoniae*. Isolates A1–4 and A6 were capsular serotype K62 and isolates A7–13 were untypable. The CHEF patterns of all 13 isolates were indistinguishable. The fourth group consisted of nine *K. pneumoniae* isolates (B1–9) from cases of sepsis detected over a 12-week period on a gastroenterology ward of a second English hospital (Hospital B). All nine isolates were capsular serotype K41. Their CHEF profiles

showed one to two band variations below the 100 kilobase mark but were otherwise indistinguishable. Over the past 2 years since the nine isolates were collected, additional cases of *K. pneumoniae* K41 sepsis have been detected at Hospital B.

All isolates were coded so that the RAPD analysis was carried out without knowledge of their capsular serotypes or CHEF profiles.

### Extraction of DNA and polymerase chain reaction

Each isolate was inoculated onto a MacConkey agar plate (Mast Diagnostics, Merseyside) which was incubated at 37 °C overnight. A colony of approximately 2 mm diameter was suspended in 30 $\mu$l of 0·2 $\mu$M filtered AnalaR water (BDH, Poole) and cooled on ice. One $\mu$l of the cell suspension was added to 10 $\mu$l of GeneReleaser (Cambio, Cambridge) and this mixture was vortexed, overlaid with filtered paraffin oil and heated in a microwave oven at 800 W for 5 min. Each heated GeneReleaser mixture was brought to 70 °C before addition of the PCR reagants.

Each PCR reaction was performed in a total volume of 50 $\mu$l. Each reaction mixture comprised 0·25 units of Super *Taq* polymerase enzyme (HT Biotechnology, Cambridge); 10 mM Tris HCl (pH 9·0), 1·5 mM-$MgCl_2$, 50 mM-KCl, 0·1% (v/v) Triton X-100 and 0·01% (w/v) gelatin; 20 $\mu$M of each dNTP (Pharmacia, Uppsala, Sweden) and 0·2 $\mu$M of primer (see below).

Amplifications were carried out in a thermal cycler (Omnigene Hybaid, Middlesex) for: 1 cycle of 3 min at 94 °C (denaturing step); followed by 40 cycles of 20 sec at 94 °C (denaturing step), 1 min at 36 °C (annealing step) and 1 min at 72 °C (synthesis step); followed by 1 cycle of 7 min at 72 °C (synthesis step).

The amplification products were fractionated by electrophoresis through 2% agarose gel (Gibco BRL, Paisley) at a constant voltage of 80 V for 2 h. The products were visualized by staining with ethidium bromide (Sigma, St Louis).

A negative control that comprised reaction mixture with no target DNA was included with each run of PCR.

### Primers

Thirteen primers were tested for their ability to produce discriminatory RAPD profiles from the *K. pneumoniae* isolates (Table 1). Primers U1, A1 and R1 were selected because they had previously been shown to generate discriminatory RAPD profiles from other organisms in our and other laboratories [13]. The remaining 10 primers were chosen at random from a collection of primers designed at our laboratory for other purposes.

### Reproducibility

A *K. pneumoniae* isolate was subcultured onto four separate MacConkey agar plates. A reaction mixture was prepared using a colony from each plate therefore yielding four sets of PCR products for the one isolate (interextract reproducibility). Quadruplicates of two other *K. pneumoniae* isolates were similarly prepared. The same cell suspension of isolate A4 was used in three separate runs of PCR to generate products for each of the gels shown in Figures 1, 2 and 4 (interamplification reproducibility).

Table 1. *Primers tested for generation of RAPD profiles from* Klebsiella pneumoniae

| Primer name | Sequence (5′ to 3′) | Tm (°C)* | Reference |
|---|---|---|---|
| U1 | CAGC(A/C)GCCGCGGTAAT(A/T)C | 58–60 | [19] |
| A1 | AGTCGTAACAAGGTAAGCCG | 60 | [20] |
| HI-FP | TGTAGTTGGTACGCTTGGTG | 60 | N/A† |
| HI-RP | AACTCGGCGACCAGCTACAA | 62 | N/A |
| NM-FP | CGATGACCAGTAACGGACGG | 64 | N/A |
| NM-RP | GCTGCCGCTTAATCCAGATA | 60 | N/A |
| APRT-FP | CTTCTTGTTCCTTCTGCGAG | 60 | N/A |
| APRT-RP | TGGCCTGAGCTGTGATGGTT | 62 | N/A |
| IS986-FP | ACGCTCAACGCCAGAGACCA | 64 | N/A |
| IS986-RP | GATGAACCACCTGACATGAC | 60 | N/A |
| MT-RP | GCCATTGCGAAGTGATTCCT | 60 | N/A |
| SPPBP-RP | CAGCACTGATGGAAATAAAC | 56 | N/A |
| R1 | TGGTCACTGA | 30 | [13] |

* Melting temperature, calculated with Gene Quant RNA/DNA calculator (Pharmacia, Cambridge).
† Not applicable, as primer designed in our laboratory.

RAPD profiles were generated from a 16-fold range of concentrations of cell suspension from one *K. pneumoniae* colony.

## RESULTS

Of the 13 primers, only 2 (U1 and HI-RP) produced discriminatory banding patterns from the *K. pneumoniae* isolates. Between 1–10 bright, major bands and up to 5 faint, minor bands were generated from each *K. pneumoniae* isolate with these 2 primers (Figs 1–4). Detail of some minor bands was lost with photographic reproduction, for example in lane 5 of Figure 3 (bottom).

There was a maximum variation of three minor bands amongst the quadruplicates from a single isolate (Fig. 1). Therefore, in all subsequent experiments, isolates were still considered indistinguishable if their RAPD profiles showed up to three minor band variations. Interamplification reproducibility with less than two minor band variation is demonstrated by comparing the RAPD profile of isolate A4 in Figures 1, 2 and 4. There was little variation in the RAPD profiles generated from the 16-fold range of concentrations of cell suspension (data not shown). For this study, *K. pneumoniae* isolates were subcultured between 5–9 times and also maintained on nutrient agar slopes over a period of 4 months. The profiles produced from each subculture and from the agar slope at the end of 4 months showed no more variation than that shown in the above reproducibility tests (data not shown).

Isolates C1–7 and A4 were easily differentiated from one another and from the other three hospitals' isolates by their RAPD profiles produced with U1 primer (Figs 1–4) and HI-RP primer (data not shown).

RAPD typing with U1 primer divided the Malaysian isolates into the same four subgroups as CHEF typing – M2; M5; M1, M3, M4 and M6; M7 and M8 – though the profiles of the latter two groups were very similar (Fig. 3). In contrast, RAPD
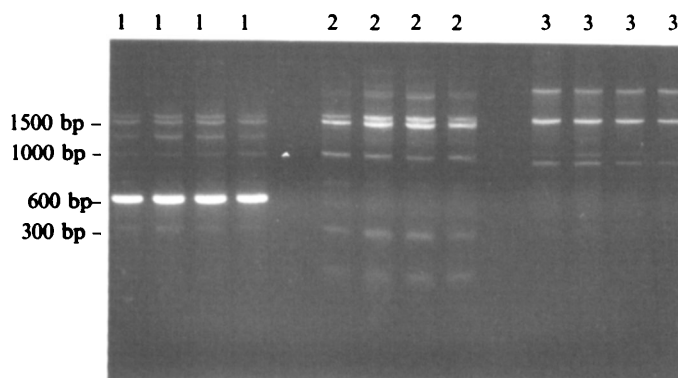
Fig. 1. Quadruplicate RAPD profiles, using U1 primer, of *K. pneumoniae* isolates A4 (lanes 1, 1, 1, 1), M4 (lanes 2, 2, 2, 2) and C4 (lanes 3, 3, 3, 3). The scale to the left indicates molecular weight in base pairs.
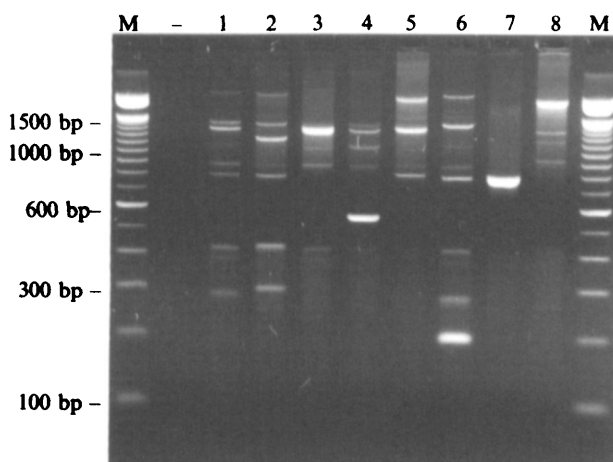


Fig. 2. RAPD profiles of *K. pneumoniae* isolates of varying capsular serotype using U1 primer. Lanes 1–3, isolates C1–3 respectively; lane 4, isolate A4; lanes 5–8, isolates C4–7 respectively; M, 100 base pair marker (Gibco BRL, Paisley); −, negative control.

typing with HI-RP primer showed these two groups to be distinct though M3 produced a different profile from the other three in its group (Fig. 3).

Aside from A5, all the Hospital A isolates had similar RAPD profiles (with less than three minor band variation) produced with primers U1 (Fig. 4) and HI-RP (data not shown). All the Hospital B isolates had similar RAPD profiles (with less than three minor band variation) using both primers (data not shown).

## DISCUSSION

An ideal typing method should be standardizable, reproducible, discriminatory, simple, readily available and of proven value to epidemiological investigations [2, 11, 21].

Randomly amplified polymorphic DNA (RAPD) profiles may be altered by changes in concentrations of primer, enzyme and target DNA and by different
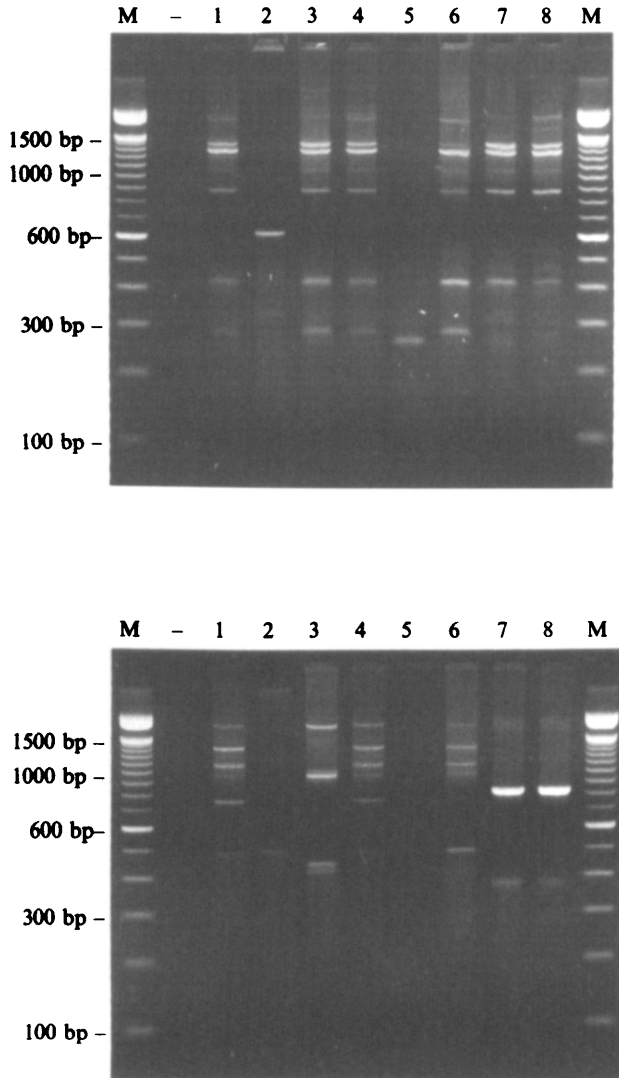
Fig. 3. RAPD profiles of *K. pneumoniae* isolates from cases of neonatal sepsis at a Malaysian hospital using U1 primer (top) and HI-RP primer (bottom). Lanes 1–8, isolates M1–8 respectively; M, 100 base pair marker (Gibco BRL, Paisley); −, negative control.

PCR cycling conditions [12, 13]. We standardized all these conditions apart from the DNA concentration. In preliminary studies, attempts to generate RAPD profiles from the supernatant of boiled cell suspension were only achieved at an acceptable level of reproducibility by standardizing DNA concentration. GeneReleaser is a commercially available preparation that blocks the action of nucleases on DNA liberated by heat cell lysis. With GeneReleaser we obtained consistent RAPD profiles for *K. pneumoniae* despite varying the concentration of cell suspension used in the reaction mixtures. Furthermore, reproducibility was not reduced by using only a crude method for standardizing the concentration of the cell suspensions. Therefore, whilst past authors have standardized target DNA
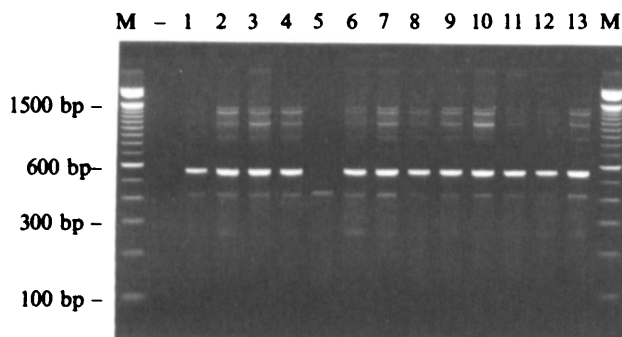
Fig. 4. RAPD profiles of klebsiella isolates from cases of sepsis at an English hospital (Hospital A) using U1 primer. Lanes 1–13, isolates A1–13 respectively; M, 100 base pair marker (Gibco BRL, Paisley); −, negative control.

concentrations for RAPD typing [10, 14–17], we were able to omit this step and further simplify the RAPD protocol.

The reproducibility of RAPD typing varies with different protocols and different microbes studied [9, 10, 14–17, 22]. As with past studies on *E. coli* [9] and *Metarhizium anisopliae* [22], our preliminary tests of interextract and inter-amplification reproducibility showed 2–3 minor band variations but consistent RAPD patterns as a whole. We generally found individual RAPD profiles to be so distinct that these variations did not complicate comparison of profiles. However, for formal numerical analysis of RAPD profiles, Fegan and colleagues have suggested a simple method for accommodating variations in band intensity, i.e. a band is only considered significantly if it is present in a duplicate profile [22]. In a recent study, seven laboratories across North America produced varied RAPD profiles from oat cultivar DNA despite using a standardized protocol [23]. If RAPD typing of *K. pneumoniae* is to have widespread use, its inter-laboratory reproducibility will therefore require further investigation. Reproducibility or stability over a long period of time is also important especially if control strains or strains from past outbreaks are to be reanalysed with newly collected isolates. We produced consistent RAPD profiles from *K. pneumoniae* isolates over 4 months and Lehmann and colleagues observed only minor differences in the RAPD profiles of 16 derivatives from two reference *Candida albicans* strains maintained over 30 years [10]. Whereas these findings are reassuring, they should be clarified by more formal study of how plasmid transfer, gene mutation and gene rearrangement may effect the long-term stability of RAPD profiles.

The ultimate test of relatedness must be chromosomal nucleic acid homology [21]. Indeed, a weakness of phenotypic typing is shown by the variation of capsular serotype amongst the 13 Hospital A isolates indicated to have genomic homology. As a 'short-cut' method of comparing DNA homology, RFLP typing is currently regarded as the most discriminatory means of typing *K. pneumoniae* [6–8, 21]. For the typing of methicillin resistant *Staphylocossus aureus* Saulnier and colleagues have shown RAPD typing to be less discriminatory than typing by pulsed field gel eletrophoresis of restriction endonuclease digest [24]. In contrast, we found that RAPD typing distinguished a *K. oxytoca* isolate from 12 *K. pneumoniae* isolates despite all 13 isolates yielding identical RFLP-based profiles.

These and other findings suggest RAPD typing of *K. pneumoniae* to be at least as discriminatory as CHEF typing. However, as shown with the Malaysian isolates, analysis with two primers was needed to achieve this discrimination. Additional primers can further increase the discriminatory power of RAPD typing [22, 25] but lengthens processing time and can complicate analysis. In epidemiological investigations, we therefore advocate two primers routinely for RAPD typing with perhaps a reserve primer for equivocal results. With two primers we achieved 100% typability, a rate unmatched by capsular serotyping, bacteriocin and bateriophage typing of *K. pneumoniae* [4–6]. There are two possible reasons why RAPD with HI-RP primer indicated M3 to be distinct from M1, M4 and M6 whereas all four isolates had identical CHEF profiles and RAPD profiles with U1 primer. The presence of a plasmid in isolate M3 may have only been detected with HI-RP primer. Alternatively, RAPD with HI-RP primer may produce a higher level of discrimination than CHEF typing or RAPD typing with U1 primer.

Perhaps the greatest attraction of RAPD typing is its simplicity and speed. Processing eight *K. pneumoniae* isolates from subculture to analysis required at least 3 days for CHEF typing (TL Pitt, personal communication) compared with under 24 h for RAPD typing. Furthermore, minimal skills or knowledge of molecular biology are required for the performance of RAPD typing. The simplicity and relatively low cost of RAPD typing make it practical for use at any teaching and large district hospital microbiology laboratory.

The practical utility of RAPD typing was tested with isolates of *K. pneumoniae* causing sepsis in three hospitals. In the case of the Malaysian hospital, neither biotyping nor capsular serotyping resolved whether the eight *K. pneumoniae* isolates had originated from a common source. RAPD typing, however, was able to demonstrate several subgroups amongst these isolates, thereby suggesting multiple reservoirs of infection. During the period when the eight isolates were collected, no strains of multi-resistant *K. pneumoniae* were isolated from swabs of the nursery environment or staff. Nevertheless, we speculate that different strains of *K. pneumoniae* are being maintained in the nursery environment and transferred between infants due to the overcrowded conditions and paucity of infection control in the nursery. RAPD typing confirmed the findings of CHEF typing which suggested that an outbreak of *K. pneumoniae* sepsis had occurred from a common source at each English hospital. At the time of preparing this paper, the outbreak at Hospital A had been controlled. In contrast, a persisting reservoir of *K. pneumoniae* K41 might explain the ongoing outbreak at Hospital B. A final requirement of a epidemiological typing method is that control isolates from epidemiologically unrelated sources should be shown to differ from the outbreak strain [11]. It is possible that the outbreak strain from each English hospital was the only strain of *K. pneumoniae* existing in the hospital. This could not be refuted as we did not have epidemiologically unrelated isolates from each hospital to compare with the outbreak strain. However, we were able to show that the RAPD profile of each English hospital strain differed from each other, from those of the Malaysian hospital isolates and the control serotyped isolates.

In summary, the findings of this preliminary study suggest that RAPD typing of *K. pneumoniae* fulfilled almost all the requirements of an ideal typing method. It was standardizable, reproducible, discriminatory and of practical use yet also

quick, simple and inexpensive to perform and thus applicable in hospital laboratories as a quick tool for the epidemiological investigation of *K. pneumoniae*.

## ACKNOWLEDGEMENTS

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