

Random Amplified Polymorphic DNA and Plasmid Analyses Used in Investigation of an Outbreak of Multiresistant *Klebsiella pneumoniae*

DAMON EISEN,^{1*} EDWARD G. RUSSELL,¹ MARTIN TYMMS,² ELISE J. ROPER,³
M. LINDSAY GRAYSON,¹ AND JOHN TURNIDGE¹

Department of Infectious Diseases and Microbiology, Monash Medical Centre,¹ and Centre for Early Human Development² and Department of Microbiology,³ Monash University, Clayton, Victoria 3168, Australia

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Multiresistant *Klebsiella pneumoniae* strains with plasmid-borne extended-spectrum beta-lactamases (ESBL) are increasingly frequent nosocomial pathogens. A major outbreak of clinical infections, mainly involving patients in the Newborn Services Unit with limited spread to adult patients, occurred at our hospital. This epidemic was investigated by typing the isolates phenotypically and with random amplified polymorphic DNA analysis (RAPD) and plasmid analysis. Forty-eight isolates, consisting of 44 consecutive clinical isolates and 4 selected surveillance isolates, were studied. A single decamer primer was used for the RAPD, and this was effective in demonstrating that the majority of isolates (45 of 48) had the same profile. Three other isolates had different RAPD patterns identifying them as nonepidemic strains. Plasmids were extracted by alkaline lysis with Magic-miniprep kits from 10 isolates selected to represent the epidemic and nonepidemic strains. This method produced small (<20-kb) plasmids; larger ESBL-carrying plasmids were not produced, but the small plasmids nonetheless allowed strain differentiation. Antibiotic susceptibility patterns alone were not reliable as strain indicators, since some isolates with the RAPD pattern characteristic of the epidemic strains did not express ESBL and therefore were susceptible to extended-spectrum cephalosporins. The investigation showed the predominance of a single epidemic strain that was transmitted between patients in the Newborn Services Unit. RAPD was the best of the methods used for detecting strain differences, and its speed and ability to type a wide variety of species suggest that it will be an increasingly useful molecular epidemiologic tool.

Nosocomial pathogens evolve with selection pressures generated by changes in medical practice and antibiotic usage. Intensive-care patients, both adult and neonatal, are prone to invasive infections with gram-negative organisms because of the degree of disruption of host defenses, frequent use of broad-spectrum antibiotics, and high prevalence of colonization with microbial pathogens. Since 1984, multiresistant *Klebsiella pneumoniae* has been increasingly recognized as a cause of hospital-acquired infections internationally (11, 13). 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 beta-lactamases (ESBL) and aminoglycoside-modifying enzymes (15). Their increasingly frequent occurrence restricts the choice of antibiotics for empiric therapy in severe nosocomial sepsis (6).

Nosocomial outbreaks of pathogens such as multiresistant *K. pneumoniae* have a significant impact on clinical practice, hospital morbidity and mortality, and overall treatment costs (10). Infection control efforts aimed at identifying a source of infection and mode of transmission are aided by typing methods showing strain differences. Traditional epidemiologic tools, including biotyping and serotyping, are not useful in distinguishing between strains of *K. pneumoniae* (12, 14). Molecular techniques, including plasmid analysis and ribotyping, when used in analyzing outbreaks of nosocomial *K. pneumoniae* infections, have suggested the involvement of more strains than are distinguishable by phenotyping (3).

Distinctive polymorphisms generated by the new PCR tech-

nique of random amplified polymorphic DNA analysis (RAPD) are now being utilized for strain differentiation in a fashion similar to ribotyping (16, 19). Species differentiation of an increasing number of bacteria is being studied by using either single or multiple overlapping, randomly designed primers (4).

This paper reports the findings of an investigation of an outbreak of nosocomial infections caused by multiresistant *K. pneumoniae* at our hospital by using the molecular typing method of RAPD. Infections caused by this organism were seen initially in patients in the Newborn Services Unit (NBS) but were later also found in other pediatric and adult patients.

MATERIALS AND METHODS

Setting. Monash Medical Centre is a 747-bed university teaching hospital providing specialist obstetrics, neonatology, pediatrics, and adult medical and surgical care to the southeast region of Melbourne, Australia. The NBS consists of a 12-bed intensive-care area and an adjoining 33-bed Special Care Nursery. Sporadic multiresistant *K. pneumoniae* infections in NBS patients were first identified in February 1991; a major outbreak of infections commenced in December 1991 and has continued to the present time.

Clinical specimens were collected when indicated by signs of infection. When the epidemic was recognized, a surveillance program was instituted. This consisted of regular rectal cultures taken to document gastrointestinal colonization in NBS patients and extensive environmental swabs performed to investigate possible initial sources and transmission modes.

Strain selection. Multiresistant *K. pneumoniae* strains were initially identified by resistance to gentamicin and/or cephalothin. Most strains were subsequently shown to have reduced susceptibilities to extended-spectrum cephalosporins. In all, 48 isolates of *K. pneumoniae* identified as having the multiresistant phenotype were studied. The isolates were collected over a 28-month period, between February 1991 and June 1993. They consisted of 44 consecutive clinical isolates (18 from urine, 10 from endotracheal aspirate, 3 from sputum, 5 from surface swabs, 4 from mucosal swabs, 3 from blood, and 1 from a central venous catheter tip) recovered from 42 patients (26 NBS patients, 8 other pediatric patients, and 8 adult patients [neurology, 3; intensive care, 2; surgical, 2, and renal, 1]), along with a group of 4 selected strains (1 each from an NBS nurse's hand, an NBS bath, an NBS sink, and an NBS patient rectal culture) from the large group of environmental cultures.

* Corresponding author. Mailing address: Department of Infectious Diseases and Microbiology, Monash Medical Centre, Clayton Rd., Clayton, Victoria 3168, Australia. Phone: 61 3 550 4564. Fax: 61 3 550 4533.

TABLE 1. Phenotypic and molecular epidemiologic typing characteristics of multiresistant *K. pneumoniae* study isolates

Isolate no.	Patient location	Specimen	ESBL ^a	MIC (μg/ml) of ^b :						Molecular typing	
				AMC	GEN	CTR	CAZ	CIP	TRI	RAPD pattern no.	Plasmid pattern
1	NBS	Clinical	Pos	16	16	64	32	2	1	1	
2	NBS	Clinical	Pos	4	16	32	4	1	0.5	1	
3	NBS	Clinical	Pos	4	16	4	4	1	4	1	
4	NBS	Clinical	Pos	8	16	4	32	4	4	1	a
5	NBS	Clinical	Pos	8	16	16	8	1	1	1	a
6	NBS	Clinical	Pos	16	16	64	32	2	1	1	a
7	NBS	Clinical	Neg	8	16	0.12	1	2	16	1	a
8	NBS	Clinical	Pos	8	16	16	8	2	0.5	1	
9	NBS	Clinical	Pos	8	16	64	16	2	4	1	
10	NBS	Clinical	Pos	4	16	16	8	2	1	1	
11	NBS	Clinical	Pos	4	16	8	2	2	0.5	1	
12	NBS	Clinical	Pos	4	16	8	4	2	1	1	
13	NBS	Clinical	Pos	4	16	2	1	2	1	1	
14	NBS	Clinical	Pos	4	16	8	4	2	1	1	
15	NBS	Clinical	Pos	4	16	16	32	2	1	1	
16	NBS	Clinical	Pos	4	16	8	4	2	1	1	
17	NBS	Clinical	Pos	4	16	8	4	2	1	1	
18	NBS	Clinical	Pos	4	16	8	4	2	1	1	
19	NBS	Clinical	Neg	2	16	0.03	0.25	2	0.5	1	a
20	NBS	Clinical	Pos	4	16	8	4	4	0.5	1	
21	NBS	Clinical	Pos	8	16	64	32	2	2	1	
22	NBS	Clinical	Pos	8	16	32	16	2	1	1	
23	NBS	Clinical	Pos	4	16	8	4	2	0.5	1	
24	NBS	Clinical	Pos	4	16	2	4	2	1	1	
25	NBS	Clinical	Neg	2	16	0.03	0.25	0.06	0.5	2	b
26	NBS	Clinical	Pos	4	16	8	4	2	1	1	
27	NBS	Clinical	Pos	8	16	32	8	2	1	1	
28	NBS	Clinical	Pos	4	16	8	4	4	8	1	
29	NBS	Clinical	Pos	4	16	8	4	2	1	1	
30	NBS	Environmental swab	Pos	4	16	16	4	2	1	1	
31	NBS	Environmental swab	Pos	4	16	8	4	2	1	1	
32	NBS	Environmental swab	Neg	2	16	0.03	0.06	0.03	1	3	c
33	NBS	Rectal culture	Pos	4	16	4	4	2	1	1	
34	Pediatrics	Clinical	Neg	8	16	0.25	0.5	2	16	1	
35	Pediatrics	Clinical	Pos	2	16	2	1	4	16	4	a
36	Pediatrics	Clinical	Pos	4	16	2	2	2	2	1	
37	Pediatrics	Clinical	Pos	4	16	16	4	2	1	1	
38	Pediatrics	Clinical	Neg	4	16	0.12	0.5	2	0.5	1	
39	Pediatrics	Clinical	Pos	4	16	16	4	2	1	1	
40	Pediatrics	Clinical	Neg	8	16	0.06	0.5	2	16	1	a
41	Pediatrics	Clinical	Pos	8	16	16	8	2	0.5	1	
42	Adult unit	Clinical	Pos	4	16	4	8	2	1	1	a
43	Adult unit	Clinical	Pos	4	16	8	4	2	1	1	
44	Adult unit	Clinical	Pos	4	16	8	4	2	1	1	
45	Adult unit	Clinical	Pos	4	16	4	4	2	0.5	1	
46	Adult unit	Clinical	Pos	16	16	2	8	2	0.5	1	
47	Adult unit	Clinical	Pos	4	16	8	4	4	0.5	1	
48	Adult unit	Clinical	Pos	4	16	4	2	2	0.5	1	

^a Pos, ESBL demonstrated by double-disc synergy test; Neg, no ESBL identified.

^b AMC, amoxicillin-clavulanate; GEN, gentamicin; CTR, cefotaxime; CAZ, ceftazidime; CIP, ciprofloxacin; TRI, trimethoprim.

Phenotyping. Routine identification and antibiotic susceptibility tests were performed with the automated Vitek system (biomerieux-Vitek, Hazelwood, Mo.). Subsequently, a panel of MIC results for selected antibiotics was determined by using Microscan broth microdilution plates (Baxter, Sacramento, Calif.). Expression of ESBL was shown by the double-disc synergy test (7). Isolates with the phenotypic characteristics typical of multiresistant *K. pneumoniae* were stored at -70°C for later study.

RAPD. RAPD was performed with cell suspensions prepared from overnight, supplemented Trypticase soy broth cultures. A 1-ml aliquot was pelleted by centrifugation ($7,000 \times g$, 5 min), and the pellet was washed twice in sterile distilled water. The resulting suspension was diluted to an optical density at 600 nm of 0.75, giving a final concentration of $4.0 \times 10^8 \pm 1.0 \times 10^8$ CFU/ml. A single primer, 5'-ACGTATCTGC-3' (first used in studies typing *Listeria monocytogenes* [9]), synthesized on a MilliGen oligosynthesizer (Millipore, Bedford, Mass.), was used for all amplification reactions. A 2.5-μl aliquot of the cell suspension was added to 20 μl of PCR mixture containing (final concentrations)

0.14% Triton X-100, 0.36 mM deoxynucleoside triphosphates (Promega, Madison, Wis.), 5.1 mM MgCl_2 , 28.6 pmol of decamer primer, and 1.4 U of *Taq* polymerase (Boehringer-Mannheim, Mannheim, Germany) in 14.3 mM Tris-HCl buffer with 71.4 mM KCl. Eighteen microliters of the final PCR solution was heat sealed into a capillary tube, denatured at 94°C for 3 min, and then amplified by 45 cycles of denaturation at 94°C for 15 s, annealing at 35°C for 20 s, and extension at 72°C for 60 s, with a ramping rate of $0.25^{\circ}\text{C}/\text{s}$ in a Corbett FTS-1S thermal cycler (Corbett Research, Sydney, Australia). PCR products were analyzed by electrophoresis in 2.0% agarose gels containing ethidium bromide and photographed under UV light.

Plasmid analysis. Plasmid DNA was extracted by using a Magic-miniprep kit (Promega) according to the manufacturer's instructions. In brief, this involved growing overnight cultures of *K. pneumoniae* in Trypticase soy broth. The cultures were then pelleted and treated with an RNase solution before alkaline lysis was performed. After centrifugation to remove the cell debris, the suspension was mixed with a DNA-binding resin and passed through a Magic-miniprep

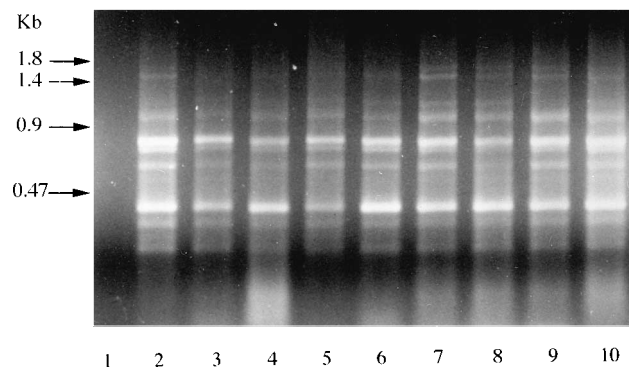


FIG. 1. RAPD patterns for representative epidemic strains. Lane 1, negative control; lanes 2 to 10, isolates 6, 7, 12, 13, 29, 30, 31, 33, and 42, respectively (see Table 1).

column, allowing the plasmid DNA to bind to the solid support. The DNA was then eluted in 30 μ l of prewarmed TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]). Digestion with the restriction endonuclease *Eco*RI (Boehringer-Mannheim) was carried out according to the manufacturer's instructions. The resulting plasmid profiles and restriction endonuclease digests were analyzed with ethidium bromide-stained 0.8% agarose electrophoresis gels which were examined under UV light.

RESULTS

Phenotype. Antibiotic susceptibility profiles determined by broth microdilution are shown in Table 1. ESBL were present in 41 isolates and absent in 7 isolates.

RAPD. All 48 isolates of multiresistant *K. pneumoniae* were studied with RAPD, with the majority of isolates showing the same pattern of bands. These included all but two of the NBS isolates. Many of the non-NBS isolates, including those from adult patients, also had the common pattern (Fig. 1). Additionally, three distinct and reproducibly different banding patterns were seen in three other isolates (from a pediatric patient catheter swab, NBS patient bag urine, and an NBS sink surveillance swab) (Fig. 2a).

Prior to testing of the multiresistant *K. pneumoniae* strains, RAPD was performed with 10 epidemiologically unrelated strains of *K. pneumoniae* that had the usual susceptibility pattern, showing resistance to ampicillin alone. The RAPD patterns produced were all unique and were dissimilar to those

seen with the multiresistant *K. pneumoniae* group. RAPD patterns for two such strains (K101 and K102) compared with the patterns for epidemic strains are shown in Fig. 2b.

Of additional interest, a cefotaxime-resistant strain of *Klebsiella oxytoca* which was initially misidentified as *K. pneumoniae* and included for study with the group of multiresistant *K. pneumoniae* strains was found to have a RAPD pattern unlike that of any of the *K. pneumoniae* strains examined (data not shown).

Plasmid profile. Ten multiresistant *K. pneumoniae* isolates, representing both epidemic and nonepidemic strains on the basis of their RAPD profiles, were selected for plasmid extraction and restriction endonuclease analysis (Fig. 3 and 4). The epidemiologically unrelated strains K101 and K102 were also subjected to plasmid analysis. Three distinct plasmid patterns were evident among the outbreak strains. Eight clinical isolates shared the same plasmid pattern. The two other plasmid profiles were seen in one clinical isolate and in an environmental isolate, both of which were distinct from the epidemic strain on the basis of ciprofloxacin susceptibility. Strains K101 and K102, studied for comparison, shared another different plasmid profile.

When RAPD and plasmid results were compared with regard to differentiation among outbreak strains, results were concordant in 9 of the 10 isolates selected for plasmid analysis. Disagreement existed for only one of the nine clinical isolates (isolate 35) with the common plasmid pattern in that the RAPD profile repeatedly showed the epidemic pattern with an additional distinct band. Environmental isolate 32 and clinical isolate 25 not only had different plasmid patterns but had RAPD profiles unlike the epidemic pattern. It is also interesting that the epidemiologically unrelated strains K101 and K102, which had the usual *K. pneumoniae* susceptibility pattern of resistance to ampicillin alone of the drug susceptibilities routinely tested, had the same plasmid profile but differing RAPD patterns, again suggesting greater discrimination by RAPD.

DISCUSSION

In this study, differentiation of strains of multiresistant *K. pneumoniae* from nosocomial infections was possible by the use of RAPD. The main epidemic strain was first isolated from patients in the NBS and was later found to be the cause of

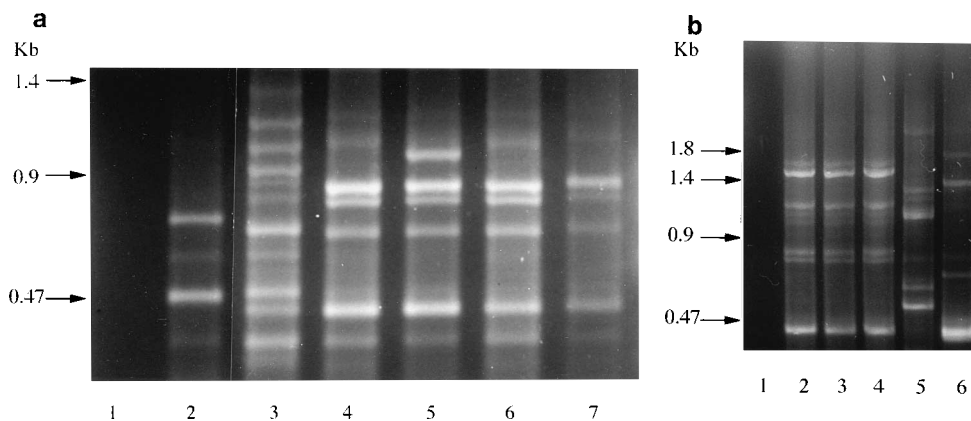


FIG. 2. (a) Comparison of RAPD patterns for epidemic strains and the three nonepidemic strains. Lanes: 1, negative control; 2, isolate 32 (nonepidemic); 3, isolate 25 (nonepidemic); 4, isolate 4 (epidemic); 5, isolate 35 (nonepidemic); 6 and 7, isolates 18 and 19, respectively (epidemic) (see Table 1). (b) Comparison of RAPD patterns for epidemic strains and epidemiologically unrelated, routinely susceptible strains. Lanes: 1, negative control; 2 to 4, epidemic strains 39, 40, and 43, respectively; 5 and 6, unrelated strains K101 and K102, respectively.

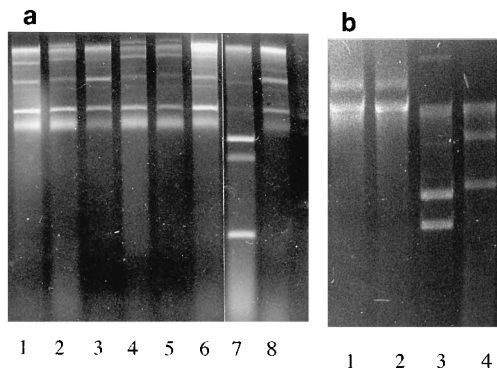


FIG. 3. (a) Whole plasmid profiles. (For plasmid patterns, see Table 1.) Lanes 1 to 4, isolates 4 to 7, respectively (pattern a); lane 5, isolate 35 (pattern a); lane 6, isolate 42 (pattern a); lane 7, isolate 32 (pattern c); lane 8, isolate 42 (pattern a). (b) Comparison of whole plasmid profiles for outbreak strains and epidemiologically unrelated, routinely susceptible strains. (For plasmid patterns, see Table 1.) Lanes: 1 and 2, strains K102 and K101, respectively; 3, strain 25 (pattern b); 4, strain 19 (pattern a).

nosocomial infections in adult patients. Three nonepidemic strains were identified on the basis of their different and discriminative RAPD patterns.

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. They are designed within constraints including (i) a length of not less than nine nucleotide residues, (ii) a GC content of $>50\%$, and (iii) a lack of palindromic sequences (19). These primers used in PCRs have been able to efficiently detect DNA polymorphisms and identify interstrain variations in an increasing number of species (1, 2). Genetic mapping and determination of the degree of relatedness between strains have been performed, with validation by ribotyping (18). The banding pattern derived from this process allows the identification of similar strains by a method significantly less complicated and time-consuming than ribotyping. When directly compared in the analysis of a *Proteus mirabilis* outbreak in a maternity unit, RAPD and ribotyping were equivalent in their abilities to discriminate between strains (1). It is of paramount importance that reaction conditions, including DNA template concentration, annealing temperature, and other PCR mixture concentrations, are strictly standardized to avoid artifactual variation in RAPD patterns (5).

While RAPD gives information regarding similarity between isolates, the precise genomic locations and functions of the amplified products remain obscure. The possibility that amplified plasmid DNAs are seen in the banding patterns from organisms carrying small plasmids has been discounted because of their short and basic DNA sequences, which provide little opportunity for primer annealing (16). It appears from this study that even the large ESBL-encoding plasmids do not contribute to the RAPD bands, since isolates with and without ESBL had the same epidemic strain RAPD pattern.

Plasmid analysis gives information on both strain identity and mechanisms of antibiotic resistance. Despite attempts at extraction of the large plasmids (>80 kb) which carry ESBL and other antibiotic resistance genes (by numerous methods, including those previously reported as being successful with *K. pneumoniae* [8, 17]), only small plasmids were purified in this study. The large plasmids in multiresistant *K. pneumoniae* that have been described are fragile and present in low copy numbers. This may explain the difficulty that we experienced in

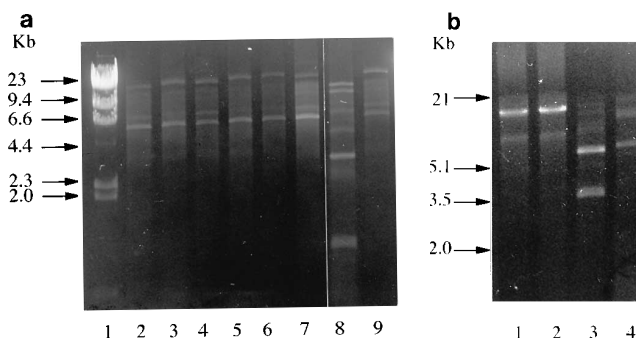


FIG. 4. (a) *Eco*RI plasmid digest profiles. (For plasmid patterns, see Table 1.) Lanes: 1, molecular weight markers (lambda phage digested by *Hind*III); 2 to 5, isolates 4 to 7, respectively (pattern a); 6, isolate 35 (pattern a); 7, isolate 40 (pattern a); 8, isolate 32 (pattern c); 9, isolate 43 (pattern a). (b) Comparison of *Eco*RI plasmid digest profiles for outbreak strains and epidemiologically unrelated, routinely susceptible strains. (For plasmid patterns, see Table 1.) Lanes: 1 and 2, strains K102 and K101, respectively; 3, strain 25 (pattern b); 4, strain 19 (pattern a).

their extraction. The plasmids identified in this study appeared to be useful as strain markers, since a common plasmid pattern in the clinical strains, which was distinct from that in the environmental strain studied, was identifiable. Overall, when plasmids in multiresistant *K. pneumoniae* involved in this outbreak were studied, there was a concordant result in strain differentiation in 9 of the 10 strains in comparison with the RAPD results. Strain 35, which shared the common plasmid profile, had a RAPD pattern distinguishable by only one additional band from the epidemic strain RAPD pattern. In addition, the two epidemiologically unrelated, routinely susceptible *K. pneumoniae* strains had the same plasmid pattern but different RAPD patterns.

Analysis of an accurate antibiogram did not always reliably differentiate between strains. Carriage of the ESBL and resistance to extended-spectrum cephalosporins were not seen in all strains identified by RAPD and plasmid analysis as representative of the epidemic. However, full susceptibility to ciprofloxacin, with MICs in the normal susceptible range, was seen only in nonepidemic strains.

No common environmental source for this multiresistant *K. pneumoniae* epidemic could be identified by this study. The babies in the NBS seemed to act as the reservoir for infection, with transmission between the babies probably the result of staff handling as suggested by the presence of the epidemic strain in surveillance cultures of the NBS bath and the hands of an NBS nurse. Nosocomial infections in the pediatric patients were in patients who had been in the NBS previously, thus establishing a link with the obvious reservoir. The means by which multiresistant *K. pneumoniae* was transmitted to adult patients in our hospital was not identified by this study.

The frequent occurrence of multiresistant *K. pneumoniae* infections in NBS patients during the epidemic necessitated changes in the empiric antibiotic regimens for suspected sepsis in this patient group from penicillin and gentamicin for early postnatal sepsis and vancomycin and cefotaxime for nosocomial sepsis to penicillin and amikacin for early postnatal sepsis and vancomycin and imipenem for nosocomial sepsis. The strains involved in the outbreak at this hospital remained susceptible to amikacin, unlike a majority of those seen elsewhere (10). Ciprofloxacin MICs for these pathogens have not been previously reported; 94% of the strains in this study were less susceptible to this agent than normal strains, with MICs of ≥ 1 $\mu\text{g/ml}$. As a result of infection control measures, of which this

molecular epidemiologic study was part, the incidence of new infections with this nosocomial pathogen has greatly decreased. The discovery of a predominant epidemic strain reinforced the need for optimal nursing practices and highlighted the requirement for careful attention to hand washing by attendant staff.

In this study of an outbreak of the serious nosocomial pathogen multiresistant *K. pneumoniae*, RAPD proved to be a useful tool in confirming the role of a single epidemic strain in NBS infections, with further infections with the epidemic strain appearing in other patient groups, including adults. In addition, this technique identified some isolates as nonepidemic, suggesting that multiple strains were present in the hospital environment. The speed, reproducibility, and simplicity of this method make it a valuable addition to current molecular epidemiological tools in the investigation of outbreaks of nosocomial infections.

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