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Physical Characterization of Ten R Plasmids Obtained from an Outbreak of Nosocomial *Klebsiella pneumoniae* Infections

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Gentamicin resistance in *Klebsiella pneumoniae* involved in an outbreak at the Minneapolis Veterans Administration Hospital was due to a transmissible R plasmid. In addition to gentamicin, this plasmid conferred resistance to tobramycin, kanamycin, ampicillin, carbenicillin, cephalothin, chloramphenicol, and sulfathiazole. R plasmids which transferred this complex antibiogram were identified in several clinical isolates, including four different serotypes of *K. pneumoniae*, *Escherichia coli*, *Enterobacter cloacae*, and *Proteus morganii*. The covalently closed circular form of all R plasmids isolated had a sedimentation coefficient of 76S to 77S, corresponding to a molecular weight of 58×10^6. The possibility that a single R plasmid was responsible for the dissemination of multiple drug resistance among all of these different clinical strains was examined by characterizing the plasmids by using *Eco*RI restriction endonuclease. The same 15 fragments were obtained from each of the 10 plasmids analyzed. Their molecular weights ranged from 4×10^5 to 11×10^6. Thus, we conclude that each of the 10 plasmids present in the various clinical strains isolated from the hospital over a 7-month period originated from a common source and that R plasmid transfer was important in their spread.

The ubiquitous presence of antibiotic-resistant bacteria in sewage and in the intestines of normal healthy people suggests that the general population is the major reservoir for these drug-resistant microorganisms or for the genes that code for antibiotic resistance (8, 19, 23, 24). Because such organisms are increasingly the cause of complications and lethal infections among hospital patients (15), extensive efforts have been made to define both the source and the method of spread of antibiotic-resistant microorganisms. These studies have been complicated by the documented abundance within hospitals of drug-resistant microorgansims which owe their resistance to the presence of transferable plasmids (R plasmids) (8, 15, 19, 23, 35). Thus, the dissemination of antibiotic resistance can occur by the spread of microorganisms or by the transfer of the drug resistance genes. Reports of both mechanisms occurring in clinical situations have been described; however, in the latter case the verification of R-plasmid transfer has been based on the identification of the plasmid in an inadequate manner. Identity among a few phenotypic markers which only represent a very small portion of the genetic capacity of the plasmid and which can in fact be determined by several genes has been used (10). As a result, the role of R-plasmid transfer in the increased frequency with which antibiotic-resistant organisms are involved in nosocomial infections has been minimized (10, 30, 31).

This report shows that R-plasmid transfer was significant in the dissemination and persistence of antibiotic resistance in a hospital environment. The epidemiology of an outbreak of gentamicin-resistant *Klebsiella pneumoniae* which occurred in late 1975 at the Minneapolis Veterans Administration Hospital (MVAH) has been studied (10a). The apparent source of the epidemic was an index case who brought the organism into the hospital. The outbreak was accompanied by an increase in frequency of all gentamicin-resistant gram-negative bacilli. The organisms were resistant to gentamicin, tobramycin, chloramphenicol, sulfathiazole, cephalothin, ampicillin, carbenicillin, and kanamycin. The multiple resistance pattern could be transferred intact to laboratory *Escherichia coli* strains, indicating that transmissible R plasmids coded for the resistance phenotype.

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This complex antibiogram was identified in four different serotypes of *K. pneumoniae* and in various other genera of the *Enterobacteriaceae*. These observations suggested that the transmission of an R plasmid played an important role in the spread and persistence of gentamicin resistance in this outbreak. To examine this possibility, we studied the physical characterization of 10 R plasmids which originated in clinical strains isolated at various times during this outbreak. Comparisons of restriction endonuclease fragments from each plasmid established without doubt that the plasmids present in the different strains at different times were identical.

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MATERIALS AND METHODS

Bacterial strains. All clinical strains were obtained from the Clinical Microbiology Laboratory at the MVAH (Table 1). A thymine-requiring *E. coli* strain, strain JC411 (1), and *E. coli* strain BK85 (JC411 *thy*$^-$ F$^+$) were obtained from Bruce Kline. *E. coli* strain M5107 (λcI857*s*7) was obtained from Jim Zissler (36). The bacterial strains were routinely stored in brain heart infusion (BHI)–0.7% agar stabs supplemented with 10 μg of thymine per ml.

Antibiotic resistance pattern. The antibiotic resistance pattern of each strain was determined by stabbing individual colonies into BHI–thymine plates containing the appropriate antibiotic. The antibiotic concentrations used were (in micrograms per milliliter): nalidixic acid, 400; tetracycline, 39; streptomycin, 10; kanamycin, 20; cephalothin, 30; ampicillin, 20; chloramphenicol, 10; tobramycin, 2; and gentamicin, 4.

Conjugation procedure. Multiple drug resistance was transferred by mixed cultivation of resistant clinical strains and the *E. coli* JC411 strain resistant only to nalidixic acid (22). This was done in BHI broth with thymine (10 μg/ml). The mating mixture (0.1 ml) was plated on BHI–thymine plates containing gentamicin (4 μg/ml) and nalidixic acid (400 μg/ml). The controls containing only the donor and recipient were always negative for growth on selective media containing both gentamicin and nalidixic acid.

Growth and radioisotopic labeling of bacteria. Bacteria were grown in BHI agar or antibiotic medium no. 3 (Difco Laboratories, Detroit, Mich.) with 10 μg of thymine per ml or in M9 medium for the preparation of radiolabeled deoxyribonucleic acid (DNA) (16). [*methyl*-^{3}H]thymidine (50 Ci/mmol; 1 mCi/ml; sterile aqueous) or [*methyl*-^{14}C]thymidine (54 mCi/mmol; 50 μCi/0.5 ml; sterile aqueous) was added to the growth media at a concentration of 10 μCi/ml.

Lysis and extraction of DNA. Cultures were harvested during mid- or late-logarithmic growth. The lysis conditions were as described for the lysozyme–Brij 58–deoxycholate-cleared lysate procedure (5), with the modification of a freeze-thaw step before the high-speed clearing step (17).

Buoyant density centrifugation. Supercoiled plasmid DNA was purified from cleared lysates by the cesium chloride–ethidium bromide (EtBr) buoyant density gradient procedure of Radloff et al. (28). EtBr was added at a final concentration of 350 μg/ml. The gradients were centrifuged with a Beckman 50 Ti rotor in nitrocellulose centrifuge tubes which had been presoaked in 0.5 M phosphate buffer (16). Centrifugation was in a Beckman L2 or L5-50 preparative ultracentrifuge for 55 to 60 h at 40,000 rpm and 15°C with the brake off. Fractions (110 μl) were collected from the bottom of the gradient. Samples of each fraction were assayed for radioactivity as previously described, except that Whatman no. 3 filter disks (2.3 cm) were used (3). The disks were counted in 2.5 ml of toluene containing 2-[(4′-*t*-butylphenyl)-5-4″-biphenyl]-1,3,4-oxadiazole (8 g/liter) and *p*-(bis-*O*-methylstyryl)-benzene (0.5 g/liter). EtBr was removed from pooled DNA fractions by extraction with isopropanol (7), and salt was removed by dialysis or by Sephadex G-50 (coarse) chromatography.

Sucrose gradient velocity centrifugation. The 4.8-ml, linear, neutral or alkaline sucrose gradients were prepared as described by Clewell (4). The plasmid F employed as a sedimentation marker was purified from *E. coli* strain BK85 by CsCl-EtBr buoyant density centrifugation.

Digestion of DNA with restriction endonuclease *Eco*RI. Before digestion all plasmid DNA was treated with (i) ribonuclease (final concentration, 50 μg/ml) for 10 min at 37°C and (ii) protease K (final

TABLE 1. *Source of clinical isolates analyzed for R plasmids*

Original strain	Patient	Date of isolation	Transconjugant	Plasmid designation
K. pneumoniae type 30	LE	9/23/75	E35	pBP4
K. pneumoniae (nontypable)	RA	9/24/75	E42	pBP1
K. pneumoniae type 17	WI	10/31/75	E43	pBP2
E. coli	WE	11/2/75	E55	pBP8
K. pneumoniae	JO	1/22/76	E56	pBP9
K. pneumoniae	GL	2/10/76	E57	pBP10
K. pneumoniae type 23	AL	3/11/76	E46	pBP3
P. morganii	AL	3/11/76	E47	pBP7
E. cloacae	AL	3/12/76	E44	pBP5
P. morganii	AL	3/12/76	E45	pBP6

concentration, 100 μg/ml) for 2 h at 37°C in the presence of 0.5% sodium lauryl sulfate. The DNA was then phenol extracted and ethanol precipitated. Phenol saturated with TES buffer [20 mM tris(hydroxymethyl)aminomethane, pH 8.0, 50 mM NaCl, 5 mM ethylenediaminetetraacetate] was used. The ribonuclease was pretreated by incubating it in a boiling water bath to eliminate deoxyribonuclease contamination (2). After ethanol precipitation, the DNA was resuspended in buffer A [100 mM tris(hydroxymethyl)aminomethane, pH 7.5, 50 mM NaCl, 0.1 mM ethylenediaminetetraacetate] or buffer B [125 mM tris(hydroxymethyl)aminomethane, pH 7.5, 125 mM NaCl, 0.1 mM ethylenediaminetetraacetate]. All *Eco*RI plasmid digestions contained at least 50 U of enzyme (Miles Laboratories, Inc., Elkhart, Ind.) and not more than 0.25 μg of DNA. The reaction mixture for DNA dissolved in buffer A has been described previously (36). The reaction mixture for DNA dissolved in buffer B contained, in addition to enzyme, 125 mM tris(hydroxymethyl)aminomethane, pH 7.5, 125 mM NaCl, 0.1 mM ethylenediaminetetraacetate, and 12.5 mM $MgCl_2$. The reactions were terminated by adding 0.25 volume of stopping buffer containing 5% sodium lauryl sulfate, 25% glycerol, and 0.025% bromophenol blue. The reaction was heated to 65°C for 10 min and then rapidly cooled on ice. The λ DNA was prepared from *E. coli* strain M5107 by the method of Thomas and Davis (36). Usually, 1 μg of λ DNA was digested with at least 5 U of *Eco*RI enzyme and then added to the plasmid digests. The DNA fragments were electrophoresed on agarose (Sigma Chemical Co., St. Louis Mo.) gels and the λ DNA fragments were detected by staining, as described by Helling et al. (13). The plasmid DNA fragments were located by slicing the gels (1.25 mm/slice) and counting the slices in 2.5 ml of a liquid scintillation cocktail containing a 3a70B complete counting cocktail (Research Products International Corp.), water (80 ml/liter), and glacial acetic acid (50 ml/liter). The slices were allowed to sit in the cocktail for at least 24 h before counting.

RESULTS

Choice of clinical strains. Ten clinical strains previously isolated from an outbreak of multiple-drug-resistant nosocomial infections at the MVAH were chosen for this study (Table 1). These isolates were all characteristically resistant to gentamicin, tobramycin, kanamycin, ampicillin, cephalothin, carbenicillin, and chloramphenicol. The strains were obtained over a 7-month period from patients present in the hospital (Table 1). This period included the peak of the epidemic and the subsequent endemic period (10a).

Because we wanted to examine the possibility that the transfer of an R plasmid had played a role in the spread of drug resistance in the hospital, the strains chosen included the various serotypes of *K. pneumoniae* and other *Enterobacteriaceae* found to have the same antibiogram.

Isolation of plasmid DNA. Previous work established that the multiple drug resistance of these clinical strains was self-transmissible to a laboratory *E. coli* strain. To facilitate the isolation of radioactively labeled DNA, the clinical strains were crossed with a laboratory *E. coli* strain (strain E33) which was thymine dependent. The presence of plasmid DNA was confirmed by centrifuging labeled DNA (obtained from cleared lysates of *E. coli* transconjugants) to equilibrium on CsCl-EtBr density gradients. A representative profile of a CsCl-EtBr buoyant density gradient of strain E35, a transconjugant resistant to all of the antibiotics, is shown in Fig. 1A. A satellite band, characteristic of supercoiled plasmid DNA, can be seen in a position of higher density than that of the chromosomal peak (Fig. 1A). DNA obtained by similar means from the antibiotic-susceptible parental strain (strain E33) contained only the chromosomal peak (Fig. 1B). Transconjugants from all 10 of the antibiotic-resistant strains yielded a similar satellite peak.

Characterization of plasmid DNA on sucrose gradients. As the first criterion of identity, the molecular weights of the plasmid DNAs were determined by sedimentation velocity centrifugation in sucrose gradients. The denser sat-

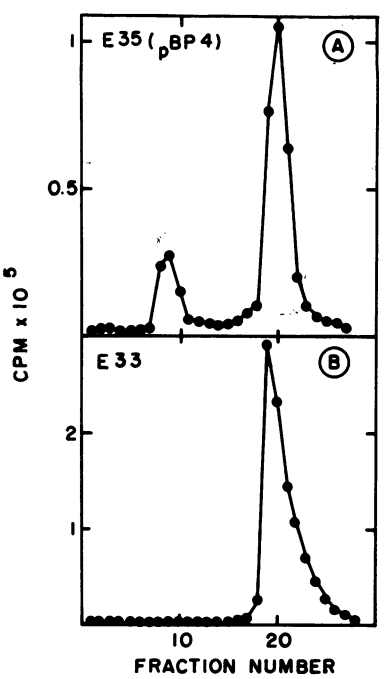

FIG. 1. *CsCl-EtBr buoyant density gradient centrifugation of cleared lysates of E. coli strains E35 (A) and E33 (B). Density increased from right to left.*

ellite DNA obtained from CsCl-EtBr gradients was sedimented in sucrose gradients; representative profiles of three different preparations are shown in Fig. 2. The fact that the fast-sedimenting peaks (76S to 77S) contained only covalently closed circular DNA was confirmed by their behavior in alkaline sucrose gradients (6; data not shown). Accordingly, the covalently closed circular DNA which sedimented as 77S material corresponded to a plasmid of 58 megadaltons when compared with F DNA, which was employed as a molecular weight marker (6).

The sedimentation velocity analysis of satellite DNA obtained from the other strains in this study (obtained from September 1975 through March 1976) also revealed the presence of 76S to 77S covalently closed circular DNA (data not shown). Thus, R plasmids from four serotypes of *K. pneumoniae* as well as from three other genera of *Enterobacteriaceae* isolated from the hospital over a span of 7 months appeared to have approximately the same molecular weight.

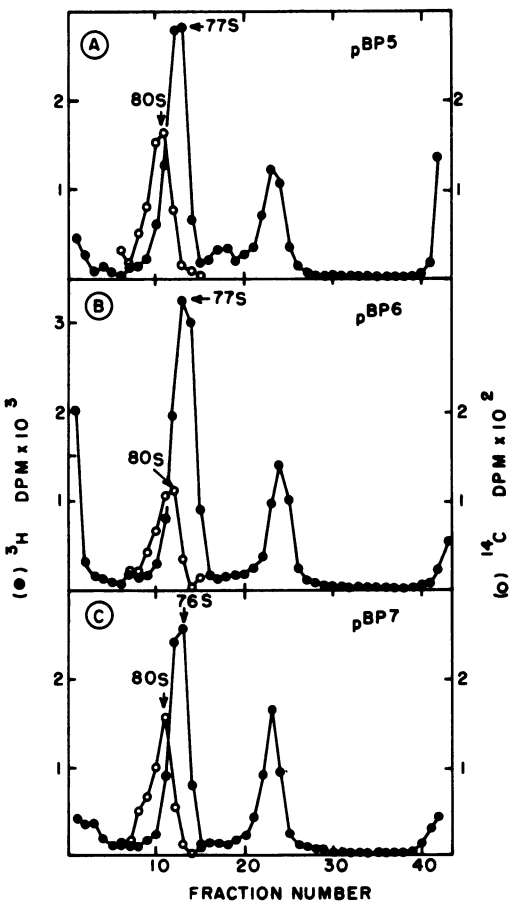

FIG. 2. *Sedimentation of satellite DNA from E. coli strains E44 (pBP5) (A), E45 (pBP6) (B), and E47 (pBP7) (C) on 5 to 20% neutral sucrose gradients. ^{14}C-labeled covalently closed circular F DNA was used as a sedimentation marker. Sedimentation was from right to left.*

Characterization of plasmid DNA by *Eco*RI restriction endonuclease digestion. To be certain that these plasmids were indeed identical, it was essential to use a criterion for comparison which would detect topological differences in the plasmid molecules (31). The analysis of restriction endonuclease DNA fragments produced when each plasmid was digested with *Eco*RI provided this information. The DNA fragments obtained from each plasmid were resolved by electrophoresis on agarose gels. The pattern and molecular weights of the fragments were then compared as a criterion of identity.

The electrophoresis and endonuclease digestion conditions were developed with plasmid pBP4. This plasmid was chosen because it was harbored by the epidemic strain of *K. pneumoniae* (type 30) and it was the earliest isolate available for the study. Agarose electrophoresis of the endonuclease digest of this plasmid on a 0.7% gel revealed 10 fragments, fragments A through K (Fig. 3A). Fragments B and C were not resolved, but peak BC (Fig. 3) was skewed to the right, and the total counts per minute in this peak indicated the presence of two fragments of heterogenous size. Fragments B and C could be resolved by extending the electrophoresis time. Examination of the total counts per minute in the other peaks indicated that they were present in equimolar amounts, confirming their homogeneity. To investigate the possibility that additional small-molecular-weight fragments were present, a digest of plasmid pBP4 was electrophoresed on a 1.4% agarose gel (Fig. 3B). Four additional fragments (fragments L, M, N, and O) were identified.

The molecular weights of the DNA fragments from the plasmid pBP4 digest were determined by using bacteriophage λ DNA *Eco*RI restriction endonuclease fragments as molecular weight standards (13). The fragments (fragments A through O) were found to vary in size from 0.4 to 11 megadaltons (Table 2), and the sum of the molecular weights of all 15 fragments represented 88% of the total molecular weight estimated from the sedimentation coefficients. Measurements of contour lengths of one population of DNA molecules by electron microscopy yielded a molecular weight of 58.6×10^6, confirming our previous determinations. We are unable to account for the 12% difference observed on the agarose gels. However, other work-

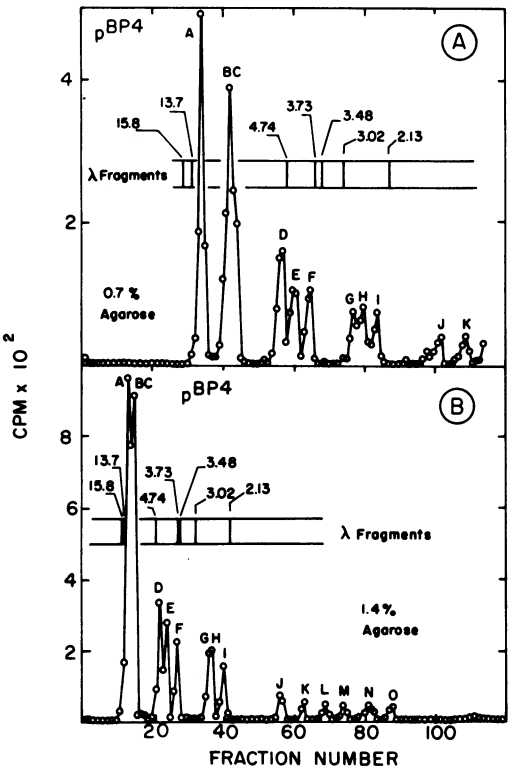

FIG. 3. *Electrophoresis of an EcoRI endonuclease digest of plasmid pBP4. The plasmid DNA was in buffer A, and the total volume was 58 μl. The reaction mixtures were incubated at 37°C for 1 h. The plasmid and λ DNAs were combined, and one-half was run on a 0.7% agarose gel (A) and the other half was run on a 1.4% agarose gel (B). Electrophoresis was for 12 h at 40 V. The λ DNA fragment sizes are in megadaltons. Migration was from left to right.*

ers have found the sum of restriction endonuclease fragment sizes to be 5 to 10% less than the known molecular weight of the intact plasmid (37).

An examination of the *Eco*RI digests of the other nine plasmids on both 0.7 and 1.4% agarose gels revealed, in each case, the presence of 15 fragments. The banding pattern of these fragments appeared to be identical to that seen for plasmid pBP4, and the molecular weights of the fragments appeared to be quite similar (Table 2).

To test the accuracy of these molecular weight determinations, fragments from a single plasmid were repeatedly electrophoresed. A range of up to 9×10^5 daltons was observed in estimating the size of fragment A. Differences of 3×10^5 daltons were observed for fragments B and C, and variations of 2×10^5 daltons were observed for the smaller fragments (data not shown). With the above considerations we feel confident that differences of one gene would be detected by the electrophoresis conditions employed in this study.

R plasmid transfer in vivo. The similarity of the molecular weights and the identical fragment patterns observed for the 10 plasmids in this study lead us to conclude that these plasmids are identical and, therefore, originated from a common source. Previous epidemiological data suggested that this outbreak was initiated in the hospital as the result of the admission of a patient harboring a multiple-drug-resistant *K. pneumoniae* strain. As this strain was not available, we cannot confirm this suggestion; however, the antibiogram of this organism was identical to those of the 10 strains in this study.

Cultures from one patient (patient AL) exemplified the infectious potential of this R plasmid in vivo. Two cultures (sputum and rectal) produced three organisms (*P. morganii, E. cloacae,* and *K. pneumoniae* type 23) which were gentamicin resistant and another strain of *K. pneumoniae* (type 23) which was antibiotic susceptible. The first three strains had similar antibiograms (Table 3) and harbored R plasmids pBP7, pBP5, and pBP3, respectively, which were identical to pBP4, the plasmid purified from the epidemic strain of *K. pneumoniae* (type 30) approximately 5 months earlier (Tables 1 and 2). The fact that one isolate from this patient was antibiotic susceptible suggests that transmission of the R plasmid did not occur during transport or cultivation of the specimen. In vitro this antibiotic-susceptible strain was able to conjugate successfully with the three antibiotic-resistant organisms isolated from this patient (Table 4). These results indicate that the colonial flora of this patient had the potential to serve as an R plasmid reservoir and provide the genes which code for antibiotic resistance to *K. pneumoniae,* the primary pathogen in the respiratory infection of patient AL.

DISCUSSION

Gentamicin has proven to be a highly effective antibiotic for the treatment of many gram-negative and gram-positive bacterial infections. Recently, however, several outbreaks of gentamicin-resistant *Klebsiella, Proteus, Serratia, Pseudomonas,* and *Staphylococcus* strains have been reported, and as a result the utility of this drug is in doubt (18, 20, 21, 26, 27, 29, 34). The nosocomial origin of these outbreaks was a feature similar to a problem which developed at the MVAH. This outbreak was thought to have been precipitated by the admission of a patient infected with a *Klebsiella* strain exhibiting a complex, yet very characteristic antibiogram. The episode included a period of increased *K.*

TABLE 2. *Comparison of the molecular weights of the EcoRI restriction endonuclease fragments from 10 plasmids*

Fragment designation	Mol wt ($\times 10^6$) in:									
	pBP1[a]	pBP2[a]	pBP3[a]	pBP4[b]	pBP5[c]	pBP6[a]	pBP7[c]	pBP8[d]	pBP9[d]	pBP10[b]
A	10.8	11.2	10.8	11.1	11.5	10.8	11.1	10.5	10.8	10.6
B	7.6	7.8	7.2[e]	7.7	8.1[e]	7.7[e]	8.0[e]	7.5	7.2[e]	7.5
C	7.2	7.0		7.2				7.0		7.1
D	4.9	4.8	4.7	4.7	4.8	4.8	4.7	4.7	4.7	4.7
E	4.3	4.3	4.3	4.3	4.4	4.3	4.3	4.3	4.2	4.3
F	3.9	3.8	3.8	3.8	3.9	3.9	3.9	3.8	3.8	3.8
G	2.8	2.7	2.7	2.7	2.7	2.7	2.7	2.6	2.7	2.6
H	2.5	2.4	2.5	2.6	2.6	2.6	2.4	2.5	2.5	2.5
I	2.2	2.1	2.2	2.3	2.3	2.3	2.2	2.2	2.2	2.2
J	1.1[f]	1.0[f]	1.1[f]	1.2[g]	1.2[f]	1.2[g]	1.1[f]	1.3[g]	1.2[g]	1.2[g]
K	0.9	0.7	0.8	0.9	1.0	0.9	0.8	1.0	1.0	0.9
L	0.7	0.5	0.7	0.7	0.7	0.7	0.6	0.8	0.8	0.8
M	0.5	0.4	0.5	0.6	0.6	0.6	0.5	0.7	0.6	0.6
N	0.4	0.3	0.4	0.5	0.4	0.5	0.4	0.5	0.5	0.5
O	0.3	0.2	0.3	0.4	0.3	0.4	0.3	0.4	0.4	0.4

[a] The molecular weights of the fragments are the averages of two gels; λ fragments co-electrophoresed in one gel, and plasmid pBP4 fragments in a parallel gel were used as the standards.

[b] As in footnote *a*, but three gels were run and λ fragments served as the molecular weight standards.

[c] As in footnote *b*, but only two gels were run.

[d] As in footnote *b*, but only one gel was run.

[e] Fragments B and C were not resolved into two distinct peaks under the electrophoresis conditions employed. The presence of both fragments in one peak is based on the sum of the counts per minute and the lack of symmetry.

[f] Restriction endonuclease fragments separated on 1.4% agarose gels, with the size estimates based on the mobility of fragments D through I.

[g] As in footnote *f*, but size estimates were based on the mobility of λ DNA fragments.

TABLE 3. *Antibiotic susceptibility patterns of five coliforms isolated from the same patient*

Bacterium	Culture source	Susceptibility pattern[a]					
		Gm	Tm	Km	Ap	Cp	Cm
E. cloacae	Sputum[b]	R	R	R	R	R	R
P. morganii	Sputum	R	R	R	R	R	R
K. pneumoniae type 23	Sputum	S	S	S	S	S	S
P. morganii	Rectum[c]	R	R	R	R	R	R
K. pneumoniae type 23	Rectum	R	R	R	R	R	R

[a] Abbreviations: Gm, gentamicin; Tm, tobramycin; Km, kanamycin; Ap, ampicillin; Cp, cephalothin; Cm, chloramphenicol; S, susceptible; R, resistant.

[b] Sputum specimen taken on 3/12/76.

[c] Rectal specimen taken on 3/11/76.

TABLE 4. *In vitro transmission of R plasmids between clinical strains isolated from a single patient*

Donor	Transfer to the following recipients:	
	K. pneumoniae type 23	*E. coli*
K. pneumoniae type 23	+[a]	+
P. morganii	+	+
E. cloacae	+	+

[a] +, Gentamicin, tobramycin, and kanamycin resistance was transferred to the recipient by conjugation.

pneumoniae (type 30) infections of epidemic proportion, which lasted for almost 1 year (10a). Whereas the epidemic period eventually subsided after the strict enforcement of isolation practices, gram-negative organisms with a similar antibiotic resistance pattern, including *Klebsiella* of differing serotypes and other genera of the *Enterobacteriaceae*, continued to be isolated from patients with various infectious disease problems for at least 2 years.

Although attempts have been made to define the epidemiology of antibiotic resistance coded by conjugal R plasmids, most studies have depended on unreliable phenotypic markers to es-

tablish the relatedness of such plasmids (31). With the increasing difficulties these molecules present in the control of infectious disease, unambiguous epidemiological data concerning their spread and persistence in human populations become highly desirable.

The thoroughly documented outbreak described above provided a collection of strains which facilitated an in depth investigation into the role of R plasmids in hospital-acquired infections (10a). Our goals were to determine whether plasmid transmission between various species was significant in the dissemination of antibiotic resistance and whether a plasmid, once introduced into a given hospital, becomes an entrenched member of the gene pool of the microbial flora of hospital residents.

This report represents the first detailed in depth molecular characterization of R plasmids involved in a single epidemic, a necessity if one is to conclude that one or more plasmid molecules are identical and therefore epidemiologically related. In a previous study concerning an epidemic of carbenicillin resistance in a burn ward, Ingram et al. utilized DNA-DNA hybridization to establish the identity of the R plasmids (14). This approach is open to question because rearrangements of DNA sequences due to translocation, inversions, and recombination are not detected. The reality of this criticism was shown by the recent study of Heffron et al, which detected by heteroduplex mapping rearrangements in plasmids of clinical origin (12). Their observations indicated that the methodology used to establish the identity of plasmids must be sensitive to topographical changes in DNA sequences.

The discovery of restriction endonucleases has provided a technique which is technically simple and overcomes the limitations of nucleic acid hybridization and genetic analysis (25). These enzymes have been shown to be particularly useful for the analysis and identification of plasmid DNA (37). Thus, plasmids which are known to be very closely related have been readily differentiated by using restriction endonucleases and agarose electrophoresis (37). However, this technique has seen very limited use in the examination of R plasmids of clinical significance (9, 11). Elwell et al. have employed restriction endonucleases to show identity between two plasmids isolated from two different enteric bacteria isolated from burn patients (9). In a more extensive study, Grinter and Barth compared the restriction endonuclease patterns of 12 epidemiologically unrelated plasmids specifying resistance to streptomycin and sulfonamide (11).

The 10 strains included in this study were isolated from various patients over a span of 7 months and included *K. pneumoniae* of various serotypes, *E. coli, E. cloacae,* and *P. morganii.* Plasmid DNA from *E. coli* transconjugants of each strain was purified, and a molecular weight of 58×10^6 was determined for each plasmid.

An analysis of the *Eco*RI restriction endonuclease fragments of plasmid pBP4, which had originated in a strain of *K. pneumoniae* type 30 isolated at the peak of the epidemic in September 1975 (Table 1), revealed 15 fragments ranging from 0.4 to 11 megadaltons in size. Although type 30 organisms were the predominant pathogen, an identical plasmid, pBP1, was also harbored by a nontypable *K. pneumoniae* strain isolated from a different patient. This and the discovery that other strains carried a pBP4-like plasmid indicate that the plasmid had already taken up residence in more than one species by the peak of the epidemic. Eight other plasmids also produced the same fingerprint upon digestion with *Eco*RI. These came from strains representing various species isolated from September 1975 to March 1976. The conclusion that these plasmids are identical and most likely originated from a common source is based on the similar pattern and number of DNA fragments obtained after restriction endonuclease digestion. This sensitive and relatively simple technique (37) cannot rule out the occurrence of minor changes confined to individual DNA fragments; but to do this unequivocally would require the sequencing of each plasmid.

Cultures from one individual yielded three genera of *Enterobacteriaceae,* and all harbored the same plasmid that resided in the original *K. pneumoniae* type 30. These findings unequivocally confirm that the plasmid had extended its host range during the period studied, and this patient may well represent a situation which is duplicated many times in a large hospital. It came as a surprise to us that a plasmid of this size, which was apparently actively engaging in promiscuous conjugation in the intestines of residents at this hospital, would persist for at least 7 months in its initial molecular state. One rare isolate was an exception. This strain was chloramphenicol susceptible, in contrast to the epidemic strains, and when the purified plasmid from this strain was compared with pBP4, it was found to be a dissociated form of the larger epidemic molecule (B. Peterson, unpublished data).

As mentioned above, the precipitating event in this epidemic is thought to have been the admission of a patient carrying a *K. pneumoniae* strain with a characteristic antibiogram. The available data do not conclusively establish

whether the R plasmid described in this study was introduced by this index case or whether a preexisting reservoir provided this R plasmid to a highly invasive strain of *Klebsiella*. The latter seems unlikely because no gentamicin-resistant *Klebsiella* strains or organisms with this extensively complex antibiogram had been isolated before the index case.

In contradiction to the belief that increased use or overuse of a given antibiotic is responsible for such an outbreak, review of pharmacy records revealed no major changes in the use of antibiotics at the MVAH during the 1-year period preceding the epidemic. No changes in the selective pressures were observed, yet the introduction of this R plasmid resulted in the epidemic spread of gentamicin-resistant bacteria. These events suggest that the careful use of antibiotics in one hospital to minimize problems with multiple-antibiotic-resistant microorganisms can be readily undone by the introduction of a complex conjugative R plasmid. Efforts to halt such outbreaks have often involved the restricted use of a given antibiotic (32). These results question the feasibility of this approach in this particular situation since the R plasmid analyzed in this study was under the selective pressure of eight antibiotics. The use of individual antibiotics varied widely at the MVAH, yet, surprisingly, the R plasmid persisted intact for months with few molecular changes.

Epidemics of antibiotic-resistant bacteria in hospitals can be controlled in most cases by isolating the infected individuals, restricting antibiotic usage, and enforcing proper sanitary practices. The problem that arises and awaits a solution is the elimination of reservoirs once an R plasmid becomes entrenched in a given hospital environment. Equally important is the prevention of such outbreaks by identifying the selective pressures both inside and outside the hospital setting which contribute to the rapid expansion of the R-plasmid gene pool.

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