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Endemic Gentamicin Resistance R Factors on a Spinal Cord Injury Unit

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Cleared lysates of gentamicin-resistant, gram-negative bacilli obtained during a prevalence survey and a subsequent prospective study on a spinal cord injury unit were analyzed. Of 105 strains obtained during the epidemiological study, 62 were analyzed for plasmid content. None of the 14 *Acinetobacter* strains carried plasmids. Of 20 strains from the initial prevalence survey, 9 carried a 36- or (in two cases) a 27-megadalton plasmid. Eight of the nine were *Providencia* strains; none were *Pseudomonas* strains. Of 28 nosocomial isolates obtained during the prospective survey, 22 carried plasmids of similar molecular weight ($P < 0.025$, compared with the prevalence survey), including 20 of 22 isolates of members of the family *Enterobacteriaceae* and 2 of 6 *Pseudomonas aeruginosa* isolates. Conjugation, curing, and transformation indicate that these plasmids carry gentamicin, tobramycin, kanamycin, ampicillin, carbenicillin, cephalothin, and, variably, chloramphenicol resistance. Restriction endonuclease digestion of purified plasmid DNA suggests that the plasmids from multiple species of the family *Enterobacteriaceae* contain common sequences, whereas those from *Pseudomonas* spp. do not. This study suggests that an endemic conjugal 36-megadalton gentamicin resistance R factor exists in many nosocomial species of the family *Enterobacteriaceae*.

We recently completed a prospective study of the epidemiology of gentamicin-resistant (Gent^r), gram-negative bacilli (GNB) on our spinal cord injury unit (SCIU) (13). On the basis of this work, we could divide patients into three groups: those colonized at the time of the initial prevalence survey (population A), those colonized on admission to the unit (population B), and those acquiring nosocomial colonization (population C). Population C included those patients in whom new organisms appeared on or after culturing day 2 (1 week after the initial culture) or in whom previously isolated organisms appeared at a new site on or after culturing day 2. Our data indicated an endemic level of nosocomial Gent^r GNB on our SCIU. This could not be easily explained by cross colonization or excessive use of antibiotics, but appeared to be most closely related to the length of stay on the unit (13). The Gent^r GNB recovered included *Providencia* spp., *Serratia* spp., *Escherichia coli, Klebsiella pneumoniae, Morganella morganii, Citrobacter amalonaticus, Proteus mirabilis, Pseudomonas* spp., *Acinetobacter calcoaceticus*, and other species. *Providencia* strains were associated with population A as opposed to B or C ($P = 0.017$ and 0.09, respectively). *Serratia* strains were associated with population C as opposed to A ($P = 0.016$) (13).

Other workers have studied the epidemiology of plasmids involved in outbreaks due to a given species or to an endemic problem with a specific organism (3, 7, 8, 11). There are few studies of the involvement of R factors in an endemic situation involving multiple species (10, 11, 13). Because we suspected that our endemic level of nosocomial Gent^r GNB could be explained in part by the dissemination of an R factor carrying gentamicin resistance among multiple species, we studied the SCIU isolates obtained during our prevalence survey and subsequent prospective study.

MATERIALS AND METHODS

Preparation of cleared lysates and agarose gel electrophoresis. We use a method obtained from L. Tompkins (personal communication and reference 14). All pH measurements for the buffers listed in this procedure should be performed using a sodium-insensitive electrode. Two milliliters of Trypticase soy broth (BBL Microbiology Systems) with 10 μg of gentamicin per ml added is inoculated with a few colonies of the test organism and incubated at 35°C overnight. The cells are washed once in TE (0.05 M Tris, 0.01 M EDTA, pH 8), suspended in 40 μl of the same buffer, and

vortexed. A 0.6-ml volume of lysis buffer (4% sodium dodecyl sulfate in TE, pH 12.4) is added to a 1.5-ml Eppendorf centrifuge tube, and the suspended cells are added to the lysis buffer. The suspension is mixed by inversion and incubated at 37°C for 20 min. Thirty microliters of 2 M Tris (pH 7) is added, and the tube is inverted until a change in viscosity is noted. The pH of the suspension should be 7 to 9. Then, 0.16 ml of 5 M NaCl is added, and the suspension is mixed by inversion and placed on ice for 1 h. The precipitate is sedimented at 11,700 × *g* for 5 min, and the supernatant is poured into a second Eppendorf tube; 0.55 ml of ice-cold isopropanol is added, and the mixture is inverted several times. The suspension is frozen solid for at least 30 min in dry ice. The tube is then allowed to begin to thaw, and the precipitate is collected by centrifugation at 11,700 × *g* for 3 min. The supernatant is poured off, and the precipitate is dried in a vacuum desiccator for 30 min. The precipitate is then suspended in 30 μl of TES (0.03 M Tris-hydrochloride, 0.005 M EDTA, 0.05 M NaCl, pH 8), and 10 μl of stop mix (0.07% bromphenol blue–7% sodium dodecyl sulfate–33% glycerol in water) is added. A sample is run on a 0.7% agarose gel (11 by 14 cm) with a submersible horizontal gel electrophoresis system (Bethesda Research Laboratories). We use a Tris-acetate (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 8.3) buffer system. For visualization of the 36-megadalton (Md) plasmids, overnight runs at 25 V are best and were used routinely. For restriction endonuclease digestion fragments, 3- to 4-h runs at 100 to 120 V are best and were used routinely. An 0.8-μg amount of ethidium bromide per ml is added to each gel, and each gel is restained with 0.8 μg of ethidium bromide per ml in water for 45 min and then destained for 20 min in distilled water before photography. A UV transilluminator, a Polaroid camera with type 57 film, and a Wratten no. 8 filter and a Tiffen no. 85 filter were used for photography.

Purification of plasmid DNA with CsCl gradients. Cleared lysates were obtained using a scaled-up procedure as described above, accommodating a 250-ml culture volume. CsCl gradients were prepared and centrifuged at 115,000 × *g* at 15°C for 48 h. Bands were visualized by visible or shortwave UV light if necessary, and the lower band(s) were removed with a 1-ml syringe and an 18-gauge needle. Ethidium bromide was removed by multiple extractions with CsCl-water-saturated 100% isopropanol. CsCl was removed by dialysis against buffer containing TES.

Conjugation. For conjugation experiments, donors were grown in Trypticase soy broth supplemented with 10 μg of gentamicin per ml, and recipients were grown similarly without antibiotics. The overnight cultures were diluted 1:100 in the morning and grown to the mid-log to late log phase in the absence of antibiotics. A total of 5×10^7 cells of each type was placed on a 25-mm-diameter, 0.45-μm membrane filter (Millipore Corp.), and the filter was placed on a Trypticase soy agar plate. The plate was incubated at 35°C overnight, and the cells were harvested from the filter in 1 ml of sterile saline by vigorous vortexing. The putative transconjugants were then plated on MacConkey agar supplemented with appropriate antibiotics. The standard *E. coli* C600 Nal^r Rif^r strain and a cured *Serratia* strain from which a Nal^r Rif^r derivative was selected were used as recipients. Donors which were Nal^s Rif^s Gent^r were used. Under these conditions, transfer of tetracycline resistance from a *Pseudomonas aeruginosa* RP1 donor occurred with a frequency of 10^{-4} per recipient.

Curing. Curing was carried out by determining minimal inhibitory concentrations, using serial twofold dilutions of ethidium bromide in Mueller-Hinton broth and plating survivors of the last tube in which turbidity was visible on nonselective agar. The survivors were replica plated to agar supplemented with 20 μg of gentamicin per ml to look for a loss of gentamicin resistance.

Transformation. CsCl gradient-purified plasmid DNA was obtained as described above and used to transform the C600 recipient. An overnight culture was diluted 1:50 in fresh LB (10 g of tryptone–5 g of yeast extract–5 g of NaCl per liter supplemented with 0.001% thiamine) broth (20 ml in a 250-ml flask) at 37°C. Cells were collected after 3 h of growth in a shaking water bath and sedimented at 3,000 × *g* for 5 min at 2°C. They were suspended in 10 ml of 50 mM $CaCl_2$ for 30 min at 0°C. They were again sedimented at 3,000 × *g* at 2°C and resuspended in 0.5 ml of 50 mM $CaCl_2$ for 30 min at 0°C. A 0.1-ml volume of cells was added to DNA in 0.1 ml of TES for 120 min at 0°C. The mixture was then heated for 2 min at 37°C, followed by 10 min at room temperature. One milliliter of LB broth was added, and the mixture was incubated at 37°C for at least 2 h; 2.5 ml of LB soft agar without antibiotics was added and poured on LB plates containing 20 μg of gentamicin per ml. This procedure yielded approximately 10^3 tetracycline-resistant transformants per μg of pBR322 DNA.

Restriction endonuclease digestion. Enzymes were obtained from Bethesda Research Laboratories and used according to the instructions of the manufacturer.

Epidemiological and microbiological procedures. The methods of patient selection, definition of cross infection, methods for microbial identification, and minimal inhibitory concentration testing have all been described previously (13).

RESULTS

Plasmid content of Gent^r GNB. Figure 1 illustrates the results of agarose gel electrophoresis of cleared lysates of Gent^r GNB from the SCIU. Although some organisms had additional smaller (1 to 6 Md in size) plasmids and some carried only these small plasmids, most strains had a 36-Md plasmid. Two *P. mirabilis* strains obtained from the same patient carried 27-Md plasmids. Plasmid DNA was not detected in any of the 14 *Acinetobacter* strains studied. Excluding *Acinetobacter* strains, 9 of 20 (45%) strains from the initial prevalence survey carried 36-Md plasmids. Eight of these nine (89%) were *Providencia* spp. None of seven *P. aeruginosa* strains from the initial survey carried a 36-Md plasmid. Of 28 nosocomial Gent^r GNB isolates, 22 (79%) carried plasmids of similar molecular weight ($P < 0.025$, compared with the prevalence survey by Fisher's exact test). This included 20 of 22 isolates of members of the family *Enterobacteriaceae* and 2 of 6 *P. aeruginosa* isolates.

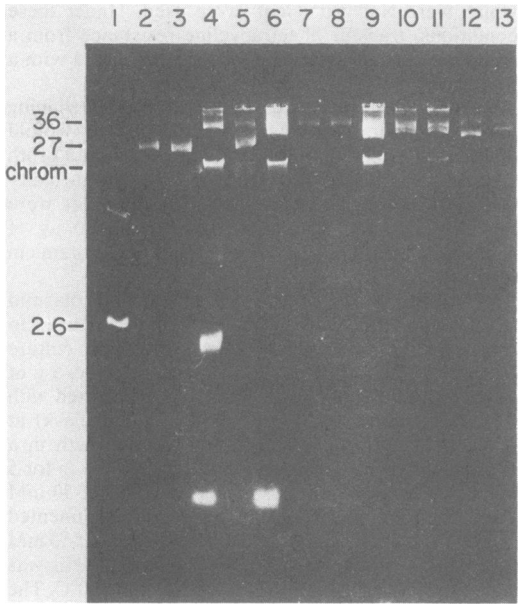

FIG. 1. Agarose gel electrophoresis of CsCl gradient-purified gentamicin resistance plasmids. Lane 1, pBR322, 2.6-Md plasmid; lanes 2 and 3, patient A, *P. mirabilis*, 27-Md plasmid; lane 4, patient E, *M. morganii*, 36-Md plasmid; lane 5, patient D, *Providencia stuartii*, 36-Md plasmid; lane 6, patient A, *S. marcescens*, 36-Md plasmid; lane 7, patient B, *S. marcescens*, 36-Md plasmid; lane 8, patient B, *S. liquefaciens*, 36-Md plasmid; lane 9, patient B, *E. coli*, 36-Md plasmid; lane 10, patient C, *E. coli*, 36- and 20-Md plasmids; lane 11, patient C, API group I, 36-Md plasmid; lane 12, patient F, *P. aeruginosa*, 36-Md plasmid; lane 13, patient G, *P. aeruginosa*, 36-Md plasmid. chrom, Chromosomal DNA.

All eight *Serratia* isolates, including seven nosocomial isolates and one from population B, carried the 36-Md plasmid. Since the *Providencia* strains were statistically associated with the initial survey population as opposed to the nosocomial population, this organism may have served as a reservoir for the spread of the plasmid to nosocomial GNB, especially *Serratia* strains.

We also noted that 5 of 10 organisms obtained from eight patients who were colonized on admission carried 36-Md plasmids. On review, two of the patients with plasmid-bearing organisms had been on the ward 4 and 6 days, respectively, before the first cultures were obtained. The other two patients with plasmid-bearing GNB were both cultured the day of arrival on the ward, but they had been transferred from other hospital areas after lengthy stays. One of these patients, transferred from a medical ward, was colonized with a 36-Md plasmid-bearing *Serratia liquefaciens* isolate. Since the patient had been transferred 3 weeks after the first nosocomial plasmid-bearing *Serratia* sp. was isolated on the SCIU, the plasmid could not have been introduced by this patient. This suggests that this size class of plasmid may be associated with Gent^r GNB throughout the hospital.

Genetic analysis of Gent^r GNB. An analysis of the minimal inhibitory concentration for Gent^r GNB suggested that the 36-Md plasmid was associated with resistance to tobramycin and kanamycin. Both plasmid-bearing *P. aeruginosa* strains were resistant to tobramycin and kanamycin, whereas some non-plasmid-bearing strains were susceptible to tobramycin. Curing experiments performed with strains of *Serratia* which were also resistant to amikacin and all other common antimicrobial agents tested confirmed this association. Other plasmid-linked markers could not be assessed because of the multiple resistance that was not associated with the 36-Md plasmid. Cured *Serratia* strains maintained their amikacin resistance (data not shown). Conjugation experiments in which an *E. coli* C600 recipient was used resulted in a frequency of transfer of gentamicin resistance of 10^{-7} and only occurred in the presence of a second 20-Md plasmid of unknown function in a multiply resistant *E. coli* donor. However, transfer of gentamicin resistance proceeded at a frequency of 10^{-4} when we used a Nal^r Rif^r derivative of the cured *Serratia* strains noted above and a plasmid-bearing *Serratia* donor. These experiments also confirmed the linkage of gentamicin, kanamycin, and tobramycin resistance with the 36-Md plasmid. Transformation yielded the results shown in Table 1, which again confirmed the previous findings and established linkage with ampicillin, carbenicillin, cephalothin, kanamycin, tobramycin, and, variably, chloramphenicol resistance.

Restriction endonuclease analysis. Figure 2 shows the *Hin*dIII and *Pst*I digestion patterns of a number of Gent^r plasmids from the SCIU. The *Pst*I digests show similar results with one or more large, common fragments among all plasmids tested. Specifically, four of the five plasmids derived from patients A and B (Fig. 2A) share a common 8.2- to 8.4-Md fragment. The plasmids of the two *Serratia* strains from patient B appear to be identical, but both are somewhat different from the other plasmids on the gel. The plasmid from the *Serratia marcescens* isolate of patient A appears to be identical to that from an *E. coli* isolate of patient B. The fifth plasmid shown, derived from an *E. coli* isolate colonizing patient C, shares a common 5.4-Md fragment with the first and fourth plasmids illustrated. The *Hin*dIII digests confirm the general similarity of these plasmids. All nine plasmids pictured in Fig. 2B share a common 15- to 16-Md frag-

TABLE 1. Phenotypes of Gent^r parents and transformants

Strain[a]	Minimal inhibitory concn (μg/ml)[b]									
	AM	CA	CE	FOX	MAN	AK	KA	TO	CAM	TE
C600	8[c]	64	8	1	8	4	4	1	4	2
048	>32	>512	>128	128	>128	>32	>64	>16	>64	16
C600:048	>32	>512	32	16	16	1	>64	16	>64	0.25
058	>32	>512	>128	128	>128	8	>64	>16	>64	8
C600:058	>32	>512	64	8	32	4	>64	>16	>64	1
121	>32	>512	16	8	4	2	>64	>16	4	>32
C600:121	>32	>512	8	2	2	2	64	>16	4	0.25
122	>32	>512	16	4	2	2	>64	>16	4	>32
C600:122	>32	>512	64	16	16	2	>64	>16	4	1

[a] Parents are three-number strains, transformants are C600 derivatives. 048, *S. marcescens*; 058, *S. liquefaciens*; 121, *E. coli*; 122, API group I.

[b] Abbreviations: AM, ampicillin; CA, carbenicillin; CE, cephalothin; FOX, cefoxitin; MAN, cefamandole; AK, amikacin; KA, kanamycin; TO, tobramycin; CAM, chloramphenicol; TE, tetracycline.

[c] Determined using microtiter trays (13).

ment. Again, the two plasmids from two *Serratia* species from patient B are more similar to each other than to other plasmids shown. Again, the plasmid from the *Serratia* isolate of patient A and the plasmid from the *E. coli* isolate of patient B appear to be identical. *Hin*dIII digestion of purified 36-Md plasmid DNA obtained from two strains of *P. aeruginosa* (data not shown) indicated that these plasmids are similar to each other, but quite different from the plasmids found in members of the family *Enterobacteriaceae*.

DISCUSSION

Our prospective study of the epidemiology of Gent^r GNB on our SCIU suggested that the spread of these organisms could not be entirely explained by cross colonization or antibiotic use. Only length of stay on the unit was correlated with nosocomial colonization (13). We suspected that one possible explanation was the occurrence of occult cross colonization with subsequent transfer of a plasmid to endogenous flora, resulting in a large number of resistant species.

Most other studies of the epidemiology of plasmid-mediated antimicrobial resistance have been performed in the setting of specific outbreaks usually due to a single species (3, 7, 8, 11). Although some workers have noted the dissemination of plasmids to species other than the epidemic organism, this has been limited to a small number of isolates (10, 11, 14). Since these studies have been of specific epidemic situations, the failure to see plasmid dissemination could be explained by the inherent bias of such a study design. Our work represents an examination of gentamicin resistance based on an endemic level of resistant organisms representing many members of the genus *Pseudomonas*, the genus *Acinetobacter*, and the family *Enterobacteriaceae*. Since the epidemiological study was prospective and specifically designed to identify Gent^r GNB, the spread of resistance determinants among many species could be appreciated.

On the basis of a comparison of the minimal inhibitory concentrations of the drugs for plasmid-bearing and non-plasmid-bearing GNB, as well as conjugation, curing, and transformation experiments, resistance to gentamicin, kanamycin, tobramycin, ampicillin, carbenicillin, cephalothin, and, variably, chloramphenicol is linked to the 36-Md plasmid.

Providencia strains were associated with the initial survey population, and *Serratia* strains were associated with the nosocomial population (13). It is tempting to speculate that the *Providencia* strains served as a reservoir for the plasmid, which was then transferred to other species serving as endogenous flora in the spinal cord injury patients. *Serratia* strains may have been emphasized in the nosocomial population because they may be more pathogenic. Such a mechanism for plasmid spread and evolution has recently been proposed by Schaberg and coworkers (12).

Although the restriction endonuclease digestion patterns indicate that these plasmids are not always identical, they appear to share large areas of sequence similarity. This includes the 27-Md plasmids from *P. mirabilis*. Even strains isolated from the same site or from different sites in a single patient may carry plasmids that are not identical. Two different patients carried an

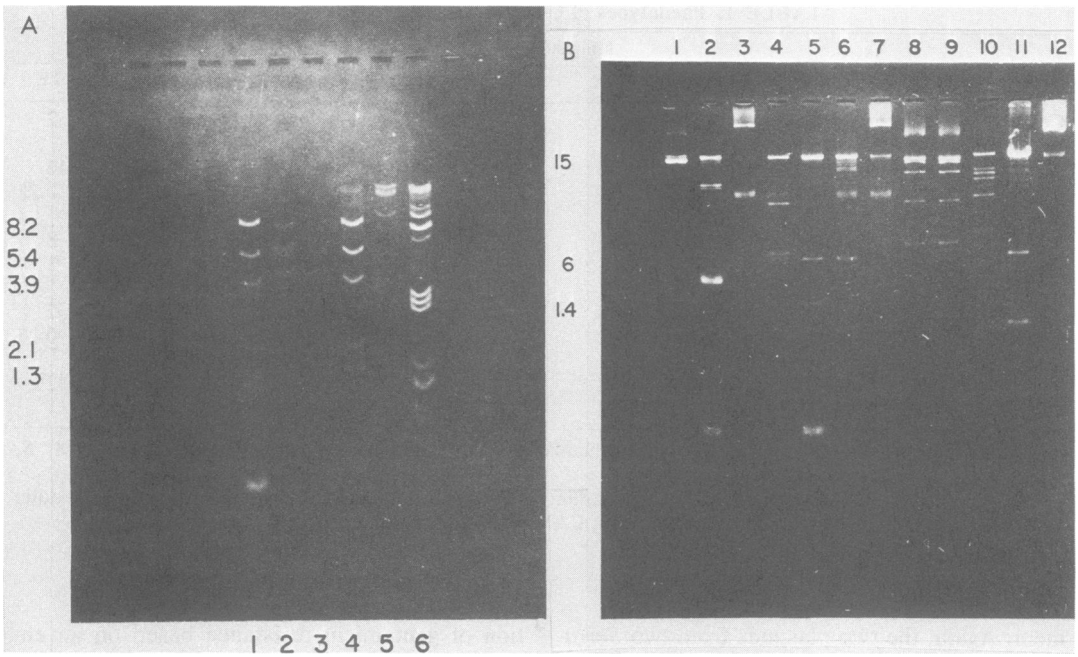

FIG. 2. Restriction endonuclease digestion of gentamicin resistance plasmids. Molecular weight markers are indicated at the left of each gel. (A) *Pst*I restriction endonuclease digestion fragments of: lane 1, patient A, *S. marcescens*, 36-Md plasmid; lane 2, patient B, *S. marcescens*, 36-Md plasmid; lane 3, patient B, *S. liquefaciens*, 36-Md plasmid; lane 4, patient B, *E. coli*, 36-Md plasmid; lane 5, patient C, *E. coli*, 36-Md plasmid; lane 6, lambda bacteriophage DNA control. (B) *Hin*dIII restriction endonuclease digestion fragments of: lane 1, patient D, *Providencia stuartii*, 36-Md plasmid; lane 2, patient E, *M. morganii*, 36-Md plasmid; lane 3, patient B, *S. liquefaciens*, undigested; lane 4, patient B, *S. liquefaciens*, *Hin*dIII digested; lane 5, patient A, *S. marcescens*, 36-Md plasmid; lane 6, patient B, *S. marcescens*, 36-Md plasmid; lane 7, patient B, *S. marcescens*, undigested; lanes 8 and 9, patient A, *P. mirabilis*, two separate isolates, 36-Md plasmids; lane 10, patient C, *E. coli*, 36-Md plasmid; lane 11, patient B, *E. coli*, 36-Md plasmid; lane 12, patient B, *E. coli*, undigested.

S. marcescens isolate and an *E. coli* isolate, respectively, with identical plasmids. We believe that the most likely explanation for these findings is occult cross colonization by a plasmid-bearing strain with dissemination to endogenous flora. There is ample precedent for this occurrence in situ in both clinical and experimental situations (1, 2, 4–6, 9). On our SCIU, this may have occurred in the absence of specific selective antibiotic pressure. Upon transmission, there may be molecular rearrangement of the plasmid within the new host strain. Thus, although the size class of the plasmid may be retained, the restriction endonuclease digestion pattern may show some differences. An alternative explanation is the existence of a variety of plasmids of a similar size class carrying aminoglycoside resistance determinants, with recombination accounting for the many similarities observed at the molecular level. We are in the process of attempting to make this distinction by using Southern blots.

Our observations have profound implications for the control of the spread of aminoglycoside resistance within our SCIU. Instead of using barrier precautions only for patients colonized with an epidemic species, we would have to establish such isolation for every patient colonized with a Gent^r GNB isolate. Since up to 35% of all GNB isolated on our SCIU are Gent^r, this solution is impractical.

In conclusion, this study suggests the spread of plasmids of a similar size class carrying resistance to gentamicin, kanamycin, tobramycin, ampicillin, carbenicillin, cephalothin, and, variably, chloramphenicol among many species of the family *Enterobacteriaceae* on a SCIU. The data indicate the possibility of molecular rearrangement of the plasmid on transfer.

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