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# Dissemination of an Antibiotic Resistance Plasmid in Hospital Patient Flora

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The 2″ aminoglycoside nucleotidyltransferase, AAD (2″), which adenylates gentamicin, tobramycin, and kanamycin, became prevalent over several months in multiple strains and species of *Enterobacteriaceae* isolated at one hospital. Eight plasmids with the gene for this enzyme purified from different strains and species isolated at different times had similar *Eco*RI digestion fragments, indicating that the gene had disseminated on one plasmid without transposition. This 56.5-megadalton plasmid of incompatibility group M, which also carried three other resistance genes, spread, at first, largely in one strain of *Klebsiella pneumoniae*, which later disappeared. It transferred to some strains which tended not to colonize other patients and later circulated predominantly in *Serratia marcescens*. Computer surveillance of routine hospital laboratory results was able to detect and trace the gene and the plasmid and measure their effect on resistance prevalence.

An antibiotic resistance gene may transpose from one plasmid to another, may transfer on a plasmid from one bacterial cell to another, or may remain in a bacterial cell which leaves one person and colonizes another (8). In clinical settings, however, it has proven difficult to observe movement of resistance genes relative to plasmids, bacteria, and people because it has been difficult to identify individual genes, plasmids, and strains of bacteria at the same time. We describe here an occasion on which such observations were possible. They show the dissemination of a single plasmid into multiple strains and species of *Enterobacteriaceae* isolated from many patients in one hospital.

## MATERIALS AND METHODS

The 330-bed general hospital was included in the International Survey of Antibiotic Resistance Prevalence, and the methods for susceptibility testing, computer filing, and analysis of results have been described previously (14, 16). Biotyping was performed by the API method (Analytab Products, Inc., Plainview, N.Y.). Serotyping was performed by the Center for Disease Control (Atlanta, Ga.). Plasmid molecular weight was determined by Alan Jacob. Aminoglycoside enzymes were identified by the method of Benveniste and Davies (1), and several of the earlier assays were performed by David I. Smith or Peter Kresel. $\beta$-lactamases were identified by isoelectric focusing in polyacrylamide gel developed with chromagenic cephalosporin (13). Methods for determining plasmid incompatibility have been described (3).

Plasmids were transferred to the *Escherichia coli* K-12 strain DR 25, an azide-resistant mutant of W3101. Plasmid DNA was isolated by the cleared-lysate method of Hansen and Olsen (10), modified for larger volumes, and further purified by CsCl-ethidium bromide centrifugation (20). Purified plasmid DNA was digested with *Eco*RI (New England Biolabs, Beverly, Mass.) in a reaction mixture containing 7 $\mu$g of deoxyribonucleic acid (DNA), 0.1 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.5), 0.05 M NaCl, 0.01 M $MgSO_4$, and 5 units of *Eco*RI in a total volume of 100 $\mu$l at 37°C for 1 h with enzyme re-added at 30 min. Digests were electrophoresed on agarose gel (21) and visualized by ethidium bromide staining. The ability of the gentamicin or ampicillin resistance genes of transconjugant 1 (Table 1) to transpose to the genome of phage $\lambda$ or the *Salmonella* phage P22 was tested as described by Kleckner et al. (11), with the plasmid also transferred to DB 6868, a plasmid-free derivative of *Salmonella typhimurium* LT2.

## RESULTS

Figure 1 shows the results of routine susceptibility testing to six antibacterial agents of all clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens* at the hospital in 1975 and 1976. Although gentamicin had been used in the hospital since 1970, strains of these or other *Enterobacteriaceae* resistant to gentamicin were rarely isolated through 1975. Very few of the 1975 isolates in Fig. 1 have zones of inhibition around the gentamicin or tobramycin disks of less than 15-mm diameter. In 1976, however, there was an appreciable population of *K. pneu-*

TABLE 1. *Isolates selected for plasmid analysis*

| Mo of isolation | Source | Species | API biotype | *E. coli* K-12 transconjugant no. |
|---|---|---|---|---|
| 2/76 | Blood | *K. pneumoniae* | 5205773 | 1 |
| 6/76 | Blood | *S. marcescens* | 5317720 | 2 |
| 8/76 | Blood | *Enterobacter aerogenes* | 5305773 | 3 |
| 9/77 | Blood | *S. marcescens* | 5317761 | 4 |
| 10/77 | Blood | *K. pneumoniae* | 5215773 | 5 |
| 3/76 | Wound | *E. coli* | 5144552 | 6 |
| 4/77 | Wound | *Morganella morganii* | 0174000 | 7 |
| 4/76 | Urine | *C. freundii* | 1404572 | 8 |

*moniae* isolates with a peak of distribution at 15 mm, which constitutes a shoulder to the left of the susceptibility peak. Minimal inhibitory concentrations of gentamicin for representative isolates of this subpopulation were in the range of 8 to 32 μg/ml. Similar but more distinctly separated populations of *K. pneumoniae* that were moderately resistant to tobramycin and kanamycin appeared in 1976, as did *S. marcescens* populations resistant to gentamicin, kanamycin, and tobramycin. In 1976 there were also appreciable increases in the percentages of these isolates resistant to chloramphenicol and sulfonamides and in the percentage of *S. marcescens* isolates resistant to carbenicillin.

The isolates of *K. pneumoniae* with reduced zones of inhibition to gentamicin are shown in Fig. 2 to be the same ones which had reduced zones to tobramycin. Also, whereas fewer than a third of the other biotypes of *K. pneumoniae* had the reduced zones, virtually all that belonged to the biotype API 5205773, a biotype distinguished principally by a negative urease reaction, had them. Eight randomly chosen isolates of this biotype serotyped as type 2, whereas seven isolates chosen randomly from among five other biotypes were of five other serotypes.

The sequence of appearance of gentamicin-resistant *Enterobacteriaceae* is shown in Fig. 3. From February through May of 1976, 43 patients had gentamicin-resistant isolates of the single biotype of *K. pneumoniae* API 5205773. Thereafter, the rate of isolation of this biotype declined abruptly, and it was isolated from only two patients in all of 1977. During the four-month period, however, the rate of isolation of other gentamicin-resistant *Enterobacteriaceae* had increased sevenfold, and it remained elevated throughout 1977 although it has subsequently declined.

In all of 1976 and 1977, 372 patients had a total of 2,020 isolates of 49 different biotypes of gentamicin-resistant *Enterobacteriaceae,* 42% of them from urine, 23% from sputum, and 3% from blood. The patients were from all of the hospital's pavilions with 31% of them from the intensive care unit. Length of hospitalization at first isolation per patient, which averaged 6 days for other *Enterobacteriaceae,* averaged 24 for the resistant isolates, suggesting that their circulation was nosocomial (15). Of the 188 isolates of gentamicin-resistant *E. coli,* 149 came from patients who also had a gentamicin-resistant isolate of another species.

A total of 23 isolates of *Enterobacteriaceae* with the characteristically reduced zone diameters to gentamicin and tobramycin were tested for aminoglycoside-inactivating enzymes, and all were found to have the 2″ aminoglycoside nucleotidyltransferase, AAD (2″), which adenylates gentamicin, tobramycin, and kanamycin (6). *Enterobacteriaceae* resistant to gentamicin were usually also resistant to chloramphenicol (92%), sulfonamides (98%), and ampicillin and carbenicillin (94%), and resistance to all of these agents proved transferable to *E. coli* K-12 in mating experiments. The eight transconjugants listed in Table 1 had all acquired the TEM 1 β-lactamase. Plasmids of 19 of the isolates were grouped and belonged to incompatibility group M. Plasmid mass was 56.5 megadaltons. Isolates of biotype API 5205773 also had transferable resistance to tetracycline (77%) and to very high concentrations of streptomycin (83%) and kanamycin (83%), which was rare in other biotypes of *K. pneumoniae* and thus further confirmed the specificity of this biotype. Resistance to all of these agents segregated when isolates of API 5205773 were passaged in vitro, but the biotype remained unchanged.

Plasmids from eight of these gentamicin-resistant isolates which differed in species or biotype and month of isolation (Table 1) were each transferred in vitro to an *E. coli* K-12 and then extracted and digested with the *Eco*RI restriction endonuclease. The resulting DNA fragments were separated by agarose gel electrophoresis, as shown in Fig. 4. The fragments of each of the plasmids of transconjugants 2 through 7 of each appear to form 13 bands which have

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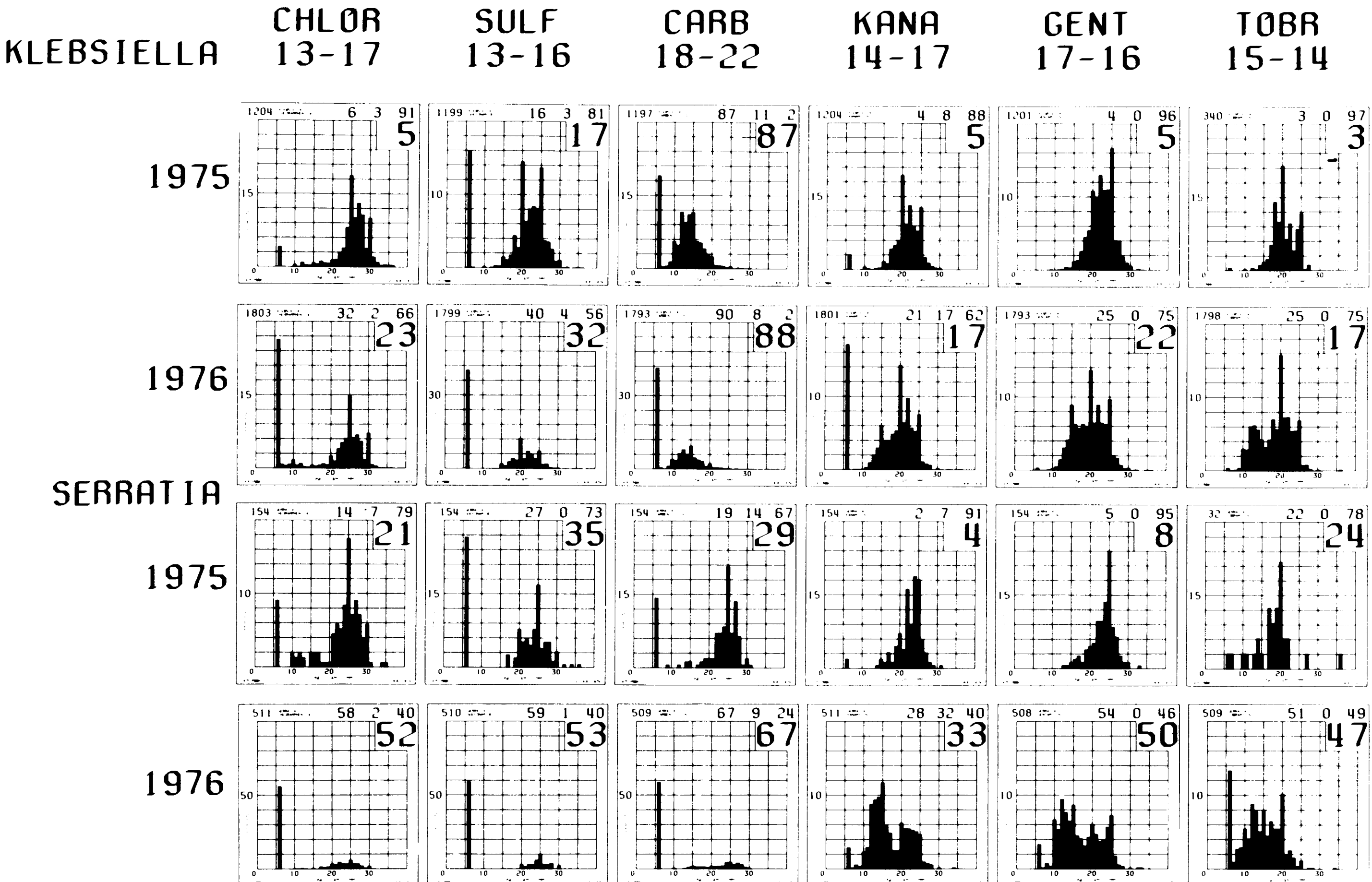


FIG. 1. *Computer-generated histograms of frequency distribution of diameters of zones of inhibition around susceptibility test disks. Vertical lines are at 5-mm intervals, and the horizontal scale (percent) shifts to accommodate data. Breakpoint limits of intermediate zone, here adjusted for thinner plates, are printed under antibacterial label. The large number in the upper right corner of each histogram is percent unrepeated isolates that are resistant.*

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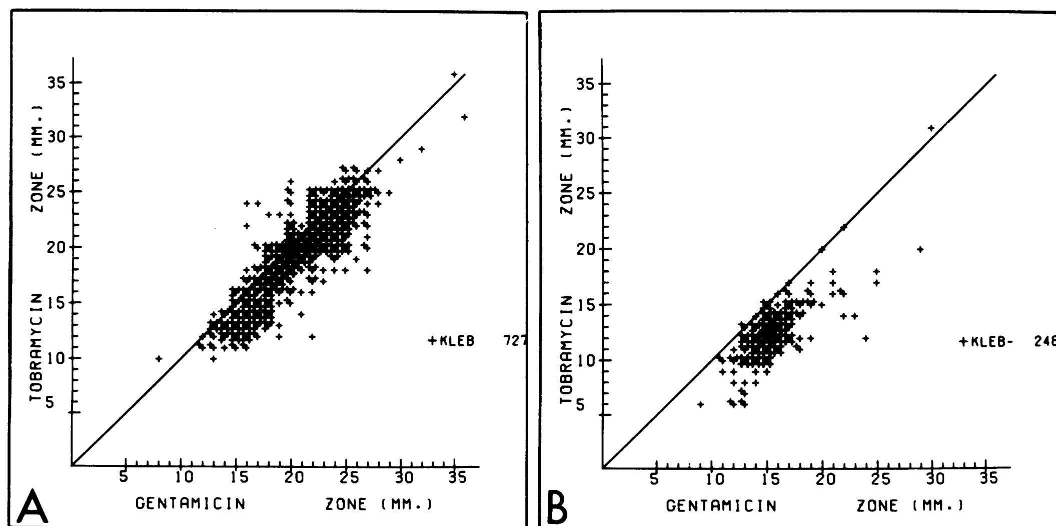


FIG. 2. *Computer-generated scatter plot of diameters of zones of inhibition around gentamicin and tobramycin disks for consecutive isolates (in 1976) of K. pneumoniae biotypes other than API 5205773 (A) and of only that biotype (B).*

corresponding positions in the gel. Transconjugant 1, which has a plasmid from one of the early isolates of API 5205773, has the same bands but has an additional large fragment band as well. Transconjugant 8, which has the plasmid from a *Citrobacter freundii* isolate carrying a tetracycline resistance gene not carried by the other isolates, has a similar additional large fragment band and is missing at least three of the smaller fragment bands which the others have.

## DISCUSSION

The abrupt emergence of gentamicin resistance observed here suggested spread of a single aminoglycoside resistance gene. This was further supported by the similar pattern of reduction of zones of inhibition around gentamicin, tobramycin, and kanamycin disks observed with all of the resistant isolates, and by the finding of the same aminoglycoside-inactivating enzyme in all that were tested. Incompatibility grouping suggested, and restriction endonuclease digestion analysis confirmed (7, 18), that the plasmids carrying the gene in different species were identical or nearly so and, by inference, that the genes were also.

Routine biotyping of all clinical isolates of *Enterobacteriaceae* showed that the plasmid spread at first mostly in one strain of *Klebsiella* with the biotype API 5205773. Later, this strain practically disappeared from the hospital, but by then the plasmid was in many other strains. The number of strains may be overestimated by the number of biotypes since the stability and reproducibility of each biotype has not been established as it has for API 5205773. Enough markers differ, however, to suggest multiple strains within most species, and at least six species were involved. In other hospitals gentamicin resistance has been observed to appear in multiple species as here (2, 5, 7, 9, 22, 23), or mostly in one species (17) or one strain (4, 19), suggesting that the extent of plasmid transfer may vary from outbreak to outbreak.

A previously rare resistance gene could become prevalent in multiple strains of *Enterobacteriaceae* in a hospital if each strain were introduced separately with individual patients admitted from a community where such strains had suddenly become widespread. Alternatively, the gene could have entered in one or a few strains and subsequently transferred to other strains circulating in hospital patient flora. Such transfer in hospital seems far more likely here. The gene was predominantly associated with species of bacteria that are commonly nosocomial and infrequently isolated from community flora. Four other hospitals in the area monitored by the same computer program had no concurrent increase in gentamicin-resistant isolates of *Enterobacteriaceae*. Patients had been hospitalized four times longer before having an isolate of *Enterobacteriaceae* with the gene than one without it. Moreover, while *E. coli* is the most commonly isolated of the *Enterobacteriaceae*, four-fifths of the isolates of *E. coli* with the gene were from patients who also had isolates of other species with the gene.

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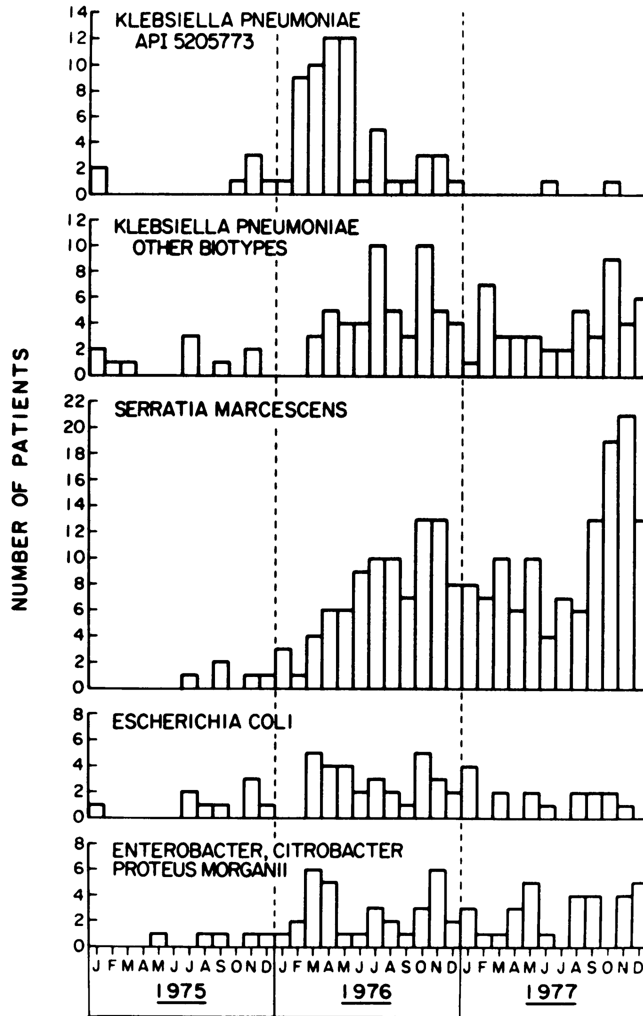


FIG. 3. *Number of patients in the hospital each month who had their first isolate of a species of gentamicin-resistant Enterobacteriaceae.*

Some strains which had the plasmid, e.g., those of *E. coli,* were usually isolated only from patients who also had other isolates with the plasmid. Epidemiologically, these strains may have been "blind-end" plasmid recipients, poor at colonizing other patients. The plasmid ultimately came to spread mostly in *S. marcescens,* which appeared to displace the *K. pneumoniae* API 5205773 in which it spread earlier. Plasmid transfer may thus eventually have produced an association of a needed resistance gene with the chromosomal traits best suited for survival and spread in the hospital. If so, there would be no further selection for transfer function, which appears to have been lost in the later phases of some other outbreaks (12, 17).

The absence from the digests of plasmids of six of the later isolates of a large band which was present in the digest of the plasmid from one of the earliest isolates of *K. pneumoniae* API 5205773 (transconjugant 1) may represent plasmid evolution during the outbreak. Whether a similar process could account for the greater differences in the plasmid from the *Citrobacter* isolate (transconjugant 8) is uncertain. In support of its being related are the bands it has in common with the other plasmids and the fact that it was isolated from a patient who also had gentamicin-resistant isolates of a number of other species, including several of API 5205773. It is of interest that Datta and her colleagues found that the plasmid which differed most from

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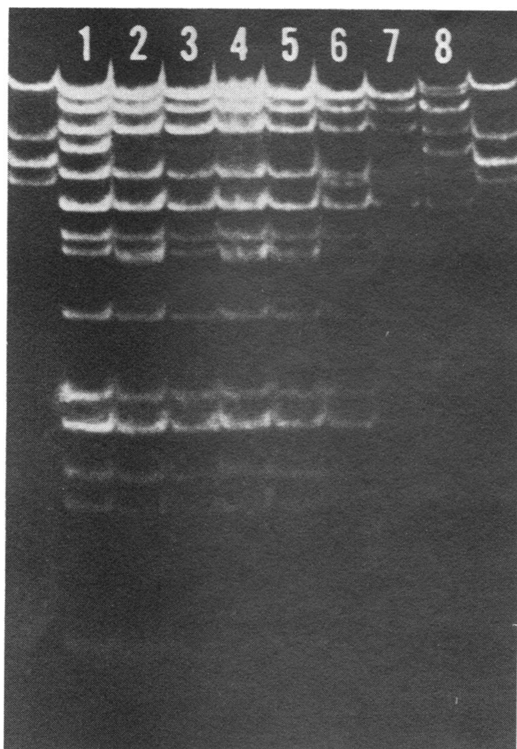


FIG. 4. *Agarose gel electrophoresis of EcoRI-digested fragments of plasmid DNA from the transconjugants described in Table 1, flanked on either side for reference by similar digests of λ phage.*

the others in the outbreak they studied was also from a *Citrobacter* (4), raising the question of host modification. There is no other evidence here that transposition may have contributed to the dissemination of the AAD (2″) gene, and it did not transpose experimentally, although the total number of both of these kinds of negative observation is small.

Although the previously rare AAD (2″) gene was the distinctive marker for this plasmid, it also carried genes for resistance to chloramphenicol, sulfonamides, and the TEM 1 β-lactamase gene. Since the latter two had been prevalent on other plasmids in the hospital and chloramphenicol, kanamycin, and tobramycin were seldom used there, gentamicin usage would seem to have been the most likely selective pressure for dissemination of the plasmid. Dissemination of the plasmid increased the prevalence of resistance to all of these agents and caused a more than threefold increase from 1976 to 1977 in the number of isolates of *Enterobacteriaceae* resistant to more than five antibacterial agents. The magnitude of this increase produced by one plasmid suggests that large differences between hospitals in prevalence of antibiotic resistance (14) might be accounted for by the circulation of a very few plasmids.

Restriction endonuclease analysis, mating experiments, and enzyme assay here confirmed dissemination of a plasmid after routine hospital laboratory results had already detected and traced it. Computer surveillance of clinical isolate files, including antibiotic inhibition zone measurements, could discriminate similar events in any hospital. Such observations in many hospitals might help to monitor and possibly to contain nosocomial or global spread of resistance genes (14, 16). These observations emphasize that resistance genes are too complex to arise often by chance mutation, so most strains of bacteria have to get them by transfer from other strains. Thus, the delivery of an antibiotic resistance gene through the world's bacterial flora to a strain exposed to that antibiotic may be as necessary for the emergence of resistance as is the antibiotic exposure.

The dissemination in patient flora of the plasmid described here is more extensive than that reported recently by Gerding (9) and Sadowski (18) and their colleagues, occurred initially in a different *K. pneumoniae* serotype (2 rather than 30), and ultimately became prevalent in another species, *S. marcescens*. The sequences of spread were similar, however, suggesting that there may be a stereotyped process through which a plasmid becomes disseminated in the patient flora of a hospital. Observation of additional plasmid incursions of this sort might further define the essentials of the process and suggest means for its prevention.

The plasmids involved in each of the outbreaks also seem similar. They are of approximately the same mass and appear to carry the same resistance genes. Moreover, although differences in method prevent direct comparison of the fragments, the two plasmids yield approximately the same number of fragments after *Eco*RI digestion. This raises the possibility that a resistance gene which spreads through the patient flora of a hospital on one plasmid may spread over a larger area or the whole world in the same way. It would be possible to detect and trace such a plasmid epidemic through computer surveillance of routine hospital laboratory files, as here, and confirm it with restriction endonuclease digestion analysis of plasmids selected by the surveillance.

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