AN R PLASMID OF BROAD HOST-RANGE, CODING FOR RESISTANCE TO NINE ANTIMICROBIAL AGENTS ENDEMIC IN GRAM-NEGATIVE NOSOCOMIAL ISOLATES

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PLATES XX–XXIII

SUMMARY. Of 3952 clinical isolates of Enterobacteriaceae, 246 exhibited resistance to at least carbenicillin, gentamicin and tobramycin. All these isolates, representing eight genera, were resistant to at least nine antimicrobial agents in common, including the three key antibiotics and streptomycin, kanamycin, sisomicin, ampicillin, cephalothin and sulphonamide. The strains could be subdivided into seven groups depending upon additional resistance traits and some were resistant to as many as 15 antibiotics. When mated with a standard strain of *Escherichia coli*, 85% of 123 randomly selected donors transferred resistance to at least the nine core antibiotics. Some donors occasionally transferred resistance to two additional antibiotics, neomycin and tetracycline, while one *Citrobacter freundii* donor always transferred linked resistance to all 11 drugs. Although many donors were found to harbour more than one species of plasmid DNA, all except a strain of *C. freundii* contained at least a plasmid of mol. wt 89×10^6 . Analysis of *E. coli* transconjugants showed this plasmid to be responsible for transferable resistance to the nine core antibiotics. Restriction-endonuclease analysis indicates that the 89×10^6 plasmids originating from different isolates were essentially identical with each other. These results show that a particular R plasmid has established itself among the Enterobacteriaceae at Hines VA Hospital. This R plasmid appears to be the predominant genetic element responsible for linked resistance to carbenicillin, gentamicin and tobramycin among these hospital-associated bacteria.

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In memoriam Hans G. Griebel, Chief of Infectious Disease, Hines V.A. Hospital, 21 February 1928–30 April 1980.

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INTRODUCTION

The introduction of new antibiotics has invariably resulted in the emergence of resistant strains of bacteria, in particular gram-negative opportunists associated with nosocomial infections (Farrar, 1979). Resistance in these organisms may be due to chromosomal mutation, but more commonly it appears to result from the acquisition of R plasmids bearing genetic resistance determinants (Falkow, 1975). The emergence and dissemination of R-plasmid-mediated resistance to new antibiotics among populations of hospital-associated bacteria can be due to any one or more of three general phenomena. Mutations may occur in genes carried by existing R plasmids which give rise to inactivating enzymes or other factors now operative on the new antibiotic. Alternatively, novel R plasmids, bearing the new resistance determinants, may be introduced into the bacterial population. In this case, the new R plasmid may either displace or co-exist with previously established genetic elements. Finally, such new R plasmids may be introduced and then eliminated but in the process donate, either by conventional recombinational mechanisms or by transposition, new resistance determinants to established endogenous plasmids. Evidence exists for each of these cases and for situations in which more than one mechanism appear to have occurred (Ingram, Richmond and Sykes, 1973; Jobanputra and Datta, 1974; Korfhagen, Loper and Ferrel, 1975; Mawer and Greenwood, 1977; Rennie and Duncan, 1977; Curie *et al.*, 1978; Elwell, Inamine and Minshew, 1978; Datta *et al.*, 1979; Gerding *et al.*, 1979; Rubens, McNeill and Farrar, 1979; Sadowski *et al.*, 1978, 1979; Datta *et al.*, 1980).

We have, for the past three years, been following the emergence of resistance to the newer penicillin and aminoglycoside antibiotics, carbenicillin (Cb), gentamicin (Gm) and tobramycin (Tm) among gram-negative clinical isolates at Hines Veterans Administration Hospital (HVAH). In 1977, we obtained an isolate of *Proteus mirabilis* resistant to 13 antibiotics including the three indicated above, and in 1978 we reported a series of isolates of *Serratia marcescens* exhibiting similar antibiograms (Olexy *et al.*, 1978, 1979). In genetic studies, these strains were shown to transfer resistance to as many as 11 antibiotics including Cb, Gm and Tm (Olexy *et al.*, 1979). Physical studies showed that a plasmid of mol. wt 89×10^6 was involved (Olexy *et al.*, 1978).

These results prompted us to examine the prevalence of resistance to Cb, Gm and Tm among all clinical gram-negative isolates. We approached this problem by screening for antibiotic resistance all clinical isolates of Enterobacteriaceae and selecting for more detailed analysis those resistant to at least Cb, Gm and Tm. In this report we show that at HVAH the emergence of resistance to these three antibiotics among Enterobacteriaceae was due in most instances to the introduction and establishment of a single R plasmid of extended host-range (Prasad *et al.*, 1978).

MATERIALS AND METHODS

Bacterial strains. Standard strains of *Escherichia coli* and *Pseudomonas aeruginosa* used in

the genetic crosses and their propagation and storage have been described (Olexy *et al.*, 1979). Clinical isolates resistant to at least Cb, Gm and Tm were obtained from the Clinical Microbiology Laboratory, HVAH. Isolates were identified and speciated in the Clinical Microbiology Laboratory, HVAH.

Culture media. Minimal media, nutrient agar and L broth were prepared as previously described (Olexy *et al.*, 1979).

Determination of resistance patterns. Clinical isolates and genetic progeny were tested for antibiotic resistance by the disc-diffusion technique of Bauer *et al.* (1966) as described by Olexy *et al.* (1979). The assays were performed on Mueller-Hinton agar and resistant and susceptible strains were always included as controls.

Mating techniques and progeny analysis. Bacterial matings were performed on nitrocellulose filters to maximise cell-cell contact essentially as described by Olexy *et al.* (1979). After matings, selections were made for only one antibiotic resistance conferred by the donor, and acquisition of unselected resistance markers was determined by the method of Bauer *et al.* (1966) as described above.

Plasmid isolation and analysis. DNA was partially purified from bacterial lysates by a technique similar to that described by Casse *et al.* (1979). These samples were analysed for plasmid content by agarose-gel electrophoresis as described by Hamada, Luckey and Farrand (1979). A mixture of five plasmids of known molecular weight was used to determine the molecular weights of plasmid species isolated from clinical isolates (Meyers *et al.*, 1976).

Restriction-endonuclease analysis. Plasmid DNA, purified by two rounds of centrifugation in CsCl-ethidium bromide (EtBr) gradients (Currier and Nester, 1976), were cleaved with the site-specific restriction endonuclease, *HincII*, in conditions described by the supplier (New England Biolabs, 283 Cabot Street, Beverly, Massachusetts, 01915, USA), and cleavage products were separated and analysed by agarose-gel electrophoresis as previously described (Farrand, Kado and Ireland, 1981).

RESULTS

Analysis of clinical isolates

During a 10-month period in 1978, 246 of 3952 (6.2%) single clinical isolates representing eight genera of Enterobacteriaceae were found to show combined resistance to at least Cb, Gm and Tm (table I). Although these multiply resistant isolates comprised only 6.2% of the total tested, as many as

TABLE I
Resistance of Enterobacteriaceae to carbenicillin, gentamicin and tobramycin

Genus	Number of strains tested	Number (and percentage) of strains resistant to Cb, Gm and Tm
<i>Escherichia</i>	1031	34 (3.3)
<i>Citrobacter</i>	177	10 (5.6)
<i>Klebsiella</i>	660	38 (5.8)
<i>Enterobacter</i>	444	26 (5.9)
<i>Serratia</i>	181	48 (26.5)
<i>Proteus</i>	932	27 (2.9)
<i>Providencia</i>	385	60 (15.6)
<i>Morganella</i>	142	3 (2.1)
All	3952	246 (6.2)

Cb = carbenicillin, Gm = gentamicin, Tm = tobramycin.

TABLE II
Additional resistance characteristics of multiply resistant clinical isolates

Group	Representative strain	Resistance pattern*
I	<i>Enterobacter aerogenes</i> <i>Klebsiella pneumoniae</i> <i>Citrobacter diversus</i>	Tp Tp Tp
II	<i>Serratia marcescens</i>	Tp Pb
III	<i>En. agglomorans</i> <i>Escherichia coli</i>	Nm Tc Nm Tc
IV	<i>En. cloacae</i> <i>C. freundii</i>	Nm Tc Tp Nm Tc Tp
V	<i>S. marcescens</i> <i>Proteus mirabilis</i>	Nm Tc Tp Pb Nm Tc Tp Pb
VI	<i>Providencia stuarti</i> (u ⁻) <i>Pr. stuarti</i> (u ⁺)	Nm Tc Tp Pb Na Nm Tc Tp Pb Na Cm
VII	<i>P. rettgeri</i>	Nm Tc Tp Pb Na Cm

Tp = trimethoprim, Pb = polymyxin B, Nm = neomycin, Tc = tetracycline, Na = nalidixic acid, Cm = chloramphenicol. (u⁻) = incapable and (u⁺) = capable of splitting urea.

* All strains were resistant to the nine core antibacterial agents: ampicillin, carbenicillin, cephalothin, streptomycin, kanamycin, gentamicin, tobramycin, sisomycin and sulphonamide.

26% of the isolates for certain genera showed resistance to the key antibiotics.

All of these 246 strains, selected for study only because they were resistant to Cb, Gm and Tm, showed common resistance to at least six additional antimicrobial agents including ampicillin (Ap), cephalothin (Ce), streptomycin (Sm), kanamycin (Km), sisomycin (Si) and sulphonamide (Su). The isolates could be divided into seven patterns based on the addition of other resistance traits to this core of nine antibiotics (table II). In total, 80 of the 246 (33%) manifested only the core pattern while 166 (67%) were resistant to as many as six additional antibiotics. Some of the eight genera represented exhibited predominantly the core pattern only, while others showed resistance to

TABLE III
Distribution of multiply resistant *Enterobacteriaceae* to core and core + other patterns

Genus	Number of strains tested	Number (and percentage) of strains resistant to	
		core antibiotics*	core + other antibiotics
<i>Escherichia</i>	34	23 (68%)	11 (32%)
<i>Citrobacter</i>	10	4 (40%)	6 (60%)
<i>Klebsiella</i>	38	31 (82%)	7 (18%)
<i>Enterobacter</i>	26	11 (42%)	15 (58%)
<i>Serratia</i>	48	5 (10%)	43 (90%)
<i>Providencia</i>	60	3 (5%)	57 (95%)
<i>Proteus</i>	27	0 (0%)	27 (100%)
<i>Morganella</i>	3	3 (100%)	0 (0%)
All	246	80 (33%)	166 (67%)

* See footnote to table II.

additional antibiotics (table III). All were sensitive to amikacin and netilmicin.

Resistance transfer

To determine whether resistance to the three key antibiotics was mediated by conjugative R plasmids, 123 (50%) randomly selected clinical isolates representing each of the eight genera were independently mated with *E. coli* strain C1200-1, a plasmidless, well-marked, rifampicin-resistant, restrictionless recipient (Olexy *et al.*, 1979). Of the donor strains, 105 (85%) were observed to transfer gentamicin resistance. Although the number of strains transferring resistance approached 100% for most genera, only five of seven (71%) of *Citrobacter* donors and 14 of 29 (48%) of *Providencia* donors could be shown to be fertile (table IV). Similar results were obtained when carbenicillin was used as the selective antibiotic.

TABLE IV
Transfer characteristics of multiply resistant clinical isolates*

Donor genus	Number of strains tested	Number (and percentage) of strains		Frequency of transfer†
		transferring‡	transferring core pattern	
<i>Escherichia</i>	14	13 (93%)	13 (100%)	10^{-1} – 10^{-9}
<i>Citrobacter</i>	7	5 (71%)	5 (100%)	10^{-2} – 10^{-9}
<i>Klebsiella</i>	15	15 (100%)	15 (100%)	10^{-1} – 10^{-5}
<i>Enterobacter</i>	15	15 (100%)	15 (100%)	10^{-1} – 10^{-4}
<i>Serratia</i>	27	27 (100%)	27 (100%)	10^{-2} – 10^{-6}
<i>Providencia</i>	29	14 (48%)	14 (100%)	10^{-2} – 10^{-9}
<i>Proteus</i>	13	13 (100%)	13 (100%)	10^{-4} – 10^{-6}
<i>Morganella</i>	3	3 (100%)	3 (100%)	10^{-4} – 10^{-6}

* *Escherichia coli* strain C1200-1 (Olexy *et al.*, 1979) was used as the recipient.

† Transconjugants were selected on nutrient agar supplemented with rifampicin (100 µg/ml) and gentamicin (50 µg/ml).

‡ Expressed as the number of transconjugants recovered per ml divided by the input titre of the donor.

When screened for the acquisition of unselected resistance markers, the vast majority of transconjugants coinherited resistance to at least the nine antibiotics comprising the core pattern. A few transconjugants showing resistance to neomycin (Nm) and tetracycline (Tc) in addition to the core pattern were isolated from matings with donors of groups III–VII (table III). However, even with donors resistant to as many as 15 antibiotics, consistent resistance transfer was observed only for the nine comprising the core pattern. One *C. freundii* donor, strain 1587, proved the exception, invariably transferring resistance to 11 antibiotics including the core pattern. Nm and Tc. Unselected transfer of resistance to trimethoprim, polymixin B, nalidixic acid, or chloramphenicol was never observed with any donor.

Plasmid analysis

The genetic evidence presented above suggested that the clinical isolates harbour at least one conjugative R plasmid coding for resistance to as many as nine antibiotics. To test this directly, samples of partially purified DNA from representative donors of each genus were subjected to agarose-gel electrophoresis. As shown in fig. 1, all strains tested harboured at least one plasmid; in some, such as *Enterobacter agglomerans* strain 1051, there were as many as four. With one exception, all isolates showed the presence of a plasmid with an estimated mol. wt of 89×10^6 . The strain of *C. freundii*, which consistently transferred resistance to 11 antibiotics, showed a single large plasmid with a mol. wt estimated at 62×10^6 (fig. 1, lane G). While some strains resistant to and transferring only the core pattern harboured a single plasmid, others contained more than one extrachromosomal element. In contrast, those resistant to additional antibiotics and occasionally transferring resistance to Nm and Tc, all contained an additional 57×10^6 plasmid, (fig. 1, lanes A, C and E).

To associate a plasmid conclusively with the transferable resistance phenotypes, partially purified DNA samples from *E. coli* transconjugants were analysed. Transconjugants representing progeny from crosses with several different donors of each genus were assayed. When such transconjugants resistant only to the nine core antibiotics were analysed, all were observed to have acquired an 89×10^6 plasmid (fig. 2). In contrast, those rare transconjugants acquiring resistance to Nm and Tc were found to harbour an 89 and a 57×10^6 plasmid identical to those seen in the donors (fig. 3, compare lanes A with B and C with D). As described above, all transconjugants derived from matings with the *C. freundii* donor acquired resistance to Nm and Tc in addition to the nine core antibiotics. These progeny were found to have inherited a 62×10^6 plasmid apparently identical in mass to the one large plasmid present in the donor (fig. 3, compare lanes E and F).

Restriction-endonuclease analysis

The data presented above suggested that the 89×10^6 plasmid common to the vast majority of the multiply resistant clinical isolates was an R plasmid coding for resistance to nine antibiotics including Cb, Gm and Tm. To confirm that these large elements from the various isolates were indeed the same plasmid, restriction-endonuclease digest fingerprints of representative DNA samples isolated and purified from *E. coli* transconjugants were compared. Such nucleolytic enzymes, which are site specific and therefore capable of generating unique digest profiles, have been used to determine the relatedness between other plasmids purified from clinical isolates (Rubens *et al.*, 1979, Sadowski *et al.*, 1978, 1979). As seen in fig. 4A, the plasmids deriving from six different genera showed essentially indistinguishable cleavage patterns. That of *P. rettgeri* (lane 3) differs from the others only in the absence of the highest molecular-weight fragment and the appearance of three new

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FIG. 1.—Agarose-gel electrophoresis of partially purified plasmid DNA from multiply resistant clinical isolates: A, *Enterobacter agglomerans* strain 1051; B, *Proteus rettgeri* strain 2035; C, *Serratia marcescens* strain 612; D, *Klebsiella pneumoniae* strain 325; E, *Escherichia coli* strain 2052; F, *Citrobacter diversus* strain 953; G, *C. freundii* strain 1587. Each sharp bright band corresponds to a plasmid of unique mol. wt (10^6) as indicated in the margins. The less defined bands, present in variable amounts in each sample, represent linear fragments of chromosomal DNA (Chr).

FIG. 2.—Agarose-gel electrophoresis of plasmid DNA from *E. coli* strain C1200-1 transconjugants showing resistance to only the nine core antibiotics and derived from matings with: A, *En. agglomerans* strain 1051; B, *P. rettgeri* strain 2035; C, *En. cloacae* strain 2119; D, *K. pneumoniae* 325; E, *E. coli* strain 2052; F, *C. diversus* strain 953, G, *Pr. stuarti* strain 1285. See legend to fig. 1 for explanation of band markings.

FIG. 3.—Agarose-gel electrophoresis of plasmid DNA from clinical isolates transferring resistance to neomycin and tetracycline in addition to the nine core antibiotics and their *E. coli* transconjugants: A, *En. agglomerans* strain 1051; B, *E. coli* strain C1200-1 (*En. agglomerans* strain 1051) $\text{Nm}^{\text{R}}\text{Tc}^{\text{R}}$; C, *E. coli* strain 2052; D, *E. coli* strain C1200-1 (*E. coli* strain 2052) $\text{Nm}^{\text{R}}\text{Tc}^{\text{R}}$; E, *C. freundii* strain 1587; F, *E. coli* strain C1200-1 (*C. freundii* strain 1587). See legend to fig. 1 for explanation of band markings.

FIG. 4.—Restriction-endonuclease analysis of R plasmid DNA. Purified plasmid DNA was subjected to digestion with *Hinc*II and the resultant DNA fragments analysed by agarose-gel electrophoresis. The lanes contain plasmid DNA isolated from (panel A) *E. coli* strain C1100-1 transconjugants derived from matings with: 1, *P. mirabilis* strain 2138; 2, *Pr. stuarti* strain 1285; 3, *P. rettgeri* strain 2035; 4, *En. agglomerans* strain 1051; 5, *C. diversus* strain 953; 6, *K. pneumoniae* strain 325; 7, *E. coli* strain 2052; 8, *C. freundii* strain 1587 and (panel B) 1, C1200-1 (*En. agglomerans* strain 1051); 2, C1200-1 (*En. agglomerans* strain 1051) $\text{Nm}^{\text{R}}\text{Tc}^{\text{R}}$.

smaller fragments. Evidently fragment 1 of this plasmic acquired two new *HincII* sites. The 62×10^6 plasmid from *C. freundii* (lane 8) represents something of an enigma. This plasmid, which codes for resistance to the nine core antibiotics, Nm and Tc has some *HincII* fragments that are unique and others similar to those of the 89×10^6 species. Digestion of plasmid DNA isolated from transconjugants harbouring the 89 and the 57×10^6 plasmids showed the presence of all of the 89×10^6 plasmid fragments and additional species presumably attributable to the 57×10^6 element (fig. 4B, lane 2). Note that the strains analysed in fig. 4B were both derived from a mating with *En. agglomerans* strain 1051. Lane 1 shows the digest pattern of plasmid DNA isolated from a $Nm^S Tc^S$ *E. coli* transconjugant harbouring the 89×10^6 plasmid only (see fig. 2 lane A). Its digestion pattern is identical to that of other isolates of the 89×10^6 plasmid (compare fig. 4B, lane 1 with fig. 4A, lanes 1, 2 and 4–7). Lane 2 of fig. 4B shows a digest of plasmid DNA from a $Nm^R Tc^R$ *E. coli* transconjugant harbouring the 89 and the 57×10^6 plasmids (see fig. 3, lane B). This digestion pattern is much more complex, but contains, as a subset, all the fragments present in the 89×10^6 element.

DISCUSSION

The physical and genetic analyses presented above indicate that a single R plasmid with a mol. wt estimated at 89×10^6 is responsible for resistance to Cb, Gm, Tm and six other antibiotics among gram-negative clinical isolates at HVAH. Similar results, showing that a single plasmid was responsible for resistance to a series of antibiotics, have been reported (Ingram *et al.*, 1973; Elwell *et al.*, 1978; Sadowski *et al.*, 1978, 1979). However, in most of these studies only a few isolates, or isolates from one particular outbreak or hospital section or showing a single homogeneous resistance pattern were studied.

In the present study, the clinical isolates were chosen solely on the basis of resistance to the three key antibiotics. However, all were also resistant to at least six other antibiotics, to complete what we call the core pattern. These 246 resistant isolates could then be divided into seven groups based on additional resistance characteristics (table II). Although some of the isolates were resistant to as many as 15 antibiotics, the vast majority tested transferred resistance only to Ap, Cb, Ce, Sm, Km, Gm, Tm, Si and Su, the nine resistance traits comprising the core pattern. Analysis of the donors and their *E. coli* transconjugants (figs. 1, 2 and 3) clearly showed this transferable resistance pattern to be associated with conjugative plasmids, all, with one exception, having a mol. wt estimated at 89×10^6 . Restriction-endonuclease analysis (figs. 4A and 4B) showed these 89×10^6 plasmids to be virtually identical regardless of the original source. Thus several criteria including resistance pattern, genetic transferability and molecular characteristics clearly show that a single plasmid has established itself within the gram-negative hospital flora. Furthermore, given the conditions by which clinical isolates were chosen for this study, this plasmid is apparently the primary agent responsible for linked

resistance to Cb, Gm and Tm among clinical isolates of the Enterobacteriaceae at HVAH.

Some of the isolates occasionally transferred resistance to Nm and Tc in addition to the nine core antibiotics. Molecular analysis showed this transfer to be associated with a 57×10^6 plasmid present in the donor strains (figs. 3 and 4). Like the larger plasmid it has also established itself in a wide range of gram-negative species isolated at HVAH (fig. 1).

However, the expression of Nm and Tc resistance among our isolates did not always correlate with presence of the 57×10^6 plasmid. For example, the group-V *S. marcescens* isolates harboured this plasmid and occasionally transferred resistance to Nm and Tc. The group-V *P. mirabilis* isolate, on the other hand, showed an identical resistance phenotype but lacked the 57×10^6 species and never transferred resistance to Nm or Tc. Thus, as has been previously emphasised (Richmond, 1975), identical resistance patterns represent insufficient criteria to prove identity of plasmids.

Some evolution of the 89×10^6 plasmid appears to be occurring. Our first isolation of this plasmid was from a strain of *P. mirabilis* in 1977 (C. Talanda-Fath, 1980). This plasmid is virtually identical to its counterparts purified from clinical isolates representing other genera obtained in 1977 and 1978 (compare fig. 4, lane 1 with lanes 2 and 4-7). However, the 89×10^6 plasmid from *P. rettgeri* (fig. 4, lane 3) appears to have gained additional *HincII* sites without any noticeable alteration in size or detectable phenotype.

By contrast, the R plasmid from *C. freundii* may represent a substantial modification of the 89×10^6 species. This 62×10^6 element apparently codes for resistance to the nine core antibiotics along with Nm and Tc. Cleavage analysis (fig. 4, lane 8) showed this plasmid to have fragments in common with the 89×10^6 species. In the absence of firm evidence we can only speculate that this R plasmid arose by some set of recombinational events between the 89 and the 57×10^6 species. Finally, we have just isolated from a strain of *Pr. stuartii* a natural derivative of the 89×10^6 plasmid which appears to code for urease production. We are now analysing this latter plasmid as to its molecular characteristics.

It is clear that the 89×10^6 plasmid described in this work is endemic within the Enterobacteriaceae associated with nosocomial infections at Hines VAH. Furthermore, this R plasmid appears to be the primary vector for resistance to carbenicillin, gentamicin and tobramycin among those isolates. We are continuing to monitor the presence and evolution of this genetic element.

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