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R-Factor Responsible for an Outbreak of Multiply Antibiotic-Resistant *Klebsiella pneumoniae*

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Seven serotypes of *Klebsiella pneumoniae* isolated from different patients demonstrated resistance to the same eight antibiotics. A plasmid carrying resistance determinants to these antibiotics and mercury salts could be transferred in toto to a plasmidless strain of *Escherichia coli*. All *E. coli* transconjugants showed the same antibiotic resistance pattern. Digestion with restriction endonucleases yielded patterns that were identical for each of the R-factors transferred from the multiply resistant serotypes. Moreover, deoxyribonucleic acid-deoxyribonucleic acid hybridization demonstrated identity between the probe, pMAC20 (an R-factor from one serotype), and all R-factors isolated from the multiply resistant strains of *K. pneumoniae* and the *E. coli* transconjugants tested.

During a 2-year period, nosocomial infections caused by multiply resistant *Klebsiella pneumoniae* appeared in the University of Louisville Hospital (M. Raff, unpublished data). R-factor transmission was suspected, since the multiply resistant character was found in several different serotypes of *K. pneumoniae* (1, 11, 17). In this study we show that a single R-factor was responsible for the epidemic and persists in our hospital environment. Deoxyribonucleic acid (DNA)-DNA hybridization was used to identify this R-factor in all the *K. pneumoniae* strains and may prove to be a useful tool in the continuing study of this and future outbreaks of multiply resistant microorganisms.

(This work is a part of the dissertation submitted by M.-A. Courtney to the Graduate Faculty of the University of Louisville in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Bacterial strains and plasmids. The antibiograms of the original *K. pneumoniae* clinical isolates, the respective *Escherichia coli* transconjugants, and the plasmid designations are given in Table 1. The Kirby-Bauer method (2) was used to determine antibiotic susceptibility. Serotypes were determined by a Quellung reaction with capsule typing sera (3). All bacterial cultures were maintained at 4°C on tryptose blood agar base (Difco) supplemented with appropriate antibiotics.

Conjugation. Cultures of donor and recipient cells in the exponential phase of growth were diluted 1:40 and 1:20, respectively, in Penassay broth (antibiotic medium no. 3, Difco). Equal volumes (1 ml) of each were mixed, and cultures were incubated at 37°C, without shaking but with proper aeration, for 2 to 4 h. Transconjugants were selected on tryptose blood agar broth or MacConkey agar (Difco) plates which contained 100 μg of rifampin per ml, 20 μg of streptomycin per ml, and 20 μg of ampicillin per ml. The putative *E. coli* transconjugants used for further studies were demonstrated to be *E. coli* by using the Corning R/B system of identification (13). The antibiograms were determined by the Kirby-Bauer method.

Plasmid isolation. Plasmid DNA from *K. pneumoniae* was isolated by the method of Hansen and Olsen (6). R-factor DNA from *E. coli* was partially purified by a cleared lysate technique (4), followed by a concentration step with NaCl and polyethyleneglycol 6000 as described by Humphreys et al. (8).

After either procedure, the resulting precipitated DNA was suspended in TES buffer [0.05 M tris(hydroxymethyl)aminomethane, pH 7.0; 0.05 M NaCl; 0.005 M ethylenediaminetetraacetate], brought to a final density of 1.55 g/ml with solid CsCl and 200 μg of ethidium bromide per ml, and purified by density equilibrium centrifugation. Covalently closed circular DNA was collected and precipitated with 95% ethanol. Purified plasmid DNA was suspended in TES buffer.

Restriction enzyme digestion and agarose gel electrophoresis. Plasmid samples (1 μg) were incubated with 10 U of one of several restriction endonucleases (Miles Laboratories, Inc.) for 1 h at 37°C under conditions (pH, salt concentration) recommended by the supplier. Endonuclease-generated restriction fragments of the plasmid DNA were then resolved by electrophoresis through a 0.8% agarose gel.

Nick translation and Southern blotting. Standard procedures were used for nick translation (9) and subsequent Southern blotting (15).

RESULTS

Nature of the epidemic. The multiply resistant *K. pneumoniae* strains used in this study were collected from several wards over a 2-year

TABLE 1. *Antibiogram of the strains used in this study and the plasmids they contain*

Strain	*Klebsiella* serotype	Plasmid[a]	Antibiotic resistances[b]	Origin[c]
LUK101	1	pMAC100	Am Cb Cf Cm Gm Km Mer Nm Tb Te	University Hospital
LUK200	2	pMAC200	Am Cb Cf Cm Gm Km Mer Nm Tb Te	University Hospital
LUK701	7	pMAC700	Am Cb Cf Cm Gm Km Mer Nm Tb Te	University Hospital
LUK1201	12	pMAC1200	Am Cb Cf Cm Gm Km Mer Nm Tb Te	University Hospital
LUK1801	18	pMAC1800	Am Cb Cf Cm Gm Km Mer Nm Tb Sm	University Hospital
LUK2501	25	pMAC2500	Am Cb Cf Cm Gm Km Mer Nm Tb	University Hospital
CSH50		—	Sm	J. R. Miller
LUE203		—	Sm Rif	M.-A. Courtney
LUE101		pMAC10	Am Cb Cf Cm Gm Km Mer Nm Rif Sm Tb Te	LUK101 × LUE203
LUE2001		pMAC20	Am Cb Cf Cm Gm Km Mer Nm Rif Sm Tb Te	LUK200 × LUE203
LUE701		pMAC70	Am Cb Cf Cm Gm Km Mer Nm Rif Sm Tb Te	LUK701 × LUE203
LUE1201		pMAC120	Am Cb Cf Cm Gm Km Mer Nm Rif Sm Tb Te	LUK1201 × LUE203
LUE1801		pMAC180	Am Cb Cf Cm Gm Km Mer Nm Rif Sm Tb Te	LUK1801 × LUE203
LUE2501		pMAC250	Am Cb Cf Cm Gm Km Mer Nm Rif Sm Tb Te	LUK2501 × LUE203

[a] LUK strains contained many plasmid species and the plasmid designation is for those that showed homology with pMAC20 by DNA-DNA hybridization.

[b] Abbreviations: Am, Ampicillin; Cb, carbencillin; Cf, cephalothin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Mer, mercuric salts; Nm, neomycin; Tb, tobramycin; Te, tetracycline; Sm, streptomycin; Rif, rifampin.

[c] LUK series were clinical isolates; LUE (except LUE203) series were obtained from crosses between LUE203 and the respective LUK strains. LUE203 is a spontaneous rifampin-resistant strain of CSH50.

period during which the multiply resistant nosocomial infection was evident. The problem was significant because the bacteria were resistant to the antibiotics routinely used in the hospital at the time. Because these cases were separated by hospital location, time, or both, it is not surprising that the isolates were different serotypes. However, because the drug resistance patterns were identical, it was evident that a transmissible R-factor might be the vehicle of resistance.

Conjugation studies. By selecting primarily with only ampicillin, it was possible to transfer the multiply resistant character in toto (i.e., resistance to ampicillin, cephalothin, chloramphenicol, gentamicin, kanamycin, mercuric salts, neomycin, tobramycin, and tetracycline) from each of the clinical *K. pneumoniae* isolates into a plasmidless *E. coli* strain, LUE203. The transconjugants obtained from these studies are described in Table 1. Although we did note great variations in the transfer frequencies among the various strains, all the clinical isolates were able to transfer the multiple resistance trait to the *E. coli* at a transfer frequency of at least 10^{-5} (M.-A. Courtney, Ph.D. thesis, University of Louisville, Louisville, Ky.). The relatively high frequency of conjugation into *E. coli* suggested to us that the spread of this R-factor to other members of the enteric bacilli could be a likely and expected event.

Molecular characterization of R-factors from transconjugants. The transconjugants contained a single plasmid species with a molecular weight of approximately 60 × 10^6. The individual restriction endonuclease digest patterns were identical for these R-factors when the enzyme *Eco*RI (Fig. 1), *Pst*I, or *Sal*I (not shown) was used. Since *Eco*RI digestion of all the R-factors isolated from the different transconjugants resulted in identical fragment patterns (a minimum of 10 fragments), we concluded that only one R-factor DNA species was responsible for the outbreak of multiply resistant *K. pneumoniae* in our hospital. A more intensive investigation using DNA homology by Southern blotting and DNA-DNA hybridization showed identity between the R-factor pMAC20 and every restriction fragment of each isolated R-factor (Fig. 2). This added evidence clearly indicates that the R-factor DNAs isolated from the different transconjugants were identical.

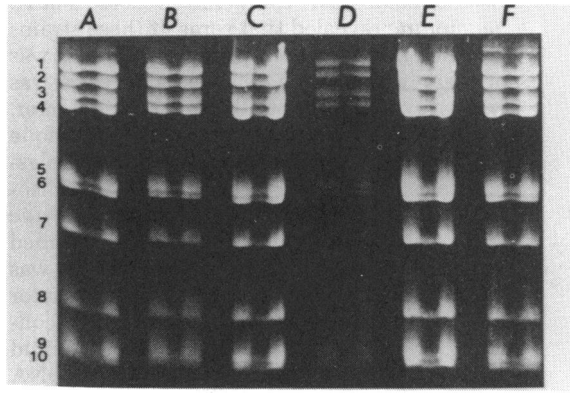

FIG. 1. *EcoRI digest of plasmids isolated from E. coli transconjugants. Procedures for DNA digestion with EcoRI and electrophoresis are described in the text. (A) pMAC20; (B) pMAC10; (C) pMAC70; (D) pMAC120; (E) pMAC180; (F) pMAC250.*

Monitoring clinical isolates for the R-fac-

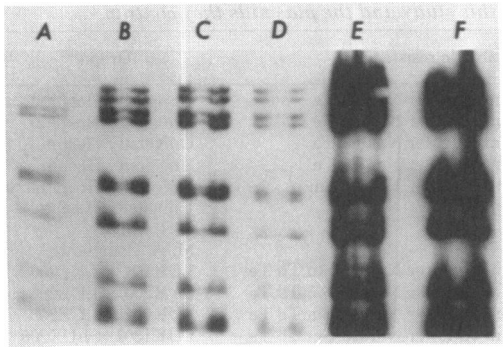

FIG. 2. *Hybridization of pMAC20 probe with DNA fragments from the gel in Fig. 1. Procedures for DNA-DNA hybridization are described fully in the text. (A) pMAC20; (B) pMAC10; (C) pMAC70; (D) pMAC120; (E) pMAC180; (F) pMAC250.*

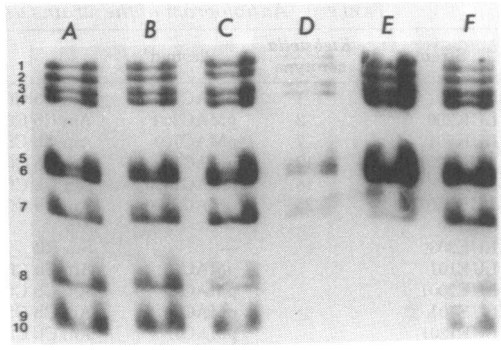

FIG. 3. *Hybridization of a pMAC20 probe with EcoRI-digested plasmids from the clinical isolates of K. pneumoniae. Procedures for plasmid isolation and DNA-DNA hybridization were as described in the text. (A) LUK101; (B) LUK200; (C) LUK701; (D) LUK1201; (E) LUK1801; (F) LUK2501.*

tor. Agarose gel electrophoresis of covalently closed circular DNA from clinical isolates of *K. pneumoniae* revealed that some of these strains contained more than one plasmid species. It was not clear which of these plasmid species was involved with the multiply resistant character. Using the sensitive Southern blotting technique (15), we were able to identify which of the plasmid species was the R-factor in the clinical isolates and to demonstrate that all *K. pneumoniae* isolates contained the identical R-factor purified from the transconjugants. Hybridization was performed using as a probe the R-factor pMAC20 that was purified from the transconjugant strain LUE2001 (Table 1). This plasmid was used as the representative R-factor DNA because of the degree of identity (homology) shown above. In alternate experiments, other R-factor isolates gave the same results as those presented for pMAC20 (data not shown). Plasmid DNA from all clinical isolates of *K. pneumoniae* had identity with pMAC20 as shown by the autoradiograph (Fig. 3). Moreover, the restriction fragment patterns were identical to those of pMAC20 itself (Fig. 1). Although many restriction fragments were present from plasmid DNA purified from clinical isolates, 10 fragments were common between these isolates and pMAC20 DNA. Since the remaining fragments did not hybridize with pMAC20, we conclude that these other plasmids were unrelated to the R-factor and were not transferred in conjugation experiments.

DISCUSSION

The emergence of antibiotic-resistant bacteria continues to pose a problem to clinicians and the pharmaceutical industry alike. Almost as soon as a new drug is introduced, microorganisms might develop resistance. This prompted Lowbury and Ayliffe (10) to suggest that we may see the decline of useful antibiotic therapy in 40 years. The problem becomes more complex for two reasons. First, the resistance is usually multiple, such that an organism may carry resistance to at least eight antibiotics (this study); therefore, the course of therapy is sometimes very difficult. Second, this multiple-resistance trait is usually an R-factor and is often self-transmissible, and thus may be harbored in many genera of the enteric bacilli, common members of hospital flora. If there is to be any hope of controlling these R-factors, the exact nature of their epidemiology must be determined.

In recent years some effort has been made to understand the epidemiology of R-factors. Elwell et al. were able to demonstrate a common plasmid isolated from two enteric species present in a burn patient (5). Sadowski et al. have found an R-factor from *K. pneumoniae* responsible for an epidemic of multiply resistant bacteria in their hospital environment (14). These workers have relied on antibiograms of the bacteria and more recently have made use of endonucleases for plasmid identification (16). In this manuscript the introduction of the Southern blotting technique in these studies has brought the ability to monitor the epidemiology of R-factors to a more advanced and accurate level. We are using this new technique to investigate more accurately the spread of R-factors in the hospital environment.

The use of DNA-DNA hybridization has allowed us to determine conclusively that the outbreak of multiply resistant *K. pneumoniae* which appeared in our hospital environment was

caused by a single plasmid. This R-factor was isolated from six different *K. pneumoniae* strains. All the isolates had a different serotype, they were isolated during a 2-year period from different wards of the hospital, and yet they all contained the same R-factor, as shown by DNA-DNA hybridization using the Southern blotting technique. In recent experiments we have demonstrated that pMAC20 has homology to the well-characterized R-factor NR1, which is also called R100 and R222 (M.-A. Courtney, J. R. Miller, and U. N. Streips, submitted for publication).

Most importantly, the reservoir of this R-factor and how it was spread through the hospital environment should be determined. We feel that the use of the sensitive techniques presented in this study will allow the constant screening of the hospital environment and even personnel for the presence of such factors. We are presently using the representative plasmid (pMAC20) and hybridization with blots of endonuclease-digested total DNA from multiply resistant bacterial isolates to perform such screening procedures (experiments in progress).

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