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Urinary isolates of apramycin-resistant *Escherichia coli* and *Klebsiella pneumoniae* from Dublin

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SUMMARY

Twenty-two gentamicin-resistant urinary isolates of *Escherichia coli* and five gentamicin-resistant urinary isolates of *Klebsiella pneumoniae* from a Dublin hospital were examined for resistance to the veterinary aminoglycoside antibiotic apramycin. Five isolates of *E. coli* and one isolate of *K. pneumoniae* were found to be resistant. The apramycin-resistant isolates, which were also resistant to the veterinary anthelmintic agent hygromycin B, hybridized with a DNA probe for the gene encoding the enzyme 3-*N*-aminoglycoside acetyltransferase type IV (AAC(3)IV). Resistance to apramycin and hygromycin B was co-transferable in four of the five isolates of *E. coli* and the isolate of *K. pneumoniae*. In one isolate of *E. coli* apramycin resistance was not transferable. On the basis of their restriction enzyme digestion profiles and the antimicrobial resistance traits encoded, the transferable plasmids encoding resistance to apramycin and hygromycin B comprised three distinct types. Genetic linkage between the gene encoding AAC(3)IV and genes encoding resistance to ampicillin and either tetracycline or trimethoprim, means that the relatively widespread use of these antimicrobial agents provides a selective pressure for the persistence of resistance to apramycin and gentamicin even in the absence of bacterial exposure to aminoglycosides.

INTRODUCTION

The aminoglycoside antibiotic apramycin has been used extensively in veterinary medicine since the early 1980s [1]. Apramycin has not, however, been used in humans. Although early studies indicated that bacterial resistance to apramycin was rare [2, 3], there have been subsequent reports of the isolation of resistant organisms, particularly *Escherichia coli* and *Salmonella* spp., from farm animals in the UK [1, 4, 5] and France [6]. These resistant organisms produce a novel aminoglycoside-modifying enzyme designated 3-*N*-aminoglycoside acetyltransferase type IV (AAC(3)IV), which acetylates not only apramycin, but also other aminoglycosides including gentamicin and tobramycin, which are used to treat serious infections in humans [7].

The finding that farm animals treated with apramycin sometimes carried and excreted bacteria resistant to gentamicin, an antimicrobial agent used to treat serious infections, gave rise to concern that these organisms might spread to humans. Since the mid-1980s, a number of reports from the UK, Belgium and Spain have documented the occurrence of human clinical isolates of Enterobacteriaceae (including *S. typhimurium*, *E. coli* and *Klebsiella pneumoniae*) that were resistant to apramycin and gentamicin due to production of AAC(3)IV [4, 8–14]. Molecular analysis indicated that the gene encoding apramycin resistance in human clinical isolates of bacteria was identical to the gene found in bacteria isolated from animals [9].

In the present study, a collection of gentamicin-resistant urinary isolates of *E. coli* and *K. pneumoniae* obtained from patients in Dublin was assessed for resistance to apramycin. Isolates resistant to apramycin were then characterized with regard to the mechanism and genetics of resistance. In addition, genetic linkage of apramycin resistance with resistance to other classes of antimicrobial agent was investigated, to determine if there was indirect evidence that usage of such agents provides a potential selective pressure for the persistence of genes encoding resistance to apramycin.

MATERIALS AND METHODS

Bacteria

Twenty-two urinary isolates of gentamicin-resistant *E. coli* and five urinary isolates of gentamicin-resistant *K. pneumoniae* isolated at the Beaumont Hospital in Dublin between 1989 and 1992 were studied.

Determination of MICs

Minimum inhibitory concentrations (MICs) of a number of antimicrobial agents including apramycin and hygromycin B (kindly provided by Lilly Research Laboratories) were determined by an agar dilution method, using Isosensitest agar supplemented with 2% (v/v) lysed horse blood. Serial twofold dilutions of each antimicrobial agent were incorporated into the medium and plates were inoculated using a multipoint inoculator (Diamed Diagnostics) with an inoculum of 10^4–10^5 organisms/spot. Apramycin resistance was defined by an MIC > 16 mg/l and resistance to hygromycin B by an MIC of > 64 mg/l. Isolates were classified as sensitive or resistant to all other antimicrobial agents using published criteria [15].

Plasmid analysis

Plasmids were extracted using the method of Kado and Liu [16] and separated by electrophoresis in agarose gels (0·7% w/v). The molecular sizes of plasmids were estimated by comparison with plasmids of known size. In some experiments extracted plasmids were digested with restriction endonucleases under conditions specified by the enzyme manufacturer (Gibco). The sizes of restriction fragments were determined by comparison with fragments of linear DNA of known size (DNA molecular-weight markers II and III; Boehringer Mannheim).

Transfer of apramycin-resistance

Conjugation experiments were performed using *E. coli* strain J62-2 (which is plasmid-free and resistant to rifampicin) as the recipient strain [17]. Equal volumes of broth-grown donor and recipient (each at a concentration of about 10^9 organisms/ml) were mixed and 200 μl were placed on the surface of a sterile Millipore filter lying on nutrient agar. After incubation at 37 °C for 3 h (in some experiments incubation was continued overnight), the organisms were washed off and plated on Isosensitest agar containing rifampicin (50 mg/l) and apramycin (16 mg/l).

Hybridization studies

The DNA probe specific for the gene encoding production of AAC(3)IV consisted of a digoxigenin-labelled 740 base-pair *Sst* I fragment of plasmid pWP701 prepared as described previously [13]. Dot-blots of whole-cell DNA and Southern blots of plasmid DNA were prepared as described previously [13].

Characterization of β-lactamases

The β-lactamase enzymes present in ultrasonically disrupted bacterial extracts were characterized by analytical isoelectric focusing using commercially-prepared polyacrylamide gels, pH 3·5–9·5 (Pharmacia-LKB) [18]. β-lactamase activity was detected using the chromogenic cephalosporin nitrocefin.

RESULTS

Antimicrobial resistance

Six (22·2%) of the 27 gentamicin-resistant isolates studied were found to be resistant to apramycin (MICs $\geqslant$ 1024 mg/l). They comprised five isolates of *E. coli* and one isolate of *K. pneumoniae* (Table 1). For the six apramycin-resistant isolates, MICs of hygromycin B were $\geqslant$ 512 mg/l, in contrast to the apramycin-sensitive isolates which had hygromycin B MICs of 32–64 mg/l. The apramycin-resistant isolates were cross-resistant to gentamicin and tobramycin but were sensitive to amikacin. There was inter-isolate variation with regard to susceptibility to ampicillin, tetracycline and trimethoprim (Table 1), but all the isolates were susceptible to ciprofloxacin, cefotaxime and ceftazidime.

Transfer of resistance to apramycin and other antimicrobial agents

The 6 apramycin-resistant clinical isolates had distinct plasmid profiles, comprising either 2 or 3 plasmids ranging in size between 100 MDa and 4 MDa. Apramycin-resistant transconjugants containing single plasmids were obtained from 5 of the 6 isolates studied, and were found to be resistant to gentamicin, tobramycin and hygromycin B (Table 1). Although transconjugants produced by four isolates (*E. coli* E16, E49, E53 and *K. pneumoniae* K19) all contained a plasmid of approximately 90 MDa which hybridized with the apramycin resistance gene probe, further studies indicated that these plasmids were not identical but comprised two groups. Restriction enzyme analysis using either *Eco*RI, *Bam*HI or *Hin*dIII showed that the plasmids in transconjugants derived from *E. coli* E49 and *K. pneumoniae* K19 were indistinguishable, but differed from the plasmids found

Table 1. *Apramycin-resistant urinary isolates from Dublin*

Species	Isolate	Year of isolation	Source of patient	Resistance pattern*	Resistances transferred
E. coli	E49	1992	Hospital	Ap, Hy, Gm, Tb, Am, Te, Tr	Ap, Hy, Gm, Tb, Am, Te
K. pneumoniae	K19	1991	Hospital	Ap, Hy, Gm, Tb, Am, Te, Tr	Ap, Hy, Gm, Tb, Am, Te
E. coli	E16	1991	Hospital	Ap, Hy, Gm, Tb, Am, Te, Tr	Ap, Hy, Gm, Tb, Am, Tr
E. coli	E53	1991	Community	Ap, Hy, Gm, Tb, Am, Tr	Ap, Hy, Gm, Tb, Am, Tr
E. coli	E87	1992	Hospital	Ap, Hy, Gm, Tb, Am, Te, Tr	Ap, Hy, Gm, Tb
E. coli	E08	1992	Community	Ap, Hy, Gm, Tb, Te, Tr	—

* Ap, apramycin; Hy, hygromycin B; Gm, gentamicin; Tb, tobramycin; Am, ampicillin; Te, tetracycline; Tr, trimethoprim.

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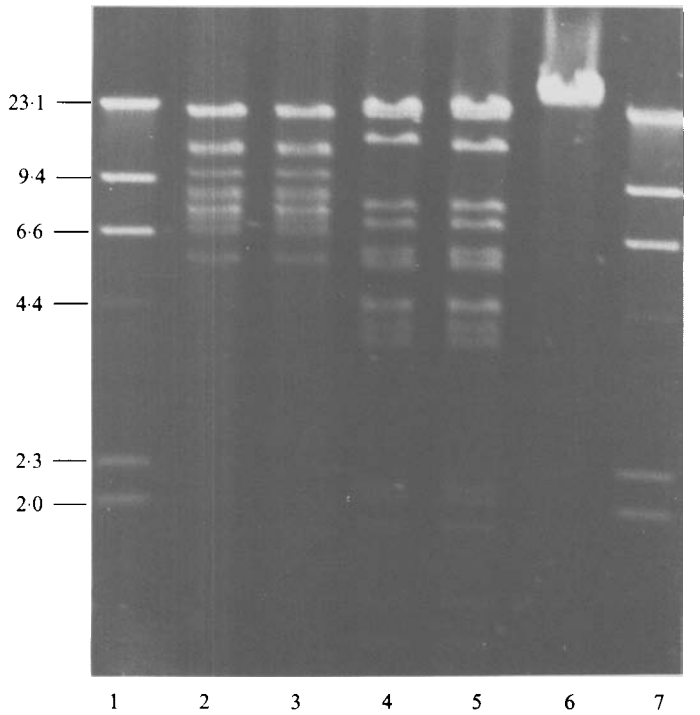

Fig. 1. *Eco*RI digests of plasmids in apramycin-resistant transconjugants. Lanes 1 and 7, molecular size markers; Lane 2, transconjugant derived from *K. pneumoniae* K19; Lane 3, transconjugant derived from *E. coli* E49; Lane 4, transconjugant derived from *E. coli* E16; Lane 5, transconjugant derived from *E. coli* E53; Lane 6, transconjugant derived from *E. coli* E87.

in transconjugants derived from *E. coli* E16 and E53 (Fig. 1). Transconjugants produced by *E. coli* E87 contained an apramycin resistance plasmid of 60 MDa, which had a unique restriction enzyme digestion profile. Hybridization of the apramycin resistance gene probe with the products obtained by digestion of each of the plasmids from the apramycin-resistant transconjugants gave patterns which confirmed the relationships outlined above (data not shown).

Further evidence for the presence of identical apramycin resistance plasmids in *E. coli* E49 and *K. pneumoniae* K19 and in *E. coli* E16 and E53, respectively, was provided by determination of other antibiotic resistances linked to apramycin resistance. Transconjugants derived from *E. coli* E49 and *K. pneumoniae* K19 exhibited linked resistance to ampicillin and tetracycline, whereas those derived from *E. coli* E16 and E53 exhibited linked resistance to ampicillin and trimethoprim (Table 1). The transconjugants produced by *E. coli* E87, which contained a unique 60 MDa plasmid, were only resistant to apramycin and other aminoglycosides.

Despite repeated attempts, transferable resistance to apramycin was not detected with *E. coli* E08. In Southern blots, the apramycin resistance gene probe failed to hybridize with plasmids present in this isolate, although faint hybridization was observed with the band of chromosomal DNA present in the plasmid preparations.

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Analysis of the β-lactamases produced by the apramycin-resistant clinical isolates and transconjugants that were also resistant to ampicillin showed that all these organisms produced a β-lactamase with an isoelectric point of 5·4 (TEM-1) except for *E. coli* E87, which failed to exhibit β-lactamase activity in isoelectric focusing studies.

DISCUSSION

Studies of apramycin-resistant Enterobacteriaceae isolated from animals have shown that resistance is due to production of the enzyme AAC(3)IV [1, 4–7] encoded by a gene closely linked to a gene encoding resistance to the aminocyclitol antibiotic hygromycin B. It was therefore of interest to note that DNA from each of the six human clinical isolates of apramycin-resistant Enterobacteriaceae described here hybridized with a DNA probe specific for the gene encoding AAC(3)IV, and that the isolates were also resistant to hygromycin B. Furthermore, apramycin-resistant transconjugants always acquired resistance to hygromycin B, indicating genetic linkage between the two resistance genes. These results are consistent with earlier reports that the mechanism and genetic basis of apramycin resistance in bacteria isolated from humans was identical to that seen in bacteria isolated from animals [9].

In the present study, resistance to apramycin was transferable from 5 of the 6 isolates. Restriction enzyme digestion analysis indicated that resistance to apramycin in *E. coli* E49 and *K. pneumoniae* K19 was encoded by an identical plasmid, indicating that inter-generic spread of the plasmid had occurred. Similarly, *E. coli* isolates E16 and E53 contained an identical plasmid, which was distinct from the plasmid seen in the former two isolates. As *E. coli* isolates E16 and E53 belonged to different O serotypes (T. Cheasty, personal communication), the presence of an identical plasmid in both isolates indicates inter-strain spread of this plasmid. Such inter-generic and inter-strain spread of apramycin resistance plasmids has been noted previously in both veterinary [5] and clinical settings [13].

Given that apramycin is not used in human medicine, the most likely explanation for the emergence of resistance to apramycin and gentamicin due to production of AAC(3)IV in human isolates of Enterobacteriaceae is that the genetic determinant of resistance has been acquired either directly or indirectly from apramycin-resistant bacteria in animals [8–14]. Although infection or colonization of humans by bacteria from animals may be transient, the transferable nature of the genetic determinant of apramycin resistance means that resistance may be transferred to other Enterobacteriaceae found in the normal human intestinal flora. The finding that the plasmids encoding apramycin resistance also contained genes encoding resistance to other commonly used antimicrobial agents such as ampicillin, tetracycline or trimethoprim, is of importance, as it indicates that the use of these agents, in addition to the use of aminoglycosides, would provide a selective pressure for the persistence of apramycin resistance plasmids.

The previously reported observations of a genetic association between the genes encoding resistance to apramycin and hygromycin B [9, 10, 13] were confirmed in the present study. However, the possible biological significance of this phenom-

enon is unknown. Although hygromycin B has activity against roundworms and has been used as an anthelmintic agent by veterinarians in the USA, its use in the same role in the UK has been negligible. Indeed, hygromycin B was withdrawn from veterinary use in the UK several decades ago. In addition, it should be noted that hygromycin B has not been available for veterinary use in Ireland. There is, therefore, little evidence to support the concept that the veterinary use of hygromycin B has proved significant selective pressure for the persistence of apramycin/hygromycin-resistant bacteria, at least in the UK and Ireland.

The results presented here show that in 6 (22·2%) of 27 gentamicin-resistant isolates of Enterobacteriaceae obtained from patients with urinary infection in Dublin, aminoglycoside resistance was due to production of the enzyme AAC(3)IV. Similar results were recently reported from a hospital in Liverpool [11], where 26% of gentamicin-resistant *E. coli* isolates were resistant to apramycin due to production of AAC(3)IV. These findings, together with the fact that human isolates of Enterobacteriaceae resistant to gentamicin and apramycin due to production of the enzyme AAC(3)IV have been reported from Belgium [8, 12] and Spain [9] suggest that such resistance may be widespread. However, because resistance to apramycin is not routinely investigated in clinical laboratories, the full extent of the problem is currently unknown. Further work involving both clinical and veterinary microbiologists will be needed if we are to fully understand the epidemiology of this important form of aminoglycoside resistance.

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