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Human isolates of apramycin-resistant *Escherichia coli* which contain the genes for the AAC(3)IV enzyme

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SUMMARY

Gentamicin-resistant *Escherichia coli* isolated at different periods from patients in two hospitals were tested for resistance to the aminoglycoside antibiotic apramycin. Twenty-four of 93 (26%) gentamicin-resistant isolates collected from the Royal Liverpool Hospital between 1981 and 1990 were resistant to apramycin. Thirteen isolates were highly resistant to apramycin (minimal inhibitory concentration (MIC) $\geqslant$ 1024 μg/ml), were also resistant to gentamicin, netilmicin and tobramycin, and hybridized with a DNA probe derived from the aminoglycoside acetyltransferase (3)IV (AAC(3)IV) gene. The proportion of gentamicin-resistant isolates which had high level resistance to apramycin increased from 7% in 1981–5 to 24% in 1986–90.

Twelve gentamicin-resistant *E. coli* from Guy's and St Thomas's Hospital isolated between 1977 and 1980 were also tested for resistance to apramycin. For five of these isolates the MICs of apramycin was 32–256 μg/ml. None was shown to have a conjugative plasmid carrying resistance to apramycin and only one hybridized with the DNA probe for the AAC(3)IV enzyme.

INTRODUCTION

Apramycin is an aminoglycoside-aminocyclitol antibiotic which was licensed in the United Kingdom in 1978 for animal use only. Resistance to apramycin has been found in *Escherichia coli* of mainly pig and calf origin and is plasmid-encoded and mediated by the AAC(3)IV enzyme [1–3].

As apramycin is used only for the treatment of animal diseases and not in human medicine it has been suggested that if apramycin resistant Enterobacteriaceae are found in humans this may be evidence of spread of resistant organisms and/or their plasmids from animals to man. The prevalence of apramycin-resistance in human Enterobacteriaceae has not been determined although some resistant isolates have been obtained from humans in Belgium [4], Spain [5] and the UK [6, 7].

Apramycin resistance has been found in human isolates of *Salmonella*

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typhimurium, *Klebsiella pneumoniae* and *E. coli* from hospitals in Belgium [4], where it was also demonstrated that resistance was mediated by the AAC(3)IV enzyme and that strains carrying resistance plasmids had spread between wards and between hospitals. Apramycin-resistant *E. coli* and *K. pneumoniae* isolated from humans in the UK have also been reported [7]. An increasing prevalence of resistance to gentamicin has been found in *S. typhimurium* phage type 204c in humans [8] and it has been suggested that use of apramycin in animals has selected for resistant salmonella isolates which then infect man [6].

Chaslus-Dancla and colleagues [2] found that multi- and apramycin-resistant isolates of *E. coli* and *S. typhimurium* from the same calves carried conjugative plasmids of different sizes but stressed the importance of this resistance being present since *S. typhimurium* of bovine origin is a common food poisoning pathogen in man. Hunter and co-workers [3] have subsequently described the presence of the same apramycin resistance plasmid in *E. coli* and *S. typhimurium* in an outbreak of salmonellosis in calves. Transfer of the plasmid from *E. coli* to *S. typhimurium* may have occurred during treatment of the calves with apramycin.

The aim of the work described in this paper was to determine whether resistance to apramycin associated with the AAC(3)IV enzyme are present in *E. coli* isolated from hospitalized patients in the UK.

MATERIALS AND METHODS

Isolates from the following sources were examined:

(a) gentamicin-resistant *E. coli* isolated from hospitalized patients at the Royal Liverpool Hospital, Liverpool and Guy's and St Thomas's Hospital, London. These were chosen because all the apramycin-resistant *E. coli* isolated by the authors from pigs and calves showed cross resistance to gentamicin [3].

(b) *E. coli* from rectal swabs from pregnant women, chosen to reflect community-associated strains.

Isolates

Ninety-three gentamicin-resistant *E. coli* isolates were collected during the period 1981–90 from blood, wound and urine samples by the Department of Medical Microbiology of the Royal Liverpool Hospital and stored at −80 °C in glycerol broth.

A total of nine freeze-dried gentamicin-resistant *E. coli* and *Klebsiella* spp. isolated from an outbreak of infection at the Royal Liverpool Hospital were also investigated [9].

Twelve gentamicin-resistant *E. coli* isolates were received from Dr A. King of Guy's and St Thomas's Hospital, London. They had been isolated over a 3-year period (1977–80), identified as *E. coli* and some contained aminoglycoside-modifying enzymes.

In addition rectal swabs collected from 64 pregnant women at the Royal Liverpool Hospital were investigated for the presence of apramycin-resistant *E. coli* by subculturing on MacConkey agar No. 3 (Oxoid CM115) incorporating 32 μg/ml apramycin sulphate (Dista Products).

All *E. coli* were identified biochemically using the API 20E kit (API System).

Antibiotic sensitivity

Antibiotic sensitivity tests were performed using the controlled disk diffusion technique [10] with Isosensitest agar (Oxoid CM471), a control *E. coli* NCTC10418 and Oxoid antibiotic disks. Initially gentamicin-resistance was confirmed and apramycin-resistance established by a preliminary sensitivity test using the following disks which contained (μg): ampicillin 10 (Am), apramycin 15 (Ap), gentamicin 5 (Ge), oxytetracylcine 30 (Tc), streptomycin 25 (Sm) and trimethoprim 5 (Tm). An isolate was considered resistant if the zone of inhibition around the disks was $\leqslant 3$ mm radius or the zone was $\geqslant 3$ mm smaller than the control zone. Apramycin-resistant isolates were subsequently tested for sensitivity to the related antibiotics; amikacin 30 (Ak), kanamycin 5 (Kn), neomycin 10 (Ne), netilmicin 10 (Nt), spectinomycin 25 (Sp) and tobramycin 10 (Tb) and also to; amoxycillin and clavulanic acid (Ac), chloramphenicol (Cm), ciprofloxacin (Cp), furazolidone (Fr), nalidixic acid (Nx) and compound sulphonamides (Su).

Minimal inhibitory concentrations (MIC) of apramycin and gentamicin were determined using a multipoint inoculator to replicate test colonies on to sheep blood agar plates incorporating dilutions of antibiotic. Dilutions used were: apramycin sulphate 32, 64, 128, 256, 512 and 1024 μg/ml and gentamicin sulphate (Sigma) 4, 8, 16, 32, 64, and 128 μg/ml.

Conjugations

Attempts were made to transfer resistance from some isolates in broth matings using a method previously described [3].

Molecular procedures

Plasmids were extracted from donors and transconjugants using the method of Kado and Liu [11]. DNA was separated by electrophoresis in 0·7% agarose gels and molecular weights of plasmids were determined by comparison with four reference plasmids carried in *E. coli* strain 39R861 [6].

The plasmid pWP701 [12] was kindly provided by W. Piepersberg (University of Munich, Germany). A 1·65 kb Pst 1 fragment of pWP701 containing the gene for AAC(3)IV was purified from agarose using glass beads (Geneclean, Bio 101, California, USA), radiolabelled by nick translation (Amersham, UK) and used as a probe in colony hybridizations.

RESULTS

Of the 93 gentamicin-resistant *E. coli* from the Royal Liverpool Hospital 24 (26%) were resistant to apramycin (Table 1). For 13 isolates the MIC of apramycin was $\geqslant 1024$ μg/ml and for 11 isolates the MIC was $\leqslant 256$ μg/ml.

All 13 isolates for which the MIC was $\geqslant 1024$ μg/ml hybridized to the probe whereas 7 strains tested for which the MIC was $\leqslant 256$ μg/ml did not.

The proportion of gentamicin-resistant isolates which were also resistant to apramycin increased from 16% in 1981–5 to 40% in 1986–90 (Table 1). Seven percent of apramycin-resistant isolates in the group 1981–5 had an MIC $\geqslant 1024$ μg/ml and this increased to 24% of isolates in 1986–90. High level

Table 1. *Numbers of apramycin-resistant* E. coli *isolated each year from 1981 to 1990 at the Royal Liverpool Hospital and MIC of apramycin*

Year isolated	Number of *E. coli* Ap^r*/ number of *E. coli* tested	Ap MIC $\geqslant$ 1024 μg/ml	AP MIC 32–256 μg/ml
1981	0/12	0	0
1982	0/15	0	0
1983	2/10	1	1
1984	3/7	2	1
1985	4/11	1	3
1986	3/10	1	2
1987	4/9	3	1
1988	3/7	1	2
1989	3/10	2	1
1990	2/2	2	0
Totals	24/93	13	11

* Ap^r, resistant to apramycin.

Table 2. *Resistance patterns of nine apramycin-resistant human* E. coli *(MIC $\geqslant$ 1024 μg/ml) isolated from the Royal Liverpool Hospital and resistance patterns of transconjugants*

Resistance of donor (in addition to Ap, Ge, Nt, Tp)	Transferred resistance of transconjugant (in addition to Ap, Ge, Nt, Tp)
Am/Sm/Su/Tm	Am/Sm
Am/Kn/Ne/Sm/Su/Tc	Am/Su
Am/Sm/Su/Tc/Tm	Sm/Su/Tm
Sm	Sm
Am/Sm/Su/Tc	Am/Sm/Su/Tc
Am/Cm/Kn/Ne/Sm/Su/Tc/Tm	Am/Tc
Am/Tc/Tm/Su	Am/Tc
Am/Tc/Tm/Su	Am/Tc
Ac/Am/Cm/Kn/Ne/Sm/Sp/Su/Tc	Kn/Ne/Sm/Tc

apramycin resistance (where the MIC was $\geqslant$ 1024 μg/ml) was first detected in 1983.

Conjugative plasmids conferring apramycin resistance were only found in isolates with high level resistance, and conferred resistance to gentamicin, netilmicin and tobramycin but not to amikacin and ciprofloxacin. The plasmids also encoded resistance to a variety of other antibiotics including ampicillin, oxytetracycline, sulphonamides and trimethoprim (Table 2). In all, seven different resistance patterns were co-transferred with apramycin resistance. Comparison of plasmids in transconjugants conferring resistance to apramycin showed that 4 of 9 had a molecular weight of approximately 140 kb.

Details were sought of those patients from whom apramycin-resistant *E. coli* with an MIC of apramycin of $\geqslant$ 1024 μg/ml and which hybridized with the AAC(3)IV probe had been isolated. Details were available for 7 patients. All were elderly, aged between 68 and 78 years, and 6 of 7 isolations had been made from urine during the years 1984–90. Repeated isolations from two patients had been made 2 days and 9 days after the initial isolation. Most of the patients had an indwelling catheter or had been catheterized and it is possible that these strains were derived from the patients' own intestinal flora [9].

The 12 gentamicin-resistant *E. coli* isolates from Guy's and St Thomas's Hospital were a random choice of known aminoglycoside-modifying enzyme producers and some which were not. For five the MIC of apramycin was 64–128 μg/ml and of gentamicin was 8–⩾ 128 μg/ml. None had conjugative plasmids carrying resistance to apramycin. Four of these isolates did not hybridize with the AAC(3)IV probe but one isolate for which the MIC of apramycin was 128 μg/ml and of gentamicin was 16 μg/ml did hybridize.

Resistance to gentamicin was confirmed in the *E. coli* and *Klebsiella* sp. isolates from the Royal Liverpool outbreaks in 1979–80 but none was resistant to apramycin. Similarly, no apramycin-resistant coliforms were isolated from the samples from pregnant women.

DISCUSSION

We have found apramycin resistance to be present in *E. coli* isolated from humans as early as 1977. This was before the introduction of the use of apramycin in animals. However, for all these strains the MIC was 64–128 μg/ml and in no instance was this resistance transferable. The first highly resistant *E. coli* (MIC ⩾ 1024 μg/ml) was isolated in 1983 (Table 1). Resistant strains were found increasingly thereafter. Subsequently, apramycin-resistant *E. coli* have been isolated each year with both high and low MIC of apramycin although none with a low MIC was isolated in 1990. Apramycin resistance in human *E. coli* conferring a high MIC was always associated with the presence of the gene for the AAC(3)IV enzyme and this was usually carried on a conjugative plasmid. This confirms the findings of Johnson and colleagues [7] that the enzyme AAC(3)IV is present in human *E. coli* in the U.K. This enzyme may be increasingly responsible for cross-resistance to gentamicin, netilmicin and tobramycin.

Since the gene encoding AAC(3)IV is present on a transferable plasmid it could spread to other strains of *E. coli* or transfer to other Enterobacteriaceae including *Salmonella* species. It has been well documented that conjugative plasmids can transfer from one strain of *E. coli* to another and also to other Enterobacteria including *Salmonella* spp. Resistance to apramycin has been reported to be transferred *in vivo* from *E. coli* to *S. typhimurium* [3] by conjugative plasmids. As gentamicin and tobramycin in particular are commonly used in hospitals to treat serious Gram-negative infections, resistance due to presence of AAC(3)IV could make successful treatment with such antibiotics difficult. Similarly, more commonly used antibiotics such as trimethoprim and ampicillin may also select for bacteria carrying the AAC(3)IV enzyme since genes encoding resistance to these antibiotics were often co-transferred on the same plasmid as the AAC(3)IV gene.

Nine of 13 isolates with a high level of resistance to apramycin (MIC ⩾ 1024 μg/ml) transferred resistance by conjugation. Not all apramycin-resistant human *E. coli* isolates from the Royal Liverpool Hospital had the same plasmid profile or carry plasmids in common but four had a transferable plasmid of approximately 140 kb. Where no transferable plasmid was demonstrated it is possible that the AAC(3)IV gene for apramycin resistance was on a non-transferable plasmid or was in the chromosome. The single isolate from the Guy's

and St Thomas's Hospital collection which reacted with the probe did not have a high MIC value for apramycin nor a transferable plasmid. It may be, however, that the gene was present but the enzyme not expressed.

Two recent reports describe a high degree of genetic homology between apramycin resistance plasmids from human and animal sources. Salauze and colleagues [13] found that isolates from both humans and animals in France were also resistant to the aminoglycoside hygromycin and that the gene for this resistance was adjacent to that for the AAC(3)IV enzyme, which together formed an operon which was transferable as a whole. As hygromycin is only used in animals in France this has been interpreted as further evidence that the AAC(3)IV gene may have originated in enteric bacteria of animals from which it has transferred to human *E. coli*. It would then be reasonable to presume that the use of apramycin, gentamicin or hygromycin in animals or the use of gentamicin, tobramycin and netilmicin in man has been the selection pressure for emergence of this resistance. Chaslus-Dancla and colleagues [14] also suggest that plasmid exchange has occurred between human and animal *E. coli* as their comparison of plasmids from animal and human isolates revealed similar restriction enzyme profiles.

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