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Apramycin resistance plasmids in *Escherichia coli*: possible transfer to *Salmonella typhimurium* in calves

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SUMMARY

An outbreak of salmonellosis in calves was monitored for persistence of *Salmonella typhimurium* excretion in faeces and the effect of treatment with apramycin. Prior to treatment apramycin-resistant *Escherichia coli* were present but all *S. typhimurium* isolates were sensitive. Following the treatment of six calves with apramycin, apramycin-resistant *S. typhimurium* were isolated from two treated calves and one untreated calf. Plasmid profiles of *E. coli* and *S. typhimurium* were compared and plasmids conferring resistance to apramycin and several other antibiotics were transferred by conjugation *in vitro* from calf *E. coli* and *S. typhimurium* isolates to *E. coli* K-12 and from *E. coli* to *S. typhimurium*. The plasmids conjugated with high frequency *in vitro* from *E. coli* to *S. typhimurium*, and hybridized to a DNA probe specific for the gene encoding aminoglycoside acetyltransferase 3-IV (AAC(3)-IV) which confers resistance to apramycin, gentamicin, netilmicin and tobramycin.

INTRODUCTION

Salmonella typhimurium phage type 204c is the serotype most commonly isolated from clinical cases of salmonellosis in calves in Great Britain [1]. *S. typhimurium* is a common cause of salmonella food poisoning in man in the UK and can cause extra-intestinal infection [2]. Since 1981, the overall incidence of *S. typhimurium* isolates from man resistant to antibiotics has not changed but the incidence of multiple-resistant strains has increased [3]. There has been recent interest in the presence of plasmids in salmonella and *E. coli* which carry the gene for the enzyme AAC(3)IV which confers resistance to apramycin and other aminoglycosides including gentamicin, tobramycin and netilmicin [4–7]. Apramycin was licensed in 1978 in the UK for use in animals but not man and since 1980 animal salmonellas and *E. coli* isolates received by the Central Veterinary Laboratory have been monitored for resistance to this antibiotic [7]. It has been suggested that the veterinary use of apramycin has led to an increase in apramycin-resistant *S. typhimurium* 204c in calves which may be transmitted to man [6, 8]. Indeed apramycin-resistant *S. typhimurium* 204c isolates have been

found in human infections in the UK [6, 8], and apramycin-resistant *S. typhimurium* has been isolated from humans in Belgium [9].

An outbreak of salmonellosis in calves caused by *S. typhimurium* phage type 204c is described in which a plasmid carrying the AAC(3)IV enzyme was apparently transferred from *E. coli* to *S. typhimurium in vivo*.

MATERIALS AND METHODS

Description of the outbreak

Eighteen 1-week-old Limousin-cross calves had been purchased from a dealer who had previously purchased the calves at various auctions. They were housed in individual pens with wooden partitions and flooring. Clinical signs of salmonellosis were recognized 1 week after the calves arrived on the farm. Signs included depression, inappetance, watery diarrhoea sometimes with mucosal shreds and blood, pyrexia, dyspnoea and ocular and nasal discharges. Four calves subsequently died despite immediate treatment with amoxycillin and intravenous fluid therapy. Treatment with apramycin of the remaining calves with enteritis and/or septicaemia commenced 17 days after their arrival on the farm when it was found that the salmonella isolates were resistant to amoxycillin but sensitive to apramycin. All six calves treated with apramycin recovered.

Isolation and identification of E. coli *and salmonella*

Isolations from rectal swabs were made either immediately or after storage in transport medium at 4 °C or −20 °C. Salmonellas were isolated either by direct inoculation on to Brilliant Green Agar (BGA) (Oxoid CM329) and incubation at 37 °C overnight or by overnight incubation in Rappaport–Vassiliadis (RV) Enrichment Broth (Oxoid CM669) at 42 °C followed by inoculation of approximately 0·2 ml on to BGA. Apramycin-resistant salmonellas were isolated on BGA incorporating 16 μg/ml apramycin sulphate (Dista Products). Apramycin-resistant *E. coli* were isolated on MacConkey Agar No. 3 (Oxoid CM115) incorporating 32 μg/ml apramycin sulphate. Both salmonellas and *E. coli* were identified biochemically using the API 20E kit (API System). Serotyping of samples was done using Difco Salmonella Antiserum (Difco Laboratories) and confirmed by G. Bennett at the Lasswade Veterinary Laboratory, Midlothian, Scotland. Phage typing was arranged by D. S. Munro at the Salmonella Reference Laboratory, Stobhill General Hospital, Glasgow.

Antibiotic sensitivity

Antibiotic sensitivity tests were done by controlled disk diffusion with Isosensitest agar (Oxoid CM471) and Oxoid disks [10]. *E. coli* NCTC 10418 was used as a sensitive control except that NCTC 11560 was used as a sensitive control for β-lactamase producers. Disks containing the following amounts of antibiotic (μg) were used: amikacin 30 (Ak), amoxycillin and clavulanic acid 30 (Ac), ampicillin 10 (Am), apramycin 15 (Ap), chloramphenicol 10 (Cm), ciprofloxacin 5 (Cp), compound sulphonamides 500 (Su), furazolidone 15 (Fr), gentamicin 5 (Ge), kanamycin 5 (Kn), nalidixic acid 30 (Nx), neomycin 10 (Ne), netilmicin 10 (Nt),

oxytetracycline 30 (Tc), spectinomycin 25 (Sp), streptomycin 25 (Sm), compound sulphonamides 500 (Su), tobramycin 10 (Tb), trimethoprim 5 (Tm). Isolates were deemed resistant if the zone of inhibition around the disks was $\leqslant 3$ mm radius or the zone was $\geqslant 3$ mm less than the control zone.

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; gentamicin sulphate (Sigma) 4, 8, 16, 32, 64, and 128 μg/ml.

Conjugations

Plasmids were transferred by conjugation in broth matings using a protocol adapted from Datta [11]. Recipients were either nalidixic acid-resistant *E. coli* K-12 or apramycin-sensitive *S. typhimurium*. Transconjugants were isolated on MacConkey Agar No. 3 incorporating nalidixic acid 30 μg/ml (Sigma) and apramycin sulphate 32 μg/ml. *S. typhimurium* transconjugants were isolated on BGA incorporating apramycin sulphate 16 μg/ml.

Plasmid analysis

Routine plasmid extractions were done using the method of Kado and Liu [12] and plasmids for restriction enzyme digestion were prepared using the method of Bennett and colleagues [13]. Restriction enzyme digestion was done as described by Sambrook and colleagues [14]. DNA was separated by electrophoresis in 0·7% agarose gels. Molecular weights of plasmids were determined by comparison with four reference plasmids carried in *E. coli* strain 39R861 [6]. Molecular weights of linear DNA were determined by comparison with *Hin*d III-digested λ phage [14].

The plasmid pWP701 [15] was kindly provided by W. Piepersburg (University of Munich, Germany). A 1·65 kb *Pst* I 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 and used as a probe in colony hybridizations [14].

RESULTS

Isolation of apramycin-resistant S. typhimurium *and* E. coli

Calves were sampled on 12 occasions between days 16 and 120 after arrival on the farm and samples were then taken monthly from days 244 to 332. Large numbers of *S. typhimurium* 204c were isolated from 10 of 18 calves between days 16 and 31 or at necropsy. No *S. typhimurium* were subsequently isolated from any calves. Apramycin-resistant *S. typhimurium* were isolated from one calf (A) on days 21, 23 and 28 and occasional apramycin-resistant colonies of *S. typhimurium* from two others calves (B and C) on days 28 and 31 respectively. Calves A and B were being treated with apramycin but calf C was not. Apramycin-resistant *S. typhimurium* were not isolated prior to commencement of treatment with apramycin on day 17.

The calves were not treated with apramycin or any other antibiotic after day 31, apart from long-acting oxytetracycline administered to five calves between days

150 and 156 and to all calves on day 174 for the treatment of pneumonia and/or interdigital dermatitis. Apramycin-resistant *E. coli* were isolated from rectal swabs taken on day 16 and could still be isolated from calf faeces on day 332. The number of calves excreting apramycin-resistant *E. coli* on any one day varied from 4 of 14 prior to treatment with apramycin to 11 of 14 after six calves had been treated each for 5–7 days during a total of 13 days. After cessation of apramycin treatment, the number of calves excreting detectable amounts of apramycin-resistant *E. coli* decreased, although on days 70 and 332, 11 of 14 and 13 of 14 calves respectively were found to be excreting apramycin-resistant *E. coli* in their faeces. Apramycin-resistant *E. coli* which persisted until slaughter at around day 332 in calves A and B were not recognized as pathogens as they possessed no specific adhesins, did not produce LT, VT, STa or CNF toxins and were untypable (courtesy of Dr C. Wray, Central Veterinary Laboratory, Weybridge).

S. typhimurium was isolated from the floor and drains of the calf unit on several occasions; even after the calf unit had been empty for 8 weeks and steam cleaning, disinfection and whitewashing had taken place twice. After cleaning and fumigation for a third time no salmonellas were isolated.

Comparison of E. coli *and* S. typhimurium

All apramycin-sensitive *S. typhimurium* isolates were found to have the same antibiotic resistance pattern (Am/Cm/Fr/Tc/Su/Sp/Tm) and the same plasmid profile, with three plasmids of approximate molecular weight 147, 92 and 12 kb (Fig. 1, lane 3). All isolates tested were *S. typhimurium* phage-type 204c. Apramycin-resistant *S. typhimurium* from the three calves A, B and C contained a fourth plasmid of approximately 100 kb (Fig. 1, lane 5). The 100 kb plasmids transferred by conjugation *in vitro* to *E. coli* K-12 with high frequency, hybridized to the AAC(3)IV-specific probe, and all conferred resistance to apramycin (MIC $\geqslant$ 1024 μg/ml), gentamicin (MIC 32 μg/ml), netilmicin and tobramycin. In addition, apramycin resistance plasmids from *E. coli* and *S. typhimurium* isolates from calves A and C conferred resistance to several other antibiotics (Table 1).

S. typhimurium and *E. coli* isolates from calf A were compared in more detail. Apramycin-resistant *S. typhimurium* differed from the sensitive isolates by having additional resistance to Ap/Ge/Kn/Ne/Nt/Sm/Tb (Table 1). An apramycin-resistant *E. coli* isolated from calf A was conjugated with *E. coli* K-12 and with an isolate of apramycin-sensitive *S. typhimurium* from calf A. The plasmid profiles of the various isolates and transconjugants are shown in Fig. 1. A plasmid of approximately 100 kb was found to transfer by conjugation readily between the calf isolate of *E. coli*, *E. coli* K-12 and *S. typhimurium*, and conferred resistance to Am/Ap/Ge/Kn/Ne/Nt/Sm/Tb. Plasmids transferred to *E. coli* K-12 from both apramycin-resistant *E. coli* and *S. typhimurium* isolated from calves were indistinguishable by digestion with the enzymes *Bam*H I and *Eco*R I.

To assess the possibility that the apramycin-resistant *S. typhimurium* may have occurred by *in vitro* conjugation during incubation of swabs in RV broth, conjugant mixtures of apramycin-resistant *E. coli* and apramycin-sensitive *S. typhimurium* from calves A and B were incubated in RV and nutrient broths overnight at both 37 and 42 °C. Conjugation occurred readily in nutrient broth at both temperatures: apramycin-resistant *S. typhimurium* were isolated overall on

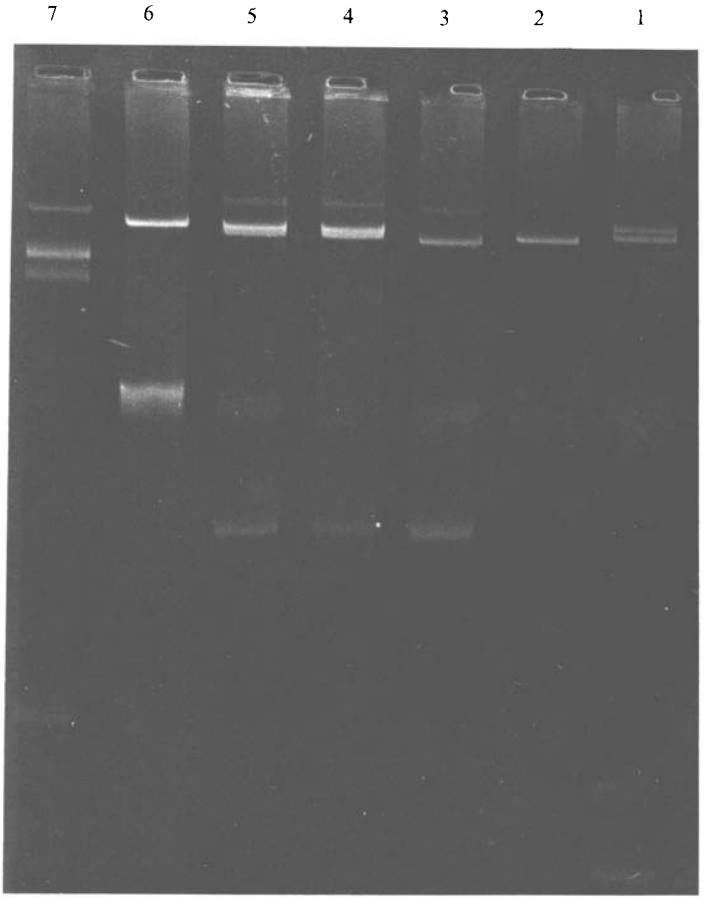

Fig. 1. Plasmid profiles of *E. coli*, *S. typhimurium* and transconjugants. Lane 1, apramycin-resistant *E. coli* (calf A); lane 2, transconjugant of lane 1; lane 3, apramycin-sensitive *S. typhimurium* (calf A); lane 4, apramycin-resistant *S. typhimurium* (calf A) transconjugant; lane 5, apramycin-resistant *S. typhimurium* isolated *in vivo* (calf A); lane 6, *E. coli* K-12 transconjugant of lane 5; lane 7, plasmid marker 39R containing plasmids of molecular weights 151, 65, 37 and 7 kb.

Table 1. *Antibiotic resistance profiles of apramycin-resistance plasmids from* S. typhimurium *isolated from calves*

Calf	Resistance profile										
A	Am	Ap	Ge	Kn	Ne	Nt	—	—	Tb	Sm	—
B	—	Ap	Ge	—	—	Nt	—	—	Tb	—	—
C	Am	Ap	Ge	Kn	Ne	Nt	Tc	Sp	Tb	Sm	Tm

77 of 80 occasions. In RV broth, however, *in vitro* conjugation occurred on only 1 of 40 occasions at 37 °C and 1 of 40 occasions at 42 °C (Table 2).

Apramycin-resistant *E. coli* were also isolated from pigs on the same farm. Conjugation of pig isolates with *E. coli* K-12 demonstrated that resistance was plasmid-mediated but that the plasmid found in pig *E. coli* was consistently smaller (92 kb) than that found in the calves.

Table 2. In vitro *conjugation and transfer of apramycin resistance from* E. coli *to* S. typhimurium *in nutrient and RV broth*

	Number of incubations producing apramycin-resistant *S. typhimurium* out of 20 attempts in total			
	Nutrient broth		RV broth	
Calf*	37 °C	42 °C	37 °C	42 °C
A	20†	20†	1	0
B	18†	19†	0	1§

* Calf from which apramycin-resistant *E. coli* was isolated.
† > 30 apramycin-resistant *S. typhimurium* colonies.
‡ One apramycin-resistant *S. typhimurium* colony.
§ Four apramycin-resistant *S. typhimurium* colonies.

DISCUSSION

In this study, treatment of calves with apramycin selected for apramycin resistance in *E. coli* and *S. typhimurium* in the intestine. The plasmids isolated from *E. coli* and *S. typhimurium* were similar as confirmed by their molecular weights (100 kb), identical antibiotic resistance, hybridization to the AAC(3)IV probe and identical profile after digestion with *Bam*H I and *Eco*R I.

It is possible that plasmid transfer from *E. coli* to *S. typhimurium* may have occurred *in vitro* during isolation in the laboratory rather than *in vivo*, and this might explain the occasional isolation of small numbers of apramycin-resistant *S. typhimurium* from calves B and C. Alternatively, isolation of *S. typhimurium* from calf C, which had not been treated with apramycin, may have been due to transmission from calf A which was housed in the next pen. However, conjugation of *E. coli* and *S. typhimurium* in RV broth proved to be very inefficient, and *in vitro* transfer therefore seems an unlikely explanation for the isolation of apramycin-resistant *S. typhimurium* in large numbers from calf A on three consecutive occasions. Rather, these findings suggest that a transfer of a multiple-resistance plasmid from *E. coli* to *S. typhimurium* may have occurred in the intestine of calf A during treatment with apramycin.

The apramycin-resistance plasmids obtained from *S. typhimurium* from the three calves were of similar size and all conferred resistance to apramycin, gentamicin, netilmicin and tobramycin. Plasmids from calves A and C conferred resistance to an additional four and seven antibiotics respectively. This variation may have been due to the movement of transposons between plasmids during the outbreak, or it may reflect the different origins of the calves and therefore several different sources of plasmids.

Despite the development of apramycin resistance in *S. typhimurium* all calves that were treated with apramycin recovered from their illness. *S. typhimurium* was not isolated from calves after day 31 and therefore transfer of the apramycin resistance plasmid to *S. typhimurium* did not enhance the ability of this organism to persist. *S. typhimurium* did not persist in the gut flora of the calves, in keeping with Kirby and Wray's observations [16] that calves have usually stopped

excreting *S. typhimurium* by the time they reach slaughter weight. Thus it would be unlikely that apramycin-resistant salmonellas would enter the food chain from an outbreak such as this.

Rectal swabs were not taken from all the calves until day 16 of the outbreak so it is not known from where the calves acquired apramycin-resistant *E. coli*. Apramycin-resistant *E. coli* had been isolated from pigs on the farm prior to the arrival of the calves, but are unlikely to have been the source of apramycin-resistance in the calves as the calves and pigs were kept physically separate, attended to by different farm workers and apramycin resistance in the pig isolates was mediated by a smaller plasmid than that found in the calves.

It is possible that the use of oxytetracycline may have encouraged the persistence of apramycin and tetracycline-resistant *E. coli* in calf C. However, from day 175 no antibiotics were given and resistant *E. coli* persisted in the faecal flora of calves for a further 5 months, possibly as a consequence of the calves being loose-housed in two pens from day 56 thereby enabling cycling of the bacterial gut population via faecal contamination of feed and bedding.

The results of this study have shown that large plasmids of *E. coli* with molecular weight of approximately 100 kb conferred resistance to apramycin. The original source(s) of *E. coli* carrying transferable apramycin resistance plasmids remains unclear since the calves were not sampled prior to the outbreak. These strains of *E. coli* were able to persist in the calf intestine for several months and could therefore act as a reservoir of transferable resistance. Transfer of a plasmid to *S. typhimurium* appeared to occur in at least one calf. Despite this, all calves including calf A (infected with apramycin-resistant *S. typhimurium*) recovered and in no case did any *S. typhimurium* persist in the calves.

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