# Antimicrobial resistance of *Salmonella enterica* Typhimurium DT104 isolates and investigation of strains with transferable apramycin resistance

J. C. LOW[1], M. ANGUS[2], G. HOPKINS[3], D. MUNRO[4] AND S. C. RANKIN[4]

[1] *SACVS Edinburgh, Bush Estate, Penicuik, Midlothian EH26 0QE*
[2] *SACVS St Boswells, Greycrook, St Boswells, Borders TD6 0EH*
[3] *SACVS Thurso, Janetstown, Thurso KW14 7XF*
[4] *Scottish Salmonella Reference Laboratory, Stobhill NHS Trust, Glasgow G21 3UW*

## SUMMARY

An examination of salmonella isolates collected by the Scottish Agricultural College Veterinary Services Division from April 1994 to May 1995 was conducted to determine the extent to which *Salmonella enterica* serotype Typhimurium phage type 104 (DT104) occurred and to investigate the antimicrobial resistance patterns of isolates. Typhimurium DT104 was the predominant salmonella and was isolated from nine species of animal. All isolates of this phage type possessed resistance to at least one antimicrobial and 98 % of the isolates were resistant to multiple antimicrobials with R-type ACTSp the predominant resistance pattern. Various other resistance patterns were identified and transferable resistance to the veterinary aminoglycoside antimicrobial apramycin was demonstrated in three strains. A retrospective study for gentamicin resistance in isolates from the Scottish Salmonella Reference Laboratory collection revealed a human isolate of Typhimurium DT104 resistant to gentamicin but sensitive to apramycin and a bovine isolate with apramycin and gentamicin resistance.

## INTRODUCTION

*Salmonella enterica* serotype Typhimurium phage type 104 (DT104) has become the most commonly isolated strain of salmonella in cattle in the UK [1]. In Scotland in 1993 it was the major phage type of Typhimurium isolated from humans, cattle, pigs and sheep [2] and many of the isolates from cattle have been shown to possess *in vitro* resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline (R-type ACSSuT). This has been attributed to chromosomal integration of resistance genes [1]. The occurrence of this strain was examined and the extent to which veterinary isolates of Typhimurium DT104 in Scotland have become antimicrobial-resistant was investigated. This study demonstrated the emergence of apramycin resistance in Typhimurium DT104 which was further examined

in isolates from both the veterinary and human populations.

Gentamicin resistance was first reported in Typhimurium strains in 1982 [3]. The resistance was shown to be transferable and the plasmids responsible for this resistance specified the enzyme 3-*N*-aminoglycoside acetyltransferase (AAC(3)IV) [4] which also conferred resistance to related amino-glycoside antibiotics including apramycin. Apramycin-resistance plasmids have been since identified in *E. coli* and some salmonella serotypes including Typhimurium [5–9]. In salmonella these plasmids have all belonged to incompatibility group I (*Inc* I) [6, 7]. Resistance to gentamicin and related amino-glycosides in Typhimurium has primarily been identified in phage type 204C (DT204c) and from 1988 the percentage of veterinary salmonella isolates, in England and Wales, resistant to apramycin, has

decreased from 10·1 to 0·7%, which results from a proportional decrease in the number of DT204c isolated during this period [10].

## MATERIALS AND METHODS

### Isolation and identification of salmonella strains

Isolates of salmonella recovered by Scottish Agricultural College Veterinary Services Division (SACVS) laboratories from clinical specimens referred by veterinary surgeons throughout Scotland from April 1994 to May 1995 were identified by routine biochemical testing in these laboratories and were serotyped and phage typed by the Scottish Salmonella Reference Laboratory (SSRL), Stobhill NHS Trust, Glasgow. Antimicrobial resistance markers were determined at SACVS St Boswells. A retrospective search of the SSRL data base for gentamicin resistance (20 $\mu$g/ml) identified two further Typhimurium DT104 isolates which were also included in the study.

### Antibiotic sensitivity tests

Antibiotic sensitivity tests were performed by controlled disk diffusion on Isosensitest agar (Oxoid CM471) with Oxoid disks. Disks contained the following amounts of antibiotic ($\mu$g): ampicillin 10 (A), apramycin 15 (Ap), chloramphenicol 10 (C), gentamicin 5 (G), tetracycline 10 (T), spectinomycin 25 (Sp), clavulanic acid potentiated amoxycillin 30 (Ac), enrofloxacin 5 (E), furazolidone 15 (F), nalidixic acid 30 (Na), neomycin 10 (N) and trimethoprim potentiated sulphamethoxazole 25 (Sxt). *Escherichia coli* NCTC 10418 was used as a sensitive control and isolates were deemed resistant if the zone of inhibition around the discs was < 3 mm radius or the zone was > 3 mm smaller than the control zone.

Minimal inhibitory concentrations (MICs) of apramycin were determined using multipoint inoculations to replicate test colonies onto Mueller–Hinton agar (Oxoid) that incorporated the following dilutions of apramycin sulphate: 32, 64, 128, 256, 512 and 1024 $\mu$g/ml. E-test strips (AB Biodisk) that produced continuous antibiotic gradients from 0·064 to 1024 $\mu$g were used to determine MICs of gentamicin. For amikacin, tobramycin and netilmicin the E-test strip gradients were from 0·016 to 256 $\mu$g.

### Conjugations

Plasmids were transferred by conjugation in nutrient broth (Oxoid) using a protocol adapted from Datta [11]. The recipient strain was nalidixic acid-resistant *E. coli* K-12 and transconjugants were isolated on MacConkey agar number 3 (Oxoid) that incorporated both nalidixic acid at 30 $\mu$g/ml and apramycin sulphate at 32 $\mu$g/ml.

### Plasmid profiles and restriction fingerprints

Plasmid DNA was examined in crude lysates by a modification of the method of Platt and Sommerville [12]. The molecular weight of plasmids was determined by reference to plasmids of known size (kb); Rts 1 (180), RA-1 (127), R1 (93), R702 (69) and RP4 (54). Supercoiled ladder (Life Technologies, Paisley, UK) was used for the molecular weight estimation of small plasmids (< 16 kb). Plasmid size values incorporated into plasmid profiles were determined on a minimum of two occasions.

Restriction endonuclease fragmentation pattern (REFP) analysis was carried out as previously described in detail [13]. Plasmid DNA was extracted and purified from isolates by an alkaline lysis, phenol extraction and ethanol precipitation method. Restriction enzymes were obtained from Life Technologies and used according to the manufacturer's instructions. Plasmids were designated using a prefix (pSRS) supplied by Dr E. Lederberg, Plasmid Reference Centre, Stanford University, USA.

## RESULTS

In the one-year period 450 salmonella isolates were collected and 302 (67%) were identified as Typhimurium DT104. Two hundred and seventy-three of the isolates of Typhimurium DT104 (90%) were from cattle, and this organism was also recovered from eight other species of domesticated animals. All DT104 isolates tested were resistant to 1 or more antimicrobial and 295 (98%) possessed resistance to 4 or more. The resistance patterns of isolates from different animal species are shown in Table 1. A single multiple resistance type, ACTSp, accounted for 289 (96%) of the DT104 isolates and was recovered from cattle, pigs, sheep, horses and an individual chicken, pigeon and dog.

As it has been established that one plasmid profile type accounts for the majority of multiple resistant DT104 isolates [1], plasmid analysis was performed on those isolates that showed antimicrobial resistance patterns other than ACTSp. Three isolates from cattle

Table 1. *Resistance patterns and source of* Salmonella enterica *serotype Typhimurium DT104 isolates from Scotland 1994–5*

| Resistance pattern | Bovine | Porcine | Ovine | Avian* | Equine | Laprine | Feline | Canine | Total |
|---|---|---|---|---|---|---|---|---|---|
| Sp | | 2 | | | | 2 | 1 | | 5 |
| AT | 2 | | | | | | | | 2 |
| ACTSp | 268 | 11 | 5 | 2 | 2 | | | 1 | 289 |
| ACTSpSxt | 3 | | | | | | | | 3 |
| ACTSpApG | | | 2 | | | | | | 2 |
| ACTSpApGSxt | | 1 | | | | | | | 1 |
| Total | 273 | 14 | 7 | 2 | 2 | 2 | 1 | 1 | 302 |

* Includes isolates from a chicken and pigeon.

Table 2. *Characteristics of* Salmonella enterica *serotype Typhimurium DT104 strains*

| Designation | Source | R-pattern | Plasmid-profile (kb) |
|---|---|---|---|
| 050/23 | Bovine | ACTSpSxt | 95:11:8 |
| 050/24 | Bovine | ACTSpSxt | 95:11:8 |
| 050/43 | Bovine | AT | 120 |
| 050/58 | Feline | Sp | 95:8 |
| 052/P51 | Porcine | ACTSpApGSxt | 95:40:7 |
| 053/P76 | Porcine | Sp | 95 |
| 053/M1828 | Laprine | Sp | 95 |
| 053/M1831 | Laprine | Sp | 95:70 |
| 054/C3192 | Bovine | ACTSpSxt | 95 |
| 055/C4501 | Bovine | AT | 95 |
| 057/S488 | Ovine | ACTSpApG | 95:95 |
| 057/S518 | Ovine | ACTSpApG | 95:95 |
| SR924676 | Bovine | ACTSpApG | 95:95 |
| SR942421 | Human | ACTSpG | 95:20:8 |

were resistant to trimethoprim potentiated sulpha-methoxazole in addition to ACTSp. There were 7 isolates, recovered from 4 animal species, that showed resistance to spectinomycin alone (5) or both ampicillin and tetracycline (2) (Table 1). Two spectinomycin resistant porcine isolates were isolated from the same batch of pigs and therefore only one strain was examined for the presence of plasmids.

Plasmid analysis of the three bovine ACTSp strains with additional trimethoprim potentiated sulpha-methoxazole resistance (Table 2) showed that 050/23 and 050/24, isolated from the same farm, were identical and harboured a 95 kb plasmid together with two additional plasmids of 11 and 8 kb. Of the spectinomycin resistant isolates, 050/58 and 053/M1831 showed the plasmid profiles 95:8 kb and 95:70 kb respectively. Isolates 053/P76, 053/M1828 and 054/C3192 all harboured a 95 kb plasmid alone. Isolate 050/43 harboured a single plasmid of 120 kb

that has been designated pSRS124. This plasmid was shown by REFP analysis to be a cointegrate of a 95 kb serotype associated plasmid (SAP) and one other plasmid (results not shown). In 055/C4501 the 95 kb plasmid was shown to be a variant SAP designated pSRS129.

Three ACTSp resistant strains were also found to be resistant to the veterinary aminoglycoside antibiotic apramycin and one of these was additionally resistant to trimethoprim potentiated sulphamethoxazole (Table 2). Two of these strains (057/S488 and 057/S518) were recovered from sheep on one farm and the isolate (052/P51), which was additionally resistant to trimethoprim potentiated sulphamethoxazole, was recovered from a pig on a different farm.

From the SSRL data base two further gentamicin resistant Typhimurium DT104 isolates were recovered. One from a human (SR942421) isolated in 1994 possessed resistance to gentamicin in addition to ACTSp. The other isolate (SR924676) originated from a bovine in 1992 and was resistant to ACTSP and both apramycin and gentamicin (Table 2).

Apramycin resistance was successfully transferred to *E. coli* K12 from each of the Typhimurium strains of veterinary origin (Table 3). Additional plasmids of 95 kb, other than an SAP, were present in strains 057/S488, 057/S518 and SR924676 and plasmids of this size were also identified in the apramycin resistant transconjugants. The parent strain 052/P51 contained two plasmids of 40 and 7 kb in addition to an SAP. Resistance to apramycin in the transconjugant was linked to transfer of the 40 kb plasmid. The strain of human origin (SR942421) showed the plasmid profile 95:20:8 kb but repeated attempts to transfer any of these plasmids to a recipient *E. coli* K12 failed. Restriction enzyme fragmentation pattern analysis showed the single plasmids present in the trans-

Table 3. *Transferable resistance to apramycin and related aminoglycosides in* Salmonella enterica *serotype* Typhimurium DT104 *strains*

| Designation | Source | A 10 | Ap 15 | C 10 | G 5 | T 10 | Sp 25 | Ac 30 | E 5 | F 15 | Na 30 | N 10 | Sxt 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 052/P51 | Porcine | R | R | R | R | R | R | S | S | S | S | S | R |
| Tx-P51 | Transcon* | S | R | S | R | S | S | S | S | S | R | S | S |
| 057/S518 | Ovine | R | R | R | R | R | R | S | S | S | S | S | S |
| Tx-S518 | Transcon | S | R | S | R | S | S | S | S | S | R | S | S |
| 057/S488 | Ovine | R | R | R | R | R | R | S | S | S | S | S | S |
| Tx-S488 | Transcon | S | R | S | R | S | S | S | S | S | R | S | S |
| SR924676 | Bovine | R | R | R | R | R | R | S | S | S | S | S | S |
| Tx-924676 | Transcon | S | R | S | R | S | S | S | S | S | R | S | S |
| SR942421 | Human | R | S | R | R | R | R | S | S | S | S | S | S |

* Transcon, transconjugant.
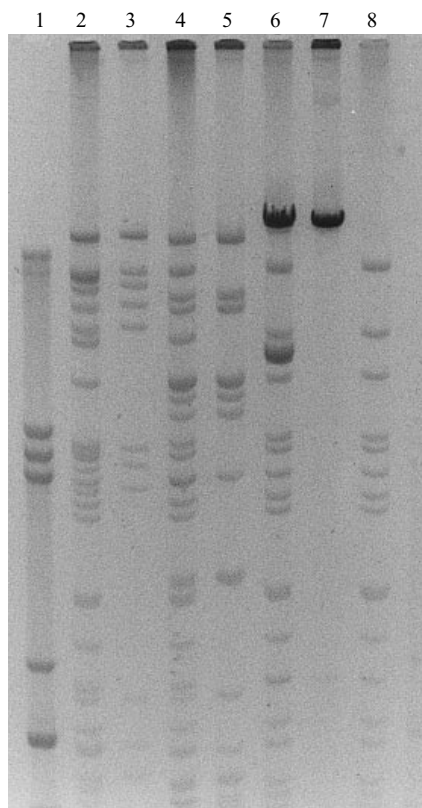


**Fig. 1.** Plasmid REFP of *Salmonella enterica* serotype Typhimurium DT104 strains and transconjugants digested with *Sma* I. Lanes: 1, Lambda phage DNA digested with *Pst* I; 2, SR924676; 3, SR924676 transconjugant; 4, 057/S518; 5, 057/S518 transconjugant; 6, 052/P51; 7, 052/P51 transconjugant; 8, 057/S165 Typhimurium DT104 plasmid control.

conjugants of 057/S488 and 057/S518 to be identical. These were different from each of the other apramycin resistance plasmids which also differed from each other (Fig. 1).

The four veterinary-associated Typhimurium strains possessed high level resistance to apramycin and were also resistant, *in vitro*, to gentamicin, tobramycin and netilmicin but sensitive to amikacin (Table 4). In each case the aminoglycoside MICs for the transconjugants were equal to or lower than the MICs for the parent strains. The Typhimurium of human origin (SR942421), although gentamicin resistant, was sensitive to apramycin, tobramycin, netilmicin and amikacin.

## DISCUSSION

In recent years *Salmonella enterica* serotype Typhimurium DT104 has become the most important salmonella to be dealt with by farm animal veterinarians. In this study Typhimurium DT104 represented 67% of all the salmonella isolates from the eight SACVS laboratories in Scotland. The dominance of isolates with multiple antimicrobial resistance was clearly demonstrated with more than 98% of isolates from cattle being resistant to ACTSp. Threlfall and colleagues [1] recorded 49% of 267 isolates from cattle as resistance-type ACSSuT and attributed this resistance to chromosomal integration of antimicrobial resistance genes. Resistance-type ACTSp strains also possess resistance to streptomycin and sulphonamides (unpublished observations) and it is probable that R-types ACTSp and ACSSuT represent a single 'epidemic strain'. Given the recognized connection of this 'epidemic strain' with human infections [14, 15] it is alarming that this strain formed such a high percentage of isolates from such wide geographic origins. The isolation of Typhimurium DT104 strains, irrespective of R-type, from nine species of animal is not representative of the incidence for each host species because of major differences in submission numbers, but the isolation

Table 4. *Salmonella enterica* serotype Typhimurium DT104 *strains and MICs* ($\mu g/ml$) *to apramycin and related aminoglycosides*

| Designation | Source | Apramycin | Gentamicin | Amikacin | Tobramycin | Netilmicin |
|---|---|---|---|---|---|---|
| 052/P51 | Porcine | > 1024 | 96 | 3 | 128 | 48 |
| Tx-P51 | Transcon* | 1024 | 24 | 0·75 | 24 | 16 |
| 057/S518 | Ovine | > 1024 | 32 | 2 | 48 | 24 |
| Tx-S518 | Transcon | > 1024 | 16 | 1 | 32 | 16 |
| 057/S488 | Ovine | 256 | 64 | 2 | 96 | 32 |
| Tx-S488 | Transcon | 256 | 12 | 0·75 | 16 | 8 |
| SR924676 | Bovine | > 1024 | 64 | 3 | 64 | 32 |
| Tx-924676 | Transcon | 1024 | 12 | 0·75 | 16 | 8 |
| 052/P28† | Porcine | < 32 | 1 | 3 | 1·5 | 1 |
| 057/S165† | Ovine | < 32 | 1 | 3 | 2 | 1 |
| SR942421 | Human | < 32 | 128 | 3 | 6 | 4 |

\* Transcon, transconjugant.
† Control apramycin sensitive ACTSp resistant strains.

of the ACTSp resistant strain from seven species of animal is evidence of its spread to many animal populations. The development of multiple antimicrobial resistance and the current dominance of this single strain has become a major issue for veterinary and medical clinicians alike and it is crucial that the epidemiology of this strain is further investigated in order that adequate control strategies can be devised.

The identification of spectinomycin resistance in 99 % of the strains and the absence of plasmid DNA, other than the SAP, in two isolates with spectinomycin resistance alone (053/P76 and 053/M1828) suggests that the chromosomal integration of antimicrobial resistance genes [1] also involves mechanisms which confer resistance to spectinomycin. This study also identified resistance patterns other than ACTSp and an examination for plasmid DNA revealed a considerable plasmid diversity in these isolates of Typhimurium DT104. The 95 kb variant SAP designated pSRS129 has been previously identified in Typhimurium strains of phage type 104b (unpublished observations). Although it is unclear where the multiple antimicrobial resistant 'epidemic' strain originated the diversity among the endemic DT104 typifies isolates which may be expected in the absence of an 'epidemic' type. Wall and colleagues [14] have also commented upon the occurrence of 20 separate plasmid profiles in Typhimurium DT104 R-type ACSSuT strains. If the isolates of the 'epidemic' type that harbour the SAP alone are regarded as one strain the discrimination achieved by plasmid analysis significantly increases and it is suggested that periodic selection of the 'epidemic' type (Typhimurium DT104 R-type ACTSp) has purged the population of much of

the genetic variation that would otherwise have accumulated. An important consequence of this is that the effective population size of a clonal organism is much less than the actual size [16].

This study included the first recorded identification of transferable resistance plasmids to aminoglycoside antimicrobials, including the veterinary antimicrobial apramycin, in multiple resistant isolates of Typhimurium DT104. The resistance patterns of the transconjugants were consistent with the possession of plasmids that carried the 3-*N*-aminoglycoside acetyltransferase (AAC(3)IV) gene [4, 6, 9]. Plasmids bearing this gene (AAC(3)IV) have been previously demonstrated in *E. coli* and other Typhimurium phage types [6–9].

The levels of antibiotic resistance in the transconjugants were consistent with MICs previously described for strains possessing AAC(3)IV [7–9, 17, 18]. However, it was an interesting feature that in all the transconjugants the MICs for aminoglycoside antimicrobials were equal to or lower than those of the salmonella parent strains. In previous investigations of aminoglycoside resistance plasmids the MICs were similar in both the donor strains and the transconjugants [7, 8, 18]. The lower MICs in the transconjugants described in this study are not thought to be related to plasmid copy but as the donor strains of Typhimurium all possessed resistance to the aminoglycoside antibiotic spectinomycin, which was not transferred with the apramycin resistance, the higher MICs in the parent strains may be the result of synergism between the two antibiotic resistance mechanisms or alternatively to less effective transcription of the resistance genes in the recipient *E. coli*.

Following the introduction of apramycin to veterinary usage resistance to apramycin in Typhimurium DT204c strains of animal origin increased from 0 to 16 % between 1981 and 1990 [19, 20]. The plasmids that bear apramycin resistance genes have been shown to be very diverse and subject to rapid evolutionary divergence [21] and it is thus remarkable that although there was no direct connection between the farms, and 8 years separated the isolation of the parent strains, one of the apramycin resistance plasmids described in this study showed identical REFPs to the Group I plasmids described by Platt and Smith [21].

No further apramycin resistant Typhimurium isolates were recovered from either of the farms in this study but further screening of faecal flora identified widespread apramycin resistance in E. coli and the records for both farms showed the antibiotic had been extensively used for treatment of enteric infections. Chaslus-Dancla and colleagues [17] previously described transmission of resistant strains between animals and Hunter and colleagues [22] described the transfer of apramycin resistance plasmids between E. coli and Typhimurium isolates. Thus it is possible that on the originating farms transfer of apramycin resistance occurred between bacterial species on an infrequent basis. The occurrence of resistance is consistent with usage of apramycin [22] and since the majority of field isolates of Typhimurium DT104 are almost invariably resistant to multiple antimicrobials it is probable that further strains with apramycin resistance will arise through the use of this antimicrobial.

A retrospective examination of isolates of Typhimurium DT104 in the collection held at SSRL identified one human isolate resistant to gentamicin. The aminoglycoside resistance pattern of this isolate was clearly different from that of the veterinary strains, with no demonstrable apramycin resistance, and was consistent with possession of the enzyme AAC(3)I [4]. Gentamicin resistance in human isolates of E. coli and salmonella as a result of the possession of genes encoding AAC(3)IV has been attributed to transfer of resistance genes from bacteria of animal origin [18, 23, 24]. To date this appears not to have occurred with human isolates of Typhimurium DT104 but the occurrence of apramycin resistance in strains with multiple antimicrobial resistance must be of major concern to both veterinary and human clinicians.

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