Epidemiological studies of human and animal *Salmonella* Typhimurium DT104 and DT104b isolates in Ireland

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SUMMARY

A total of 122 human and animal *Salmonella* Typhimurium DT104 isolates and 6 epidemiologically related DT104b isolates from human and animal products were analysed by pulsed-field gel electrophoresis (PFGE). Genomic DNA was subjected to macrorestriction with three enzymes, *Spe*I, *Sfi*I and *Xba*I. A total of 14 restriction fragment length polymorphism (RFLP) profiles were identified when the PFGE patterns from the three enzymes were combined. The majority of isolates (81·2%) exhibited the same RFLP profile. Six animal DT104 isolates, susceptible to enrofloxacin and resistant to naladixic acid, were identified from the antibiotic susceptibility test. Four of these isolates had a different PFGE profile from the common RFLP. In addition, 4 of the 6 isolates were geographically clustered in one region. It was concluded that there was one predominant strain of *S.* Typhimurium DT104 in Ireland and that the potential and selection pressures for emergence of fluoroquinolone-resistant isolates were present.

INTRODUCTION

Infections with the multiple antibiotic resistant *Salmonella* Typhimurium DT104 occur with increasing frequency in animals and man [1] and the hospitalization and mortality rates in humans associated with this serotype are higher than for other *Salmonella* serotypes [2]. *S.* Typhimurium DT104 is primarily a pathogen of cattle but species such as cats, horses, pigs and sheep can also be affected [3, 4]. Infection in all species is unusually persistent and contact with infected animals or consumption of

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contaminated animal products are the most common sources of infection for humans [5, 6].

The ability to characterize and subtype *S.* Typhimurium DT104 isolates is necessary from an epidemiological perspective in order to trace infections in different animal populations and their relationship to human cases. Pulsed-field gel electrophoresis (PFGE) of macrorestricted genomic DNA has proved to be a highly reproducible and discriminating tool for typing *S.* Typhimurium [7–9]. It has also been shown to be able to discriminate between bacterial strains of the same phage type [10, 11].

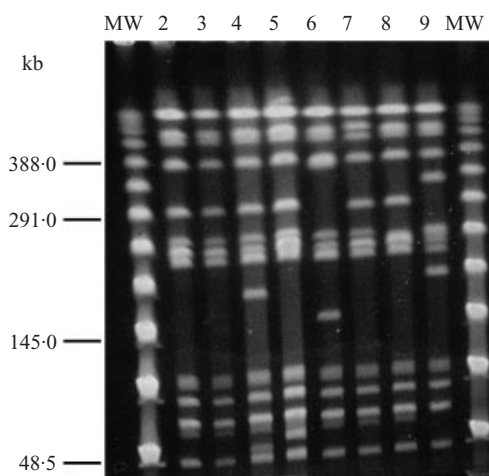The isolation of DT104 strains with resistance to

Fig. 1. PFGE patterns of *Xba*I DNA digests from human and animal *S*. Typhimurium DT104 isolates. Lane 1, DNA marker; 2, X1 (human); 3, X1 (animal); 4, X2; 5, X3; 6, X4; 7, X1 (avian); 8, X5; 9, X6; 10, DNA marker.

fluoroquinolones from animals and humans is a public health concern as fluoroquinolones are the drugs of choice for treating salmonellosis in humans [4]. Resistance to nalidixic acid, a non-fluorinated narrow spectrum quinolone, has been shown to be an indication of resistance or decreased sensitivity to fluoroquinolone compounds amongst *Salmonella* species [12–14]. A survey of *S*. Typhimurium isolates in England and Wales showed that all isolates resistant to nalidixic acid (14%) were also resistant to ciprofloxacin [4]. In Ireland human *S*. Typhimurium DT104 isolates remained susceptible to ciprofloxacin despite a related drug, enrofloxacin, being licensed for use in animals [15].

S. Typhimurium DT104 was first isolated from cattle in Ireland in 1995. Since then its prevalence has increased to a level where in 1998 it represented 94% of all isolates of this serotype from cattle [16]. We compared the PFGE pattern of human, farm animal and food isolates and determined their resistance to antibiotics.

MATERIAL AND METHODS

Bacterial strains

A total of 120 *S*. Typhimurium DT104 isolates from humans, cooked foods, farm animals and poultry (including environmental samples from poultry houses) were used in this study. The farm animal isolates consisted of 63 bovine, 2 equine, 6 ovine and 27 porcine isolates from sporadic cases and were isolated from either diseased carcasses or clinical specimens submitted by veterinary surgeons to the Central Veterinary Laboratory (CVL) and Regional Veterinary Laboratories (RVL) at Cork, Dublin, Kilkenny, Limerick and Sligo during 1997 and 1998. Eleven poultry isolates were from carcasses and environmental samples of avian fluff and litter submitted to the CVL as part of the 'National Poultry Monitoring Programme' during the same period. An isolate from a zoo animal and another from a domestic cat were also included. Eleven human isolates of *S*. Typhimurium DT104 were studied and these were from sporadic clinical cases referred to the Public Health Laboratory of the Eastern Health Board at Cherry Orchard Hospital, Dublin during 1998. A further six epidemiologically related *S*. Typhimurium DT104b isolates (3 from clinical cases, 2 from cooked meats and 1 from a healthy food handler) from a major outbreak of food poisoning in 1998 were also examined. Isolates were identified as *Salmonella* species using triple sugar iron agar (TSI, Oxoid, UK, CM277) and urea agar (Oxoid, UK, CM53) or the Api 20E kit (BioMerieux SA, France). All isolates were serotyped using the Kauffmann–White scheme [17] and *S*. Typhimurium isolates were phage typed by the Laboratory for Enteric Pathogens, Central Public Health Laboratory, Colindale, UK. Isolates were maintained on Dorset Egg slopes and stored at 4 °C.

Preparation of DNA, restriction digestion and PFGE

Isolates were grown on MacConkey agar and incubated at 37 °C overnight. One colony forming unit was subcultured into Luria–Bertani broth and incubated at 37 °C for a further 24 h. The broth cultures were incorporated into agarose plugs by the method described by Corbett-Feeney and Ni Riain [8] with minor modifications. PIV buffer (10 mM Tris, 1 M NaCl, pH 7·6) was used instead of 0·8% saline and the bacterial/agar solution was made by mixing 24 μl of bacterial suspension with 450 μl PIV buffer which was then added to 474 μl of molten 1·3% agarose (Sigma, UK). Genomic DNA was prepared and stored according to the procedures of Maslow and colleagues [18]. DNA was digested by *Xba*I (40 U), *Spe*I (40 U) and *Sfi*I (30 U) (New England Biolabs, UK) according to the manufacturer's instructions. Fragments were separated on 1% agarose gel (Sigma, UK) in a contour-clamped homogenous electric field
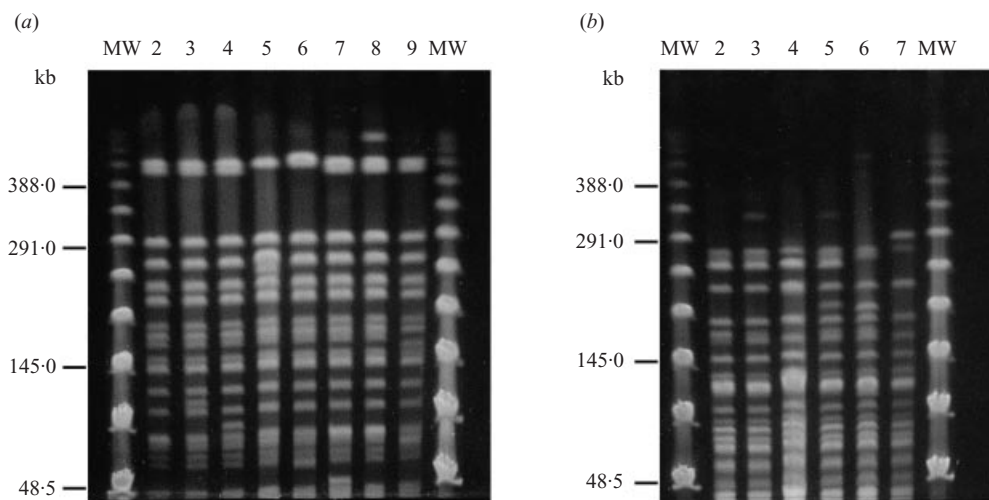
Fig. 2. PFGE patterns of *Spe*I and *Sfi*I DNA digests from human and animal *S.* Typhimurium DT104 isolates. (*a*) *Spe*I patterns. Lane 1, DNA marker; 2, Sp1; 3, Sp2; 4, Sp3; 5, Sp4; 6, Sp5; 7, Sp6; 8, Sp7; 9, Sp8; 10, DNA marker. (*b*) *Sfi*I patterns. Lane 1, DNA marker; 2, Sf1; 3, Sf3; 4, Sf2; 5, Sf4; 6, Sf5; 7, Sf6; 8, DNA marker.

(CHEF-DRII system, BioRad Laboratories, USA) using lambda concatamers (Sigma, UK) as molecular weight markers. The gels were run at 6 V/cm, 14 °C for 20 h with ramp pulse times of 3–28 s, stained in ethidium bromide, and viewed and photographed under UV light with a Polaroid DS34 camera. The DNA banding patterns were compared visually and interpreted according to the criteria of Tenover and colleagues [19].

Antibiotic susceptibility and minimum inhibitory concentrations tests

Antibiotic sensitivity tests were performed on 91 farm animal and 17 human isolates by controlled disk diffusion on Isosensitest agar (Oxoid, UK, CM471). Antibiotic concentrations (μg) in disks (Oxoid, UK) were ampicillin 10 (A), apramycin 15 (Ap), chloramphenicol 10 (C), gentamicin 5 (G), tetracycline 10 (T), clavulanic acid-amoxycillin 30 (Amc), enrofloxacin 5 (En), nalidixic acid 30 (Nal), neomycin 10 (N), framycetin 100 (Fr) and trimethoprim-sulphonamethoxazole 25 (Sxt). Disks containing florfenicol 30 μg (Fc) and spectinomycin 25 μg (Spt) were purchased from Mast Diagnostics, UK. *Escherichia coli* NCTC 10418 was used as a reference control. Isolates were considered resistant if the radius of the zone of inhibition around the discs was < 3 mm after incubation in air at 37 °C for 18 h.

The minimum inhibitory concentrations (MIC) of Sxt, Nal, ciprofloxacin (Cip), and En were determined for 86 isolates using Etest strips (AB Biodisk, Sweden) according to the manufacturer's instructions.

RESULTS

The 111 animal isolates in this study constituted 80·7% of the total *S.* Typhimurium DT104 isolates submitted to the Veterinary Laboratory Services during 1997 and 1998.

Pulsed-field gel electrophoresis of restriction digests of genomic DNA from 128 isolates with the three enzymes *Xba*I, *Spe*I and *Sfi*I generated respectively 13–14, 15–16 and 16–18 well separated and clearly resolvable fragments. Each enzyme produced a distinct banding pattern. A total of 6, 8 and 6 DNA restriction fragment profiles was generated by *Xba*I, *Spe*I and *Sfi*I respectively (Figs. 1, 2) and digest patterns for each enzyme differed from one another by 1–4 fragments. The PFGE patterns for *Xba*I, *Spe*I and *Sfi*I were designated X1, X2, X3, etc., Sp1, Sp2, Sp3, etc., and Sf1, Sf2, Sf3, etc., respectively depending on the number of different patterns. When the banding patterns of the three restriction enzymes were combined, 14 different fragment profiles were identified (Table 1). These combined restriction fragment profiles differed from one another by 1–6 fragments. The majority (78·9%) of bovine, equine, porcine and all avian isolates exhibited pattern 'X1 Sp1 Sf1' (Table 1). This combined RFLP profile was also displayed by isolates from the cat and zoo animal as well as the 11 sporadic human DT104 and the 6 epidemiologically related DT104b isolates. This pattern was widely

Table 1. *PFGE profiles of* Salmonella *Typhimurium DT 104 isolates*

Restriction pattern			Source of isolates					
*Xba*I	*Spe*I	*Sfi*I	Avian	Bovine	Equine	Ovine	Porcine	Environment
X1	Sp1	Sf1	7	46	1	3	24	4
X1	Sp6	Sf2		1				
X1	Sp1	Sf4		1				
X1	Sp3	Sf1		4				
X1	Sp1	Sf3		4			1	
X1	Sp6	Sf1		1				
X1	Sp2	Sf1		1				
X1	Sp7	Sf1		1				
X1	Sp5	Sf2		2	1			
X5	Sp8	Sf1					1	
X6	Sp5	Sf6					1	
X2	Sp4	Sf1		1		2		
X3	Sp1	Sf1				1		
X4	Sp1	Sf5		1				

Table 2. *The distribution of PFGE profiles of* Salmonella *Typhimurium DT 104 from animal carcasses and veterinary clinical specimens*

Restriction pattern			Veterinary Laboratory*				
*Xba*I	*Spe*I	*Sfi*I	Cork	Dublin	Kilkenny	Limerick	Sligo
X1	Sp1	Sf1	8	46	6	23	2
X1	Sp6	Sf2		1			
X1	Sp1	Sf4		1			
X1	Sp3	Sf1	2	1		1	
X1	Sp1	Sf3	4	1			
X1	Sp6	Sf1	1				
X1	Sp2	Sf1	1				
X1	Sp7	Sf1	1				
X1	Sp5	Sf2		3			
X5	Sp8	Sf1		1			
X6	Sp5	Sf6		1			
X2	Sp4	Sf1				3	
X3	Sp1	Sf1					1
X4	Sp1	Sf5			1		

* Isolates submitted to Central and Regional Veterinary laboratories.

distributed throughout the country (Table 2). In the strains with different profiles the greatest diversity was observed amongst those from cattle, sheep and pigs (Table 1) and these strains were mostly confined to specific areas (Table 2).

A number of the animal isolates failed to grow after storage and so antibiotic sensitivity tests were performed on 108 isolates. All isolates exhibited antibiotic resistance type (R-Type) TACSpt. One hundred and seven (99·1%) isolates were also resistant to Fc. In addition, 11 isolates (10·2%) were resistant to Sxt. All isolates were sensitive to Amc, Ap, Fr, N, G and

to the fluoroquinolone En. However, despite being sensitive to the latter, six DT104 animal isolates (5·6%) exhibited resistance to Nal.

The MICs of Sxt, Nal, Cip and En were determined for 17 isolates, which had exhibited resistance to either Sxt or Nal in disk diffusion assays, and 69 randomly selected animal and human isolates. Eleven proved to be resistant to Sxt with an MIC of > 32 μg/ml. These comprised 4 animal and 1 human DT104 isolate and the 6 epidemiologically related DT104b isolates. The MIC values of Cip and En for the Nal sensitive isolates were 0.02 ± 0.01 μg/ml and 0.06 ± 0.01 μg/ml

Table 3. *Characterization of nalidixic acid-resistant* Salmonella Typhimurium *DT104 isolates*

Isolate no.	RVL*	Source	PFGE pattern	MIC (µg/ml)		
				Nal†	Cip†	En†
L1413	Limerick	Ovine	X2 Sp4 Sf1	> 256	0·19	0·50
L276	Limerick	Ovine	X2 Sp4 Sf1	> 256	0·13	0·50
L1553	Limerick	Bovine	X2 Sp4 Sf1	> 256	0·25	0·50
L1801	Limerick	Bovine	X1 Sp1 Sf1	> 256	0·19	0·50
C2558	Cork	Bovine	X1 Sp1 Sf3	> 256	0·38	0·75
ST3	Dublin	Bovine	X1 Sp1 Sf1	> 256	0·25	0·50

* Regional Veterinary Laboratory.
† Nal, nalidixic acid; Cip, ciprofloxacin; En, enrofloxacin.

respectively. The six Nal resistant isolates had a ten-fold increase in MIC values for Cip and En (Table 3). The Nal resistant isolates were from cattle and sheep and the majority had been isolated at the RVL in Limerick (Table 3). Although epidemiologically unrelated, three of them had the same X1 Sp4 Sf1, PFGE pattern, two were of the common X1 Sp1 Sf1 profile and one the X1 Sp1 Sf3 profile.

DISCUSSION

Pulsed-field gel electrophoresis and antimicrobial susceptibility techniques were used to differentiate *S.* Typhimurium isolates of phage type DT104 and DT104b. Considerable genetic homogeneity was demonstrated amongst epidemiological unrelated *S.* Typhimurium DT104 isolates, the majority having a common DNA profile (X1 Sp1 Sf1), which appeared to be stable for the duration of the study. The high prevalence of this genotype, and its widespread distribution throughout the country, suggest it may be endemic amongst animals in Ireland. A comparison of French *S.* Typhimurium strains revealed a clonal similarity between human and bovine strains and diversity between human, poultry and pig strains [20]. However, the 11 human sporadic *S.* Typhimurium DT104 isolates in this study exhibited the same PFGE fragment profile as the endemic strain indicating the spread of a genetically identical clone in humans and animal species. Epidemiologically related isolates from humans and cooked food products also had a similar DNA pattern (X1 Sp1 Sf1) and may also be clonally related to the endemic strain.

A high natural mutation rate has been demonstrated in salmonella [21]. This study identified some genetic diversity within the DT104 phage type but the extent to which mutation, deletion or chromosomal rearrangements were responsible for the minor changes observed after macrorestriction with *Xba*I, *Sfi*I and *Spe*I is not known. Diversity was most prevalent amongst isolates from cattle and to a lesser extent in pigs and sheep. The isolates differed from the combined DNA fragment profile of the main strain type by the presence or absence of 1–6 bands. These differences in PFGE patterns are consistent with relatively minor variations (1–2 genetic events) and consequently the isolates may be considered closely related to, or represent a subtype of, the endemic strain [19].

There is evidence to support the spread of a DT104 clone as isolates from within a country and from different countries sometimes have similar *Xba*I profiles [7, 9, 22]. However, it is difficult to compare published PFGE profiles for DT104 due to different run conditions and pulse times used. The run conditions employed here did not separate *Xba*I restriction fragments smaller than > 48·5 kb and thus were unable to resolve a 10 kb fragment containing antibiotic resistance genes identified by Ridley and Threlfall [9]. Notwithstanding this limitation, the size and number of fragments > 48·5 kb in the *Xba*I RFLP type X1 appeared similar to the Xtm1 PFGE profile of the majority of multiresistant DT104 isolates in the UK [9] and Denmark [7].

It has been reported that the combination of results from two enzymes (*Xba*I and *Spe*I) enhanced the ability of PFGE to characterize *S.* Enteriditis isolates [23]. The introduction of two additional restriction enzymes (*Sfi*I and *Spe*I) with *Xba*I and the combination of their results proved to be more discriminatory than the use of a single enzyme. In addition to the PFGE pattern of the most prevalent strain a further 13 RFLP types, albeit with minor differences, were identified.

Antimicrobial susceptibility test results indicate that the prevalence of antibiotic resistance in Ireland amongst *S.* Typhimurium isolates has increased for ampicillin, chloramphenicol and tetracycline since the previous report [15]. Sulphonamide was not included here as it is not used by veterinary practitioners to treat bacterial diseases. Reports from the UK have shown that salmonella isolates from animals have decreased susceptibility to fluoroquinolones [4, 24]. We found no evidence of resistance to fluoroquinolones although the low numbers of nalidixic acid resistant isolates suggest the potential for the emergence of resistance to ciprofloxacin and enrofloxacin in the future. According to recommended breakpoints none of the nalidixic acid resistant strains could be classified as resistant to ciprofloxacin and enrofloxacin [25, 26]. However, the MIC of enrofloxacin for one of the isolates was 0·75 μg/ml which was close to the intermediate resistant (1 μg/ml) breakpoint [26]. There was no correlation between PFGE profile and fluoroquinolone resistance despite evidence of minor genetic heterogeneity in 4 of the 6 nalidixic acid resistant isolates. The selection pressures that result in fluoroquinolone resistance may be greater in the mid-west region of Ireland as the majority of the nalidixic acid resistant isolates originated from this region.

In conclusion, this study has shown that the majority of *S.* Typhimurium DT104 animal isolates have a common PFGE profile. The results also suggest that at present fluoroquinolone resistance amongst DT104 and DT104b isolates is not a problem in Ireland. However, this requires monitoring as the identification of a small number of nalidixic acid resistant isolates from cattle and sheep particularly in the mid-west region indicates the presence of selection pressures and these may induce further increases in MIC values for fluoroquinolones.

ACKNOWLEDGEMENT

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