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Multiresistant *Salmonella* Typhimurium DT104 infections of humans and domestic animals in the Pacific Northwest of the United States

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SUMMARY

Salmonella Typhimurium definitive type 104 with chromosomally encoded resistance to five or more antimicrobial drugs (R-type ACSSuT+) has been reported increasingly frequently as the cause of human and animal salmonellosis since 1990. Among animal isolates from the northwestern United States (NWUS), R-type ACSSuT+ Typhimurium isolates increased through the early 1990s to comprise 73% of Typhimurium isolates by 1995, but subsequently decreased to comprise only 30% of isolates during 1998. NWUS *S.* Typhimurium R-type ACSSuT+ were consistently (99%) phage typed as DT104 or the closely related DTu302. *S.* Typhimurium isolates from cattle with primary salmonellosis, randomly selected from a national repository, from NWUS were more likely to exhibit R-type ACSSuT+ (19/24, 79%) compared to isolates from other quadrants (17/71, 24%; $P < 0.01$). Human patients infected with R-type ACSSuT+ resided in postal zip code polygons of above average cattle farm density ($P < 0.05$), while patients infected with other R-types showed no similar tendency. Furthermore, humans infected with R-type ACSSuT+ Typhimurium were more likely to report direct contact with livestock ($P < 0.01$) than humans infected with other R-types.

INTRODUCTION

Salmonella enterica serotype Typhimurium phage type DT104 was first reported to be epidemic in both humans and domestic animals in England and Wales. The epidemic strain of DT104 is consistently resistant to at least five antimicrobial drugs, ampicillin, chloramphenicol, streptomycin, sulphonamide, and tetracycline. Increased reports of the multiresistant DT104 strain in humans and agricultural animals have occurred since 1990, and consumption of certain food items and direct contact with animals, particularly ill cattle, were reported as risk factors for human infection [1–4]. We reported the appearance of multiresistant DT104 in wild and domestic animals in the northwestern United States, and showed that humans infected with MR-DT104 tended to live in counties with larger livestock populations [5]. Nationwide, *S.* Typhimurium isolates resistant to the five antimicrobials typical of MR-DT104 or more (R-type ACSSuT+) increased during the early 1990s to comprise approximately one third of all human isolates at CDC surveillance sites in 1996 [6]. The investigations reported here were designed to document further the emergence of this epidemic strain in

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the United States and to evaluate the association between animal and human infections with this agent.

METHODS

Salmonella Typhimurium strains

S. enterica, serovars Typhimurium or Typhimurium (Copenhagen) isolates in this study included: (1) 676 isolates from diagnostic submissions of animal faecal specimens to the Washington Animal Disease Diagnostic Laboratory from 1986 to 1998; all subjected to antimicrobial susceptibility testing and 86 to phage typing; (2) 25 isolates from cattle with clinical salmonellosis randomly selected from each of the four geographic quadrants of the United States obtained from the National Veterinary Services Laboratory (USDA, Ames IA, courtesy of Dr David Miller) during the period 1995–6; of which the 95 viable isolates were subjected to antimicrobial susceptibility testing and to pulsed field gel electrophoresis; (3) 188 isolates from human infections in Washington State in 1994 (courtesy of Jay Lewis, Washington State Department of Health); all subjected to antimicrobial susceptibility testing, and 48 to phage typing.

Geospatial comparisons

Since the population-adjusted rates of human infection with MR-DT104 and the populations of livestock are positively correlated by county in Washington State [5], it was logical to determine if this spatial association held at the finer resolution of zip code polygons. The zip codes of residence of human cases of *S.* Typhimurium in Washington State in 1994 were obtained from the Washington State Department of Health Communicable Diseases reportable disease surveillance system. The zip code polygons in Washington State were classified as to whether they contained above or below the mean number of dairy cattle farms for the state, using data from the 1992 Agricultural census (http://govinfo.kerr.orst.edu/agstateis.html), χ^2 analyses were used to test for association between dairy farm numbers per zip code and the occurrence of R-type ACSSuT+ and other R-type human Typhimurium cases.

Case-control comparison of R-type ACSSuT+ *vs.* other R-types

A one-page questionnaire covering several potential risk factors for human salmonellosis was prepared for self administration to all patients identified as the source of *S.* Typhimurium isolates in 1994. The questionnaire was mailed in December 1995, with a subsequent reminder letter to non-respondents. Data from returned questionnaires were analysed in Epi-Info v. 6.0. Cases were defined as human cases of Typhimurium with the characteristic R-type ACSSuT+. For the purposes of identifying risks specific for R-type ACSSuT+, all *S.* Typhimurium cases from 1994 that were not R-type ACSSuT+ were used as the control population. Data from questionnaires lacking responses to variables under analysis were excluded. For statistically significant associations, stratified analyses were performed, but no evidence of significant interaction or confounding was observed.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by disk diffusion on Mueller–Hinton agar for ampicillin (10 μg), chloramphenicol (30 μg), kanamycin (30 μg), gentamicin (10 μg), trimethoprim (5 μg), streptomycin (10 μg), trimethoprim-sulphamethoxazole (1.25–23.75 μg), tetracycline (30 μg), and sulphonamide (300 μg) (Difco Laboratories, Detroit, MI) [7].

Plasmid profiles

Plasmid DNA was extracted by alkaline lysis [8], electrophoresed in 0.7% agarose gel at 100 V constant current for 4 h in TBE buffer, stained with ethidium bromide, and photographed on an ultraviolet transilluminator.

Phage typing

Eighty-six animal isolates from diagnostic laboratory submissions from the northwestern United States and 48 human isolates from Washington State were phage typed by one of us (R.K.) who was blinded to the source of the isolates [9]. The isolates to be typed were selected to include predominantly those with multiple antimicrobial resistance (30 human and 74 animal isolates with R-type ACSSuT+) as well as isolates representing the range of human ($n = 18$) and animal ($n = 12$) R-types within the study.

Restriction endonuclease digestion pattern (REDP) determination

Chromosomal DNA was prepared by modifications of the method developed by Dr Persing, Mayo Clinic

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[10], as follows. After isolates were grown in 3 ml Luria–Bertani broth (Difco) at 37 °C to 50% T (540 nm), 1 ml was centrifuged (14000 ***g***, 2 min), and the pellet was re-suspended in 250 μl EET buffer (100 mM EDTA, 10 mM EGTA, 10 mM Tris, pH 8.0). This was mixed with 350 μl of melted 1.6% chromosomal grade agarose (BioRad, Hercules, CA) in EET, pipetted into agarose plugs molds (BioRad) and cooled. Plugs were placed in 50 ml centrifuge tubes with 1 ml EET, 200 μg/ml lysozyme, and 0.05% *N*-lauroyl sarcosine sodium (EET-LS) and incubated at 30 °C for 4 h. The EET-LS was replaced with 1 ml of EET containing proteinase K (1 mg/ml) and SDS (1% w/v) and the plugs were incubated at 50 °C overnight. The plugs were rinsed with TE buffer (four 30 min washes in 40 ml 10 mM Tris, 1 mM EDTA, pH 8.0), and stored at 4 °C until analysed. A 3 mm slice of plug from each isolate was pre-incubated in 150 μl of restriction enzyme buffer for 15 min at room temperature. The buffer was replaced with 150 μl of restriction enzyme buffer containing 20 U of *Xba*I (Life Technologies, Gaithersburg, MD) and incubated at 37 °C for 24 h. Restriction fragments were separated by electrophoresis through 1% pulsed-field gel electrophoresis (PFGE) agarose (BioRad) in 0.5× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 13 °C in a CHEF-DRII apparatus (BioRad). Gels were run for 22–25 h at 6 V/cm and a linear ramped pulse time of 5–50 s.

RESULTS

Emergence of DT104, R-type ACSSuT in animals with salmonellosis

Isolates of *S.* Typhimurium and *S.* Typhimurium (Copenhagen) obtained by the Washington Animal Disease Diagnostic Laboratory from cattle, other livestock, and other domestic animals were found frequently to carry multiple antimicrobial resistance traits. From 1993 through 1996, > 60% of such isolates were R-type ACSSuT+, characteristic of MR-DT104. Since 1995, the percentage of isolates with R-type ACSSuT+ decreased to 50% in 1997 and to 30% in 1998 (Fig. 1). Of 104 human and animal origin R-type ACSSuT+ Typhimurium isolates tested, 97 (93.3%) were phage type DT104 and 6 (5.8%) were the closely related provisional phage type DTu302. Most (115/121, 95.0%) isolates of phage type DT104 or u302 exhibited a single REDP following *Xba*I digestion and separation of fragments by PFGE, irrespective of R-type (Table 1).

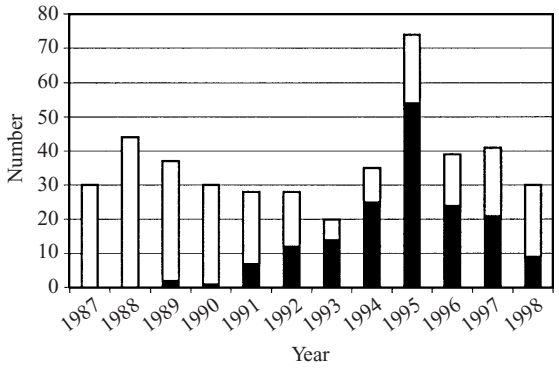

Fig. 1. Annual incidence of isolation of *S.* Typhimurium R-type ACSSuT+ (solid bars) and other R-types (hollow bars) from animals in Washington State from 1988–98.

United States distribution of R-type ACSSuT in bovine salmonellosis

One hundred *S.* Typhimurium isolates from primary cattle disease that had been serotyped at the USDA National Veterinary Services Laboratories in 1995–6 were selected by stratified random sampling such that 25 isolates were requested from each of the four geographic quadrants of the United States. Of the isolates requested, 24, 22, 25 and 24 isolates from the northwestern, northeastern, southwestern, and southeastern United States, respectively, were viable and available for analysis. R-type ACSSuT+ was more frequent in isolates from the northwestern quadrant (19/24, 79%), than the other three quadrants (7/22, 32%; 4/25, 16%; and 6/24, 25%, respectively (4×2 χ^2 24.5, $P < 0.01$). Sixteen of the 19 (84%) R-type ACSSuT+ isolates from cattle in the NW quadrant exhibited the REDP most frequently found in Washington state DT104/u302 isolates (Table 1), while only 7 of the 17 (41%) R-type ACSSuT+ isolates from the other quadrants exhibited this REDP (2×2 χ^2 5.46, $P < 0.05$), indicating that R-type ACSSuT isolates from the NW quadrant were more homogeneous than those from other quadrants.

Geographical associations of Washington state human infections with *S.* Typhimurium

Population-adjusted county-by-county infection rates of humans with *S.* Typhimurium R-type ACSSuT+ were previously found to correlate positively with several agricultural animal and farm census statistics, while non-ACSSuT+ R-types were not significantly associated with any agricultural census statistic

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Table 1. *REDP, phage type (DT) and plasmid analysis of human and animal* Salmonella *Typhimurium isolates selected by R-type*

R-type*	Human†	Animal†	REDP‡	DT§	Plasmid (kb)‖
ACSSuT	13	44	1	104	92
	2		1c	104	92
	2	1	1	104	92, 2·1
		1	1	104	92, 5·0
	1		1	104	92, 40
ACKSSuT	8	22	1	104	92, 2·1
	1		2	104	92
		1	1	104	92, 3·0
ACGKSSuSxtTTm	1		8	10	124
		1	1	u302	92
		1	1a	u302	92, 2·1
		1	1	104	124, 92, 2·1
ACKSSuSxtTTm	2	1	1	u302	92, 85, 5·0, 3·0, 2·1
		1	1b	u302	92, 2·1
ASSuT	5	6	1	104	92
AGKSSuT	1		4	121	92, 3·5
	1		5	193	2·7
ACSSu		2	1	104	92
ACSSuTm	1		1	104	92
ACKSSu	1		1	104	92, 2·1
AKSSuT	1		5	193	124, 3·5
	1		7	193	124, 3·2
		1	1	104	92, 2·1
		1	12	208	92
AKSu	1		1	104	92
ASSu	1		13	UT§	ND
KSSuT	1		11	3	124
S	1		10	132	92, 85
Ssu	1		4	811	ND
SuT	1		3	u302	12
Su	1		9	1	92
Susceptible		1	6	160	ND
		1	8	10	124, $< 2{\cdot}1$, $< 2{\cdot}1$

* R-type is an acronym of the concentrated resistances to A (ampicillin), C (chloramphenicol), G (gentamicin), K (kanamycin), S (streptomycin), Su (triple sulpha), Sxt (trimethoprim–sulphamethoxazole), T (tetracycline) and Tm (trimethoprim).

† Number of isolates tested.

‡ Restriction endonuclease digestion pattern (REDP), where different patterns are indicated by different numbers. Closely related REDP indicated by letters differ from type 1 by one (1a, 1b) or two bands (1c) (32).

§ Phage type (definitive type or DT). UT was untypable.

‖ Plasmid content by molecular size (kilobases).

evaluated [5]. Similar associations between human *S.* Typhimurium R-types and farms were observed in this study at the finer spatial resolution of postal zip code polygons. Agricultural data were available for 379 of the 493 zip codes in the state of Washington in 1994, since the other 114 zip codes were located in non-agricultural areas. These 379 zip codes contained an average of 4.5 farms each (range, 0–153 farms per zip code; 214 or 56% contained one or fewer farms). Of the 91 zip codes containing 5 or more dairy farms, 23 (25%) reported 1 or more human infections with ACSSuT+ isolates during 1994, while only 36 (12.5%) of the 288 zip codes with below average dairy farm numbers reported ACSSuT infections (χ^2 8.59;

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Table 2. *Case* (*R-type ACSSuT+*) – *control* (*other R-types*) *analysis of exposures associated with human cases of* S. *Typhimurium infection in 1994 in Washington State*

Exposure	ACSSuT+	Other	Odds ratio	95% CI	*P*
Undercooked meats	10/26	16/37	0.82	0.26–2.56	0.71
Undercooked eggs	11/30	5/39	3.94	1.04–16.40	0.02
Unpasteurized milk	2/34	0/40	Undefined	Undefined	0.21*
Raw milk dairy products	3/33	1/37	3.60	0.27–194	0.34*
Travel out of US/Canada	0/37	4/43	0.0	0.0–1.72	0.12*
Contact with livestock	12/43	3/52	6.32	1.51–37.0	0.003
Poultry	3/43	2/52	1.88	0.20–23.3	0.66*
Pet birds	3/43	2/52	1.88	0.20–23.3	0.66*
Reptiles	3/43	2/52	1.88	0.20–23.3	0.66*
Dogs, cats, etc.	27/43	24/52	1.56	0.63–3.91	0.11

* Fisher's Exact test.

$P < 0.005$). Non-R-type ACSSuT+ Typhimurium isolates were not significantly correlated to farm numbers within zip codes during this same year.

Risk factors for acquiring DT104 infection

All human cases of salmonellosis from calendar year 1994 were mailed a brief questionnaire regarding possible exposures in December 1995. Ninety-eight (52%) questionnaires with partial or full information were returned. R-type was used to designate cases (ACSSuT+) and controls (non ACSSuT+) in order to identify specific exposures associated with MR-DT104 infection. Exposure to livestock and eating runny or undercooked eggs were associated with MR-DT104 infection (odds ratios > 4, $P < 0.01$) (Table 2)

DISCUSSION

MR-DT104, in all significant respects identical to the strain epidemic in England and Wales, was isolated in increasing numbers from infected domestic animals in Washington State since 1991. In both locations, the prevalence of MR-DT104 relative to other strains increased dramatically to become the predominant type by 1993–4 [1, 5]. In both locations, the agent was isolated from many different host species [1, 11], and contact with cattle was identified as a particular risk for human infection [2].

The emergence of MR-DT104 demonstrates the value of routine surveillance to identify and monitor new strains even of long established pathogens such as Typhimurium. While the MR-DT104 epidemic was initially detected by phage typing in combination with R-typing and plasmid analysis [2], other widely available methods, particularly PFGE, would also have served this purpose [6]. The shortcomings of current global surveillance are also clearly illustrated by the emergence of MR-DT104. After this strain was documented in the United Kingdom, it was identified retrospectively in additional countries [5,12]. Molecular evidence, based on antimicrobial resistance gene cassettes in isolates from Europe, North and South America, and Africa, clearly show MR-DT104 to be a global epidemic clone [13], and the data reported here indicate that increased numbers of reports of infections with this clone first appeared in the northwestern United States and in England and Wales almost simultaneously. The global location of the origin of this clone is not known, but it is of interest that *S*. Typhimurium DT104 R-type ACSSuT (but with plasmid-encoded, rather than chromosomal-encoded antimicrobial resistance traits) comprised a significant percentage of antimicrobial resistant strains in human patients in Hong Kong as early as 1975–1980 [14]. It has long been understood that the tendency for pathogenic salmonellae to circulate as epidemic clones can result in wide swings in the frequency of antimicrobial resistance in local surveillance [15]. MR-DT104 extends this observation to demonstrate that the emergence of a single pathogenic clone can result in global changes in the frequency of antimicrobial resistance traits.

Antimicrobial usage is thought to underlie the origin of bacterial strains that, like MR-DT104, demonstrate multiple antimicrobial resistance traits. If MR-DT104 originated in the developed world, where significant amounts of antimicrobials are used

in livestock and where human infections with *S. enterica* are most commonly foodborne, it is reasonable to attribute its origin to antimicrobial use in livestock [6]. However, if MR-DT104 originated elsewhere in the world, this logic is less convincing. Compared to the developed countries, in much of the rest of the world use of antimicrobials in livestock is less likely to occur, nosocomial and other human-to-human transmission of salmonella strains with multiple resistance are more frequent [16], and access to antimicrobials for therapeutic uses in either humans or domestic animals is poorly controlled [17].

The epidemic of MR-DT104 shows that the (currently unknown) factors that enabled rapid global dissemination of this multiresistant strain are critically important. Livestock are implausible vectors for global dissemination of *S. enterica*, but international human travel, wildlife migration and the global commerce in meat and other human foods and animal feedstuffs all occur at high volume and are therefore more likely candidates. Antimicrobial use on farm premises leads to local amplification and perhaps persistence of resistant strains [18]. There is, however, no clear link between local amplification and global dissemination. Multiple antimicrobial resistance is unnecessary for dissemination of enteric bacteria among livestock, as shown by widely disseminated organisms such as *Escherichia coli* O157:H7 [7] and *Salmonella* Typhimurium DT10 [19] that lack any consistent antimicrobial resistance. Sub-therapeutic antimicrobial use in livestock cannot be linked to the British MR-DT104 epidemic, as the antimicrobials to which it is resistant were banned for use as growth promotants in the United Kingdom following the 1969 Swann report [20]. Antimicrobial use in a host species is unnecessary for infection of that host with a strain carrying multiple antimicrobial resistance, as demonstrated by the frequency of infection of wild birds and mammals by MR-DT104 [4, 5]. Furthermore, the global dissemination of multiresistant *S. enterica* serotype Typhi strains (R-type ACSSuT+) demonstrates that even pathogens limited to humans can emerge and circulate globally, although the failure of this agent to propagate in developed countries attests to the effective control of human-to-human transmission of salmonellosis in these areas [21, 22]. Lastly, there seem to be large differences in the scope of MR-DT104 infection in geographically adjacent areas not clearly linked to differences in antimicrobial use patterns. Like the difference between the northwestern and other areas of the United States shown here for bovine infections in 1995, geographically related areas as near as England and France compared to Denmark and Germany have reported greatly dissimilar rates of human infection following the emergence of MR-DT104 [23–26].

A worldwide consensus is emerging in support of reducing the total amount of antimicrobials used, eliminating inappropriate and non-essential antimicrobial uses in both human and animal medicine, and reserving specific antimicrobials for critical human applications [27]. Rapid global dissemination of bacterial strains with multiple antimicrobial resistance threatens the efficacy of these measures even if the controls are uniformly applied around the world, but especially if they are only locally or sporadically applied [17].

MR-DT104 may have specific biological traits that contribute to its broad dissemination. Previous Typhimurium strains epidemic in livestock populations, such as DT-204c which carried antimicrobial resistance traits similar to those of MR-DT104, did not exceed 5% of human Typhimurium reports despite accounting for $>50\%$ of cattle infections [28], suggesting that MR-DT104 may have a uniquely high human infectivity. In addition, MR-DT104 may have an unusual ability to cause persistent asymptomatic infections in animals, compared to most Typhimurium strains [29,30]. In the United Kingdom, an early report based on a small number of cases indicated that MR-DT104 infection had higher human hospitalization and case-fatality rates than other Typhimurium strains [2]. Similarly, based on a relatively small number of cases, the CDC reported that R-type ACSSuT was more frequently isolated from the blood than other Typhimurium R-types [31]. However, in a recent study of more than 10000 isolations, MR-DT104 was no more likely than other strains of Typhimurium or *S. enterica* serotype Enteriditis to result in bacteraemia [32].

There has been no clear-cut trend in the total annual reports of human Typhimurium infections in the United States or the United Kingdom over the last decade, and the annual reports of infections with the serotype have generally failed to rise despite large increases in MR-DT104 reports. For example, in England and Wales, despite the addition of several thousand annual reports of MR-DT104, the annual *S.* Typhimurium reports for the period 1982–97 showed no consistent upward trend, ranging from 4778 (1993 and 1997) to 7785 (1983) [23]. In contrast, a large increase in the total number of annual *S.*

enterica serotype Enteriditis reports closely paralleled the emergence of phage type 4 during this same time period [23]. Moreover, the total annual reports in cattle have remained stable during the previous emergence and wide dissemination of epidemic Typhimurium strains such as DT204c and DT10 [19, 28]. We analysed all isolates from symptomatic infection available from passive laboratory based surveillance for the included periods, and while this is expected to underestimate substantially the actual incidence of *S.* Typhimurium infection within the populations studied [33], we are unaware of any factors likely to have strongly affected the intensity of surveillance for either human or animal infections from year-to-year within the period studied. Therefore, these data indicate a tendency for newly emergent *S.* Typhimurium strains to displace, rather than add to, previously extant strains, and so raise fundamental questions about the nature of the reservoir resulting in both human and animal Typhimurium infections.

In this study, humans infected with R-type ACSSuT *S.* Typhimurium were more likely to live in zip codes with more cattle farms and were more likely to have had direct contact with livestock, compared to humans infected with other strains of *S.* Typhimurium. While these data are consistent with higher infectivity of MR-DT104 for humans compared to other *S.* Typhimurium strains, it is also possible that these associations simply mark a bovine origin of infection. MR-DT104 accounted for nearly 75% of bovine *S.* Typhimurium infections in 1995, so a conservative interpretation of these associations is that proximity or contact with cattle is a risk for zoonotic *S.* Typhimurium infection and that such infections reflect the predominant strains infecting cattle, which often carry multiple antimicrobial resistance traits. The association between reported consumption of under-cooked eggs and R-type ACSSuT reported here cannot be evaluated in comparison with the frequency of this R-type in poultry infections, as no poultry isolates were available for analysis.

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