Genetic and Phenotypic Variability among *Salmonella enterica* Serovar Typhimurium Isolates from California Dairy Cattle and Humans

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Fifty-six human and 24 adult dairy cattle isolates of *Salmonella enterica* serovar Typhimurium from a single county in California were compared using ribotyping, insertion sequence typing (IS200), pulsed-field gel electrophoresis, plasmid typing, phage typing, and antimicrobial resistance testing. The majority of the isolates fell into one of two groups which were phage types DT104 and DT193. Combining the information from all typing methods, a total of 45 different “clusters” were defined, with 35 of those including only a single isolate. The library of isolates had a high degree of variability, but antibiotic resistance and plasmid typing each defined single clusters in which human or bovine isolates predominated (χ^2 , $P < 0.05$).

Historically, the classification of *Salmonella enterica* into strains has been based on methods such as biotyping, serotyping, antibiotic resistance profiles, phage typing, and plasmid typing. In the past 30 years, the use of molecular techniques to evaluate the relationships between isolates has become common. Often, these methods have been used to investigate disease outbreaks to clarify the relationship between the outbreak source and cases (8). *Salmonella enterica* subsp. *enterica* serovar Typhimurium (serovar Typhimurium) is a common serovar obtained from both humans and animals and is often associated with enteritis and occasionally systemic disease (17). In humans, disease outbreaks due to serovar Typhimurium are commonly associated with foods of animal origin, but the source of *Salmonella* in sporadic cases is often unknown (16). Usually, sporadic cases receive minimal attention, and it is uncommon for a wide array of typing methods to be applied to a library of isolates from these cases. The relative lack of investigations of serovar Typhimurium isolates from sporadic cases results in a significant gap in our knowledge of the ecology of *Salmonella*. We hypothesized that serovar Typhimurium isolated from sporadic cases in humans have some degree of host preference for humans. Furthermore, this preference could be observed by comparing a library of isolates obtained from human and cattle clinical cases and categorizing them using a combination of genotypic and phenotypic methods.

MATERIALS AND METHODS

Isolates. A total of 80 *Salmonella* isolates, 68 serovar Typhimurium and 12 serovar Typhimurium var. Copenhagen (var. 5–), 56 from humans and 24 from animals, were included in this study. Unless otherwise noted, the isolates are collectively referred to as serovar Typhimurium. The bacterial isolates originated from Tulare County, California, between 1996 and 1999. Human isolates were provided by the Tulare County Public Health Laboratory. These had been

submitted from hospitals that made the original isolation, usually from feces, from humans with clinical disease and were serotyped by the California Health and Human Services Laboratory. The animal isolates were from adult dairy cattle with clinical diarrhea and originated from routine submissions to the Tulare branch of the California Animal Health and Food Safety Laboratory. Isolates originated primarily from feces, but a small number were isolated from culture of submitted tissues or tissue from submission of whole animals. All isolates used in this study had been collected previously and stored at -70°C in Microbank cryopreservative beads (Pro-lab diagnostics, Richmond Hill, Ontario, Canada).

Antimicrobial resistance testing. Prior to testing, each frozen stock was streaked for isolation on MacConkey agar plates and incubated at 37°C for ~ 16 h. The antimicrobial resistance of serovar Typhimurium strains was tested using the disk diffusion assay (5) with diameters of the inhibition zones as the outcome variable used in cluster analysis. Briefly, a single isolated colony of each isolate was transferred from the agar plate to brain heart infusion broth and incubated for 5 h at 37°C . Following incubation, the inoculum was diluted to 0.5 McFarland units with 0.85% sterile NaCl solution. A sterile, nontoxic swab was used to streak to a cation-adjusted Mueller-Hinton agar plate (150 by 15 mm) to form a uniform lawn of bacterial growth. Drug-impregnated disks (Table 1) (Fisher Scientific, Pittsburgh, PA) were placed on the agar surface using a disk dispenser and incubated for 16 h at 37°C . Following incubation, the diameters of the inhibition zones around the antibiotic disks (“clear zones”) were measured and recorded. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 23853 were used as control strains.

Isolation of DNA. From the freezer samples, a sterile wooden stick was used to remove a small amount of the frozen liquid media or a single bead from the Microbank tube and place it onto a small area of a blood agar plate. Streaking for isolation was performed with a sterile loop, and the plate was incubated at 37°C overnight. Following incubation, a single colony of each isolate was inoculated in 5 ml of brain heart infusion broth and incubated overnight at 37°C . Genomic DNA was purified as described by Ausubel et al. (2). The DNA pellet was resuspended in 50 μl DNA grade water, the concentration of which was quantified by spectrophotometry and by comparison to standards in an agarose gel, and stored at -20°C .

Ribotyping and IS200. Chromosomal DNA (5 μg) from each isolate was digested with PvuII or HincII to completion to determine the band pattern of rRNA sequence-containing fragments or ribotypes. HincII or PstI digestions were used individually for IS200 mapping. All enzymes used were purchased from Gibco BRL, Life Technologies, Rockville, Md. Southern blots were performed as previously described (2). Briefly, the DNA fragments were separated in a 0.8% agarose gel and transferred by capillary action to a positively charged nylon membrane. Digoxigenin-labeled λ DNA/HindIII fragments were used as molecular weight markers. Southern blot hybridization, labeling, and detection were performed with the DIG-High prime DNA labeling and detection starter kit II (Roche Applied Science, Germany). Films were developed, and images were imported into Diversity Database software (Bio-Rad Laboratories, Hercules CA).

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TABLE 1. Antimicrobial compounds included and codes used in the disk diffusion assay and concentrations of antibiotics in the disks

Antimicrobial	Code	Concn (μg)
Amoxicillin-clavulanic acid	AMC	20, 10
Ampicillin	AMP	10
Cephalothin	CEF	30
Ceftiofur	XNL	30
Ceftriaxone	CRO	30
Chloramphenicol	CHL	30
Florfenicol	FFC	30
Amikacin	AMK	30
Gentamicin	GEN	10
Kanamycin	KAN	30
Neomycin	NEO	30
Streptomycin	STR	10
Sulfisoxazole	SULF	250
Sulfamethoxazole-trimethoprim	SXT	23.75, 1.25
Tetracycline	TET	30
Nalidixic acid	NAL	30
Ciprofloxacin	CIP	5
Spectinomycin	SPT	100

DNA probes. The DNA probe for ribotyping was obtained from the plasmid pKK3535 supplied by M. Mendoza (University of Oviedo, Oviedo, Spain). This plasmid carried a 7.5-kb BamHI fragment encoding the *rmB* operon from *Escherichia coli*-cloned pBR322. The 7.5-kb BamHI fragment was isolated from an agarose gel by using the Gene Clean method (Qbiogene) and labeled with the DIG-High Prime labeling system. Plasmid pIZ46, supplied by J. Casadesus (University of Sevilla, Seville, Spain), was used as the source of IS200 DNA. A 600-bp EcoRI fragment containing IS200 was labeled as described above and used as a hybridization probe.

Pulsed-field gel electrophoresis. DNA samples for pulse-field gel electrophoresis (PFGE) were prepared using a method adopted from the Washington State Department of Health, Public Health Laboratories, and the CDC (9). Electrophoresis was carried out using a Bio-Rad CHEF-DRII system and the following parameters: 6 V/cm; 120° angle, ramp time of 5 to 50 s, run time of 22 h; 14°C. PFGE gels were scanned by using a digital gel documentation system, and images were imported into Diversity Database software (Bio-Rad Laboratories, Hercules CA). A 0.05- to 1-Mb lambda ladder was used as a molecular size standard (Bio-Rad Laboratories, Hercules CA).

Plasmid typing. Plasmids were isolated as described previously (7). Electrophoresis was carried out in a 0.8% agarose gel in Tris-acetate-EDTA buffer at 80 V for approximately 10 cm. The gels were stained with ethidium bromide. Virulence plasmids were identified by comparison with plasmids carried by reference strains (serovar Typhimurium X3000, containing the 100-kb plasmid,

provided by Roy Curtis, Washington University, St Louis, Missouri) and *E. coli* 39R361 carrying plasmids with a molecular size range of 5 to 90 kb (supplied by Linda Ward, Central Public Health Laboratory, United Kingdom). The gels were scanned and documented as described previously.

Phage typing. Phage typing was performed by the National Veterinary Services Laboratory on a fee-for-service basis. For isolates that failed to be clearly categorized, the terms UT and RDNC were utilized. UT indicated that the isolate was untypeable and the tested strain was not lysed by any of the typing phages, and RDNC indicated that the tested bacterial strain reacted with some of the typing phages but did not conform to a standard phage type.

Data analysis. Each of three restriction enzyme-based methods (IS200, ribotype, and PFGE) was initially treated as an independent evaluation. For each of the methods, the complete family of observed electrophoretic restriction fragments utilizing data from all the isolates was determined. The restriction fragment pattern of each isolate was then compared to the complete family of bands. The result of the comparison was a set of binary outcomes reflecting the presence or absence of bands in the isolate compared to the complete set of bands. Similarity clusters were formed and contained only isolates with identity, i.e., within a cluster, the restriction fragments of the isolates were identical.

Using the susceptibility profiles from the antimicrobial sensitivity testing, cluster analysis using zone sizes was performed as described previously (6). Isolates with similar inhibition zone patterns for the tested antimicrobials were formed into clusters. The squared Euclidean distance was used as a measure of dissimilarity, and clusters were determined using the average linkage algorithm. Clusters that consisted of a single isolate were considered "singleton clusters." For descriptive purposes, disk diffusion breakpoint values as described by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (15) for human *E. coli* were used to describe isolates as sensitive, intermediate, or resistant.

The classification of plasmid types was based on the presence or absence of any plasmid and, if plasmids were present, whether they were small plasmids and/or an approximately 90-kb plasmid. The 90-kb plasmid was assumed, based solely on the approximate size, to be the so-called "virulence plasmid" (pSLT), but the presence of *spv* genes was not confirmed. This resulted in four categories: no plasmid, small plasmid(s) only, virulence plasmid only, and virulence plasmid and one or more small plasmids.

The classification clusters from all of the methods were cross-classified against the source of the isolates (human and bovine). For each classification or typing method, independence of source and cluster were evaluated using χ^2 tests for independence (Excel, Microsoft, Redmond, WA).

RESULTS

Antimicrobial resistance. A total of 9 clusters (ABRT) were defined (Table 2), with seven additional isolates being placed in "singleton clusters." ABRT 1, 2, and 3 contained 57 of the 80 isolates in the library. ABRT 1 and 2 were both resistant to a large percentage of the antimicrobials tested, while isolates

TABLE 2. Antimicrobial resistance patterns of serovar Typhimurium isolates grouped into antibiotic resistance (ABRT) clusters 1 through 9 and number of isolates belonging to each cluster^a

ABRT cluster ^c	No. of isolates resistant or sensitive to antibiotic ^b :																		Total no. of isolates (human:bovine)
	AMC	AMP	CEF	XNL	CRO	CHL	FFC	AMK	GEN	KAN	NEO	STR	SULF	SXT	TET	NAL	CIP	SPT	
1	<u>15</u>	6	20	23	29	6	11	21	20	20	17	6	6	21	9	22	33	6	10:11
2	18	6	18	23	27	6	26	22	6	7	10	<u>12</u>	6	6	6	20	34	10	7:2
3	25	23	22	25	28	24	24	23	21	19	18	15	18	25	18	22	31	20	21:6
4	24	22	19	23	29	23	25	20	20	6	7	6	6	19	6	20	33	6	2:0
5	18	6	19	22	26	6	25	22	6	18	18	15	6	6	6	21	32	20	3:1
6	19	6	18	24	27	24	22	23	20	6	10	6	6	22	6	21	29	6	1:1
7	<u>15</u>	6	20	23	28	6	13	23	22	20	18	6	6	20	11	6	26	6	4:0
8	19	6	18	23	31	6	6	23	9	6	7	6	6	20	6	19	35	20	1:1
9	19	6	18	23	26	24	24	22	21	6	10	6	6	21	18	21	32	6	1:1

^a The susceptibility pattern of each cluster is described by the mean inhibition zone size of the isolates to the antibiotics as measured by the disk diffusion assay. The zone sizes in boldface type indicate that the bacteria are resistant to the antibiotic, and the underlined zone sizes indicate that the isolates exhibit intermediate resistance to the antibiotic according to the CLSI guidelines for human *E. coli*. The zone sizes in regular type indicate sensitivity according to the same standard.

^b AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CEF, cephalothin; XNL, ceftiofur; CRO, ceftriaxone; CHL, chloramphenicol; FFC, florfenicol; AMK, amikacin; GEN, gentamicin; KAN, kanamycin; NEO, neomycin; STR, streptomycin; SULF, sulfisoxazole; SXT, sulfamethoxazole-trimethoprim; TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; SPT, spectinomycin.

^c Seven isolates did not group with any other isolates, were classified as "singleton" isolates, and are not included in the table.

within ABRT 3 were pansusceptible. Human isolates were significantly less likely to occur in ABRT 1 than were bovine isolates ($P = 0.009$) (Table 3).

Ribotyping. Based on HincII restriction digests, isolates were classified into 5 different restriction clusters (ribotypes). From the PstI digestion of the genomic DNA, isolates were classified into 6 different ribotypes. The ribotype classifications from the methods were identical, with the exception that one of the ribotypes defined by HincII (containing 46 isolates) was split into two ribotypes (RT1 and RT3) containing 4 and 42 isolates, respectively, by PstI digestion. Given the high degree of concurrence between the two enzymes and the additional ribotype observed using PstI digestion, the classification scheme based on PstI digestion was used in subsequent analyses. The overwhelming majority of the isolates belonged to RT3 and RT4 (69/80). The χ^2 test for independence indicated that human source isolates tended to occur within RT3 at a higher frequency than bovine isolates ($P = 0.079$) (Table 3).

Insertion sequence typing. Based on HincII restriction digests, isolates were grouped into 6 different insertion sequence (IS) clusters. From the PvuII digestion, 8 IS clusters were defined. The IS classifications between the two methods were identical, with the exception that an IS type defined by the HincII digests was subdivided by PvuII digestion to form three groups designated IS1, IS2, and IS6. The IS designations from PvuII digestion were used for all subsequent analyses (Table 3). Eighty percent of the isolates were designated as either IS3 or IS4. Within these IS types, neither of the individual clusters had a statistical association with the source.

Pulsed-field gel electrophoresis. A total of 24 different-sized restriction fragments were found in the overall isolate set following restriction with XbaI. Using this as the basis for classification, 17 PFGE clusters were created. Each PFGE cluster contained 9 to 12 restriction fragments. PFGE types 3, 4, and 9 accounted for nearly 70% of the isolates. PFGE typing did not discriminate between human and bovine sources (Table 3).

Plasmid typing. The majority of the isolates had either no plasmids found (40 isolates) or only the 90-kb (virulence) plasmid found (33 isolates). One or more small plasmids were found in 7 isolates, and in two of those, the 90-kb plasmid was also found. Human isolates accounted for 77.5% (31/40) of the isolates lacking plasmids and 57.6% (19/33) of the isolates with the 90-kb plasmid. Bovine isolates were statistically more likely to have just the 90-kb plasmid rather than other plasmid compositions ($P = 0.042$) (Table 3). Within RT3, 37 of 42 isolates had no plasmid detected, while in the isolates within RT4, all 27 isolates had the 90-kb plasmid present (Table 4).

Phage typing. The isolate set was categorized into 9 phage types not including UT or RDNC. Two phage types, DT104 ($n = 27$, 34% of library) and DT193 ($n = 37$, 46% of library), predominated (Table 3).

Classification summary. The typing methods that returned the largest number of classification clusters were PFGE (17 clusters), phage typing (11 clusters), antibiotic resistance testing (9 clusters plus 7 singleton isolates), and IS typing (8 clusters). Combining the classification results from the 7 methods (ribotyping, IS typing, plasmid typing, phage typing, PFGE, antibiotic resistance testing, and serotyping) used to characterize the 80 serovar Typhimurium isolates, 45 different "clusters" completely described the isolate set. Thirty-five of

TABLE 3. Distribution frequency of isolates of *Salmonella* serovar Typhimurium from sporadic adult dairy cattle and humans in a single California county

Strain type	No. (%) of isolates ^a		<i>P</i> value for χ^2 test for human vs bovine isolates
	Human (<i>n</i> = 56)	Bovine (<i>n</i> = 24)	
Antibiotic resistance clusters			
ABRT 1	10 (17.9)	11 (45.8)*	0.009
ABRT 2	7 (12.5)	2 (8.3)	0.589
ABRT 3	21 (37.5)	6 (25.0)	0.278
ABRT 4	2 (3.6)	0	0.348
ABRT 5	3 (5.4)	1 (4.2)	0.823
ABRT 6	1 (1.8)	1 (4.2)	0.532
ABRT 7	4 (7.1)	0	0.179
ABRT 8	1 (1.8)	1 (4.2)	0.532
ABRT 9	1 (1.8)	1 (4.2)	0.532
Singleton	6 (10.7)	1 (4.2)	0.342
Ribotypes			
RT1	2 (3.6)	2 (8.3)	0.370
RT2	3 (5.4)	1 (4.2)	0.823
RT3	33 (58.9)	9 (37.5)	0.079
RT4	16 (28.6)	11 (45.8)	0.135
RT5	1 (1.8)	0	0.51
RT6	1 (1.8)	1 (4.2)	0.532
IS200 types			
IS1	2 (3.6)	2 (8.3)	0.370
IS2	3 (5.4)	1 (4.2)	0.823
IS3	29 (51.8)	9 (37.5)	0.241
IS4	16 (28.6)	10 (41.7)	0.251
IS5	1 (1.8)	0	0.510
IS6	4 (7.1)	0	0.179
IS7	1 (1.8)	1 (4.2)	0.532
IS8	0	1 (4.2)	0.124
PFGE types			
1	2 (3.6)	2 (8.3)	0.370
2	2 (3.6)	1 (4.2)	0.898
3	12 (21.4)	5 (20.8)	0.952
4	16 (28.6)	11 (45.8)	0.135
5	1 (1.8)	0	0.510
6	1 (1.8)	0	0.510
7	3 (5.4)	0	0.248
8	1 (1.8)	0	0.510
9	9 (16.1)	2 (8.3)	0.357
10	3 (5.4)	2 (8.3)	0.614
11	1 (1.8)	0	0.510
12	1 (1.8)	0	0.510
13	1 (1.8)	0	0.510
14	1 (1.8)	0	0.510
15	1 (1.8)	0	0.510
16	1 (1.8)	0	0.510
17	0	1 (4.2)	0.124
Plasmid types			
Virulence plasmid only	19 (33.9)	14 (58.3)*	0.042
No plasmids	31 (55.4)	9 (37.5)	0.143
Small plasmids only	4 (7.1)	1 (4.2)	0.614
Virulence and small plasmid(s)	2 (3.6)	0	0.348
Phage types			
DT12	1 (1.8)	0	0.510
DT82	0	1 (4.2)	0.124
DT104	16 (28.6)	11 (45.8)	0.135
DT105	1 (1.8)	0	0.510
DT126	1 (1.8)	0	0.510
DT146	1 (1.8)	0	0.510
DT164	1 (1.8)	0	0.510
DT193	29 (51.8)	8 (33.3)	0.129
U302	2 (3.6)	1 (4.2)	0.898
RDNC	1 (1.8)	0	0.510
Untypeable	3 (5.4)	3 (12.5)	0.266

^a *, indicates the host species with significantly larger numbers of isolates from a particular cluster.

TABLE 4. Numbers of isolates with clusters of serovar Typhimurium based on ribotype, IS type, plasmid type, phage type, PFGE type, antibiotic cluster (ABRT cluster), serotype, and host species^a

Ribotype	IS200 type	Plasmid type(s)	Phage type	PFGE cluster	ABRT cluster	Serotype	No. of human:bovine isolates	Total no. of isolates
1	1	None	Untype	1	9	var. Copenhagen (5-)	0:1	1
1	1	Vir	Untype	1	9	var. Copenhagen (5-)	1:0	1
1	1	Vir	Untype	1	6	var. Copenhagen (5-)	1:0	1
1	1	Vir	Untype	1	6	Typhimurium	0:1	1
2	2	Single small	DT193	14	3	var. Copenhagen (5-)	1:0	1
2	2	Single small	DT193	2	4	Typhimurium	1:0	1
2	2	Multiple small	DT193	2	4	Typhimurium	1:0	1
2	2	Vir	DT193	2	Single	Typhimurium	0:1	1
3	3	Multiple small	DT193	10	2	Typhimurium	0:1	1
3	3	None	DT164	3	Single	Typhimurium	1:0	1
3	3	None	DT193	3	2	Typhimurium	1:0	1
3	3	None	DT193	3	3	Typhimurium	3:3	6
3	3	None	DT193	3	3	var. Copenhagen (5-)	3:0	3
3	3	None	DT193	3	8	Typhimurium	0:1	1
3	3	None	DT193	3	Single	Typhimurium	1:0	1
3	3	None	DT193	5	3	var. Copenhagen (5-)	1:0	1
3	3	None	DT193	7	2	Typhimurium	1:0	1
3	3	None	DT193	7	3	Typhimurium	2:0	2
3	3	None	DT193	9	2	Typhimurium	1:0	1
3	3	None	DT193	9	3	Typhimurium	3:1	4
3	3	None	DT193	9	5	Typhimurium	3:0	3
3	3	None	DT193	9	Single	Typhimurium	1:0	1
3	3	None	DT193	10	2	Typhimurium	1:0	1
3	3	None	DT193	10	3	Typhimurium	1:1	2
3	3	None	DT193	11	3	Typhimurium	1:0	1
3	3	None	U302	3	2	Typhimurium	1:1	2
3	3	None	U302	10	2	Typhimurium	1:0	1
3	3	None	Untype	9	2	Typhimurium	1:0	1
3	3	None	Untype	9	5	Typhimurium	0:1	1
3	3	Vir	DT193	3	8	Typhimurium	1:0	1
3	3	Vir	DT193	16	3	Typhimurium	1:0	1
3	6	None	DT193	3	3	Typhimurium	1:0	1
3	6	None	DT12	15	3	Typhimurium	1:0	1
3	6	None	DT105	12	3	Typhimurium	1:0	1
3	6	Multiple small	DT146	13	Single	Typhimurium	1:0	1
4	4	Multiple small + vir	DT104	4	1	Typhimurium	1:0	1
4	4	Single small + vir	DT104	4	Single	Typhimurium	1:0	1
4	4	Vir	DT104	4	1	Typhimurium	8:8	16
4	4	Vir	DT104	4	1	var. Copenhagen (5-)	1:2	3
4	4	Vir	DT104	4	7	Typhimurium	4:0	4
4	4	Vir	DT104	4	Single	Typhimurium	1:0	1
4	8	Vir	DT104	4	1	Typhimurium	0:1	1
5	5	Vir	DT126	6	3	var. Copenhagen (5-)	1:0	1
6	7	None	DT82	8	3	Typhimurium	1:0	1
6	7	Vir	RDNC	17	3	Typhimurium	0:1	1
Total								80

^a Vir, virulence plasmid; untype, untypeable; single, "singleton" cluster.

these clusters contained only one isolate (Table 4). Ribotyping, IS typing, and PFGE typing resulted in differing numbers of classification clusters, but there was a strong tendency for isolates within a given set of clusters defined by PFGE to group within a single cluster as defined by IS typing and, in turn, for those to group within a single cluster as defined by ribotyping.

Based on results from all seven typing methods, the largest single cluster contained 20% of the isolates (16/80) and had equal numbers ($n = 8$) of human and bovine isolates, although proportionately, bovine isolates (8/24, 33%) were more likely to be in this cluster than human isolates (8/56, 14%). Isolates in this cluster belonged to a larger group of 27 isolates which, with the exception of one isolate with a unique IS type, were

characterized by being ribotype 4, IS type 4, PFGE type 4, and phage type DT104. All of the isolates within this larger group have the 90-kb plasmid and all 21 of the isolates within ABRT 1 were contained in this group. Most of the DT193 isolates (29/37) were of human origin. These isolates were largely ribotype and IS type 3 and belonged to ABRT 3. A smaller number of the DT193 isolates belonged to ABRT 2.

DISCUSSION

The methods used in this study segregated isolates into clusters based on different characteristics. Each of the methods provided different levels of discrimination, with ribotyping cre-

ating the fewest clusters and PFGE producing the largest number of clusters. Combining the information obtained from all the methods resulted in the 80 isolates being distributed among 45 clusters and producing many clusters that contained a single isolate. None of the methods was able to detect any evidence of absolute host specificity for these isolates and, in all the methods, human and bovine isolates shared characteristics. This implies that either no host-specific subtypes exist or that the various typing techniques used in this study were unable to detect the differences responsible for such absolute specificity. Within individual methods, however, the majority of the isolates tended to group in a few categories, and there was evidence of possible host preference, as opposed to absolute specificity, from the results of plasmid typing and antimicrobial resistance typing. Each of these methods had individual clusters with significantly larger numbers of bovine isolates than expected. Within the largest clusters, phage typing, ribotyping, and IS typing identified clusters that contained bovine or human isolates at approximately two times the prevalence found in the comparison cluster, but these were not statistically significant at a *P* value of 0.05.

Two groups of isolates predominated in the library, DT104 and DT193, and these two groups were associated to some degree with cattle and humans, respectively, and had somewhat uniform characteristics within the groups by ribotyping, IS typing, and antimicrobial resistance testing. The uniformity of these isolates likely explains why host origin tended to correlate with cluster defined by each of these methods.

The intermethod correlations between ribotyping, IS typing, and PFGE are not surprising given that all three methods have the same underlying principle, restriction enzyme digestion and agarose separation, and vary only in the way in which the information from the resulting DNA fragments is characterized. In the library of isolates evaluated by all three techniques, only two isolates did not support the idea that PFGE was a more discriminating technique than IS typing. One of these isolates is the sole member of IS type 8 and the second was placed in IS 6, which included a variety of PFGE types, but PFGE cluster 6, with this one exception, includes only IS type 3 isolates.

In the study reported here, the DT104 isolates showed near clonality and were all placed within single ribotype, IS type, and PFGE cluster (cluster 4 in all 3 methods). This apparent genetic uniformity in the DT104 isolates is in agreement with a study reported by Baggesen et al. in which they characterized 136 isolates of DT104 and found that, with PFGE using *Xba*I restriction enzyme digestion, all but two isolates could be placed in a single cluster with a single banding pattern. By digesting the DNA with *Bln*I, however, a total of 7 PFGE banding patterns were found (3). In contrast, a different study found 5 PFGE patterns using *Xba*I digestion of 14 DT104 or DT104b isolates (14). The clonal or diverse nature of DT104 can be difficult to discern, as illustrated by a study that used a variable number of tandem repeat-based fingerprinting method which did not group DT104 as a distinct cluster but instead spread it out into groupings with several other phage types and also gave identical fingerprints for U302 and DT104 strains in some cases (13). There were three U302 isolates in this study, and these did not occur within the same cluster as DT104 isolates by any of the methods used. Another large

cluster in the study reported here contained the DT193 isolates and, in agreement with another study which stated that "DT193 is a composite phage type containing several distinct clones and hybrid lines" (4), we found this to be a more diverse group than DT104. In our library of serovar Typhimurium, there were 23 isolates that were DT193; within this group, there were two ribotypes, three IS types, and nine PFGE types. Interestingly, a Spanish study of a diverse library of serovar Typhimurium isolates using *Xba*I-cleaved DNA for PFGE found 26 profiles among 68 isolates (a larger number of profiles in fewer isolates than reported here) and they had several clusters that contained both DT104 and DT193 (11). This is in contrast to the results reported here in which there was no overlap between the PFGE patterns of these two phage types. Previous work has discussed the possibility that the international dissemination of DT104 in the 1980s and 1990s was more likely due to the transmission of a few clonal subtypes rather than reduced antimicrobial sensitivity (10). The finding that two groups within DT193, one that was multiple-drug resistant (ABRT 2) and one that was pansusceptible (ABRT 3), occurred at rates within the human isolates similar to those within the cattle isolates implies that antimicrobial resistance by itself does not result in the presence and dissemination of particular strains of serovar Typhimurium within a given host or population and that factors involved in host preference may play a larger role. The majority of the IS3, RT3 DT193 isolates lacked any plasmids in our assays. The lack of plasmids is unusual, since the *spv* genes of serovar Typhimurium are generally required for virulence and are encoded on the pSLT or related plasmid. It has been reported that the virulence plasmid may exist in either an autonomous or an integrated form, and it is possible that these isolates had integrated the plasmid genes into the chromosome (1, 12). It is also possible that our plasmid assay failed, but this seems unlikely, since the virulence plasmid was identified in other strains.

The grouping or clustering of isolates can be done with a variety of equally valid methods which may provide different clusters, but ultimately, those clusters are the basis on which most conclusions are drawn. In the study reported here, we created clusters in ribotyping, IS typing, and PFGE based on identity between isolates within the same cluster. Thus, the decisions underlying the creation of the clusters were strict, and because isolates were often placed in separate clusters even if they differed by only a single band within the overall pattern, the result may have been the artificial separation of isolates that were closely related. Conversely, the goal of this work was to determine if there was any indication of host preference, and the use of less strict clustering guidelines may have resulted in such evidence being unapparent. Techniques using other criteria, such as the presence or absence of virulence or colonization factors would not suffer from this constraint and might provide for better resolution of the question of host preference. However, the search for particular, known genetic elements would suffer from a failure to detect genetic elements not included in the search strategy nor would novel, previously uncharacterized genetic factors be detected.

The study reported here used multiple genotypic and phenotypic methods to create clusters within a library of 80 serovar Typhimurium isolates that were isolated from adult dairy cattle and sporadic cases of disease in humans. The sample size was

obviously small and the isolates all came from a limited geographical area; therefore, the results may have been confounded by unidentified epidemiologic links between isolates and cannot be extrapolated to other or larger geographical areas. The goal was to determine whether any of the clusters would cosegregate with the host of origin. The only classification methods that identified a statistical relationship at a P value of ≤ 0.05 were antibiotic resistance typing and plasmid typing. Given the small number of bovine isolates, it is possible that the statistical relationships are due to a clonal spread in a limited geographical area rather than identifying host preference. Since humans tend to travel more and consume products imported into the region from elsewhere, this association may not have been as strong in the human source isolates.

When the typing methods were used in aggregate to define clusters, there was no clear correlation between the host from which isolates originated and the cluster(s) into which the isolate was grouped. It is likely that the small numbers of isolates in each of the clusters, as a result of the small size of the library and the large number of clusters generated when the data from all the typing methods was combined, resulted in our inability to find a relationship between host species and any particular cluster if one existed. Another possible explanation for the failure to find any host association when the results from each of the methods were used in aggregate is that no such association exists, and that serovar Typhimurium is a true generalist with no host preferences among subtypes or strains of this serovar. The existence of statistically significant relationships between clusters of isolates (as defined by individual methods) and the host from which they were isolated suggests, however, that host preference by serovar Typhimurium may occur. Studies with a larger number of isolates from a larger variety of host species are warranted.

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