Applied and Environmental Microbiology, Jan. 2010, p. 69–74 Vol. 76, No. 1
0099-2240/10/$12.00 doi:10.1128/AEM.00210-09
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Multilocus Variable-Number Tandem-Repeat Analysis and Plasmid Profiling To Study the Occurrence of *bla*$_{CMY-2}$ within a Pulsed-Field Gel Electrophoresis-Defined Clade of *Salmonella enterica* Serovar Typhimurium▽†

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Received 29 January 2009/Accepted 26 October 2009

***Salmonella enterica* serovar Typhimurium circulating in food animal populations and carrying resistance to antimicrobial agents represents a human health risk. Recently, a new clade of *S.* Typhimurium, WA-TYP035/187, was reported in cattle and humans in the Pacific Northwest, United States of America. The objective of this study was to describe a possible mechanism of acquisition of expanded-spectrum cephalosporin resistance in this clade. Ceftazidime resistance increased steadily among WA-TYP035/187 isolates, from 0% (0/2) in 1999 to 77.8% (28/36) in 2006 (χ^2 for linear trend, *P* value of <0.001). Among 112 bovine-source and 18 human-source isolates, 49 (43.8%) and 12 (66.7%) were resistant to ceftazidime, respectively. Multiple-locus variable-number tandem-repeat analysis (MLVA) and plasmid profiling suggested that resistance was acquired by multiple independent genetic events within the WA-TYP035/187 clade. Given the lack of an obvious reservoir in species other than cattle and a parallel rise in ceftiofur resistance in the bovine-specific serovar *Salmonella enterica* serovar Dublin in the same time frame and region, selection pressure due to the use of the expanded-spectrum cephalosporin drug ceftiofur in cattle is a likely factor driving the increasing cephalosporin resistance of WA-TYP035/187.**

Salmonella enterica serovar Typhimurium is one of the leading causes of food-borne bacterial disease in the United States (5). The proportion of human clinical isolates with resistance to ceftiofur, an expanded-spectrum cephalosporin, increased from 0% in 1996 to 4.5% in 2004 (5). Expanded-spectrum cephalosporin resistance in *Salmonella* is of particular concern because these drugs are commonly used in treatment of pediatric salmonellosis. In addition to limiting the effectiveness of antibiotics in treating multidrug-resistant (MDR) *Salmonella* infections, these infections may be more likely to cause prolonged or severe illnesses than are infections with antimicrobial-susceptible strains (31). Cattle are known to be a reservoir for pathogenic nontyphoidal *Salmonella* and most likely contribute to human cases of MDR salmonellosis (1, 24, 26). Furthermore, recent studies of *Salmonella* isolates from veterinary diagnostic laboratory submissions suggest that antimicrobial resistance, particularly resistance to expanded-spectrum cephalosporins, has increased among northwestern U.S. cattle isolates (11, 12).

We have observed that humans and cattle frequently have infections with the same strains as defined by PFGE and that many of these shared strains have multidrug resistance, including resistance to the expanded-spectrum cephalosporin ceftazidime. One of these shared strains (designated TYP035 at the Washington State Department of Health Public Health Laboratories) emerged among cattle in the late 1990s and was subsequently detected in human infections. In 2004 a *Salmonella* Typhimurium pulsed-field gel electrophoresis (PFGE) type designated TYP187 was detected in cattle (and subsequently in humans); it was distinguishable from TYP035 by only one band and shared a similar resistance phenotype, and its SpeI-PFGE profile was similar to or indistinguishable from that of TYP035 (11). This cattle-associated MDR clade is referred to hereafter as WA-TYP035/187.

These observations indicate that cattle contribute to MDR *Salmonella* infections in humans and that an understanding of the mechanisms of emergence, dissemination, or acquisition of MDR would be of public health importance. This study investigates the increasing incidence of expanded-spectrum cephalosporin resistance in WA-TYP035/187. Variable-number tandem-repeat (VNTR) loci provide polymorphic markers that are the basis of a powerful molecular technique to discriminate isolates within clonal complexes (17, 18, 29, 32). Therefore, we used a combination of multilocus VNTR analysis (MLVA) and *bla*$_{CMY-2}$ plasmid profiling to investigate antimicrobial resistance within the WA-TYP035/187 clade.

MATERIALS AND METHODS

Bacterial isolates. A total of 130 *Salmonella enterica* serovar Typhimurium isolates were used in this study. These were obtained from three sources: the Washington Animal Disease Diagnostic Laboratory (WADDL), the Zoonoses

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 30 October 2009.

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Research Unit (ZRU), and the Field Disease Investigation Unit (FDIU). The FDIU has been banking isolates of *Salmonella* derived from field research projects conducted in cattle herds across the Pacific Northwest for over 20 years. Since 2004, the ZRU has obtained human clinical isolates of *Salmonella* from the Washington State Department of Public Health for comparison with strains circulating in the animal reservoirs.

Phenotypic characterization. All confirmed *S.* Typhimurium isolates were serotyped at the USDA National Veterinary Services Laboratory, Ames, IA. To test for antimicrobial resistance, the Kirby-Bauer disk diffusion method (2) was used following Clinical and Laboratory Standards Institute (CLSI) guidelines (8). The following antimicrobial disks were used: ampicillin (10 μg), ceftazidime (30 μg), chloramphenicol (30 μg), gentamicin (10 μg), amoxicillin-clavulanic acid (20/10 μg, respectively), kanamycin (30 μg), streptomycin (10 μg), tetracycline (30 μg), triple-sulfa (a combination of sulfadiazine, sulfamethazine, and sulfamerazine; 250 μg), and trimethoprim-sulfamethoxazole (1.25/23.75 μg, respectively) (BD Diagnostics, Sparks, MD). Isolates resistant to two or more antimicrobials were considered MDR. The following quality control organisms were used: *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 29212), and *Escherichia coli* (ATCC 25922). Forty-four isolates that were obtained from WADDL were also tested using a standard broth microdilution procedure (9) to measure MICs for clinically relevant antimicrobials, including ampicillin, ceftiofur, chlortetracycline, florfenicol, gentamicin, neomycin, oxytetracycline, spectinomycin, sulfachlorpyridazime, sulfadimethoxine, sulfathiazole, and trimethoprim-sulfamethoxazole. The same quality control organisms were used for this protocol except that *S. aureus* ATCC 29213 was used instead of *S. aureus* ATCC 25923.

Pulsed-field gel electrophoresis. *S.* Typhimurium isolates were assayed by PFGE following XbaI restriction using the PulseNet protocol for *Salmonella enterica* (23). WA-TYP035/TYP187 PFGE profiles were identified by comparing PFGE profiles among *S.* Typhimurium isolates using BioNumerics 3.5 software (Applied Maths, Sint-Martens-Latem, Belgium).

Plasmid profiles. Plasmids were detected by a previously described method (15). Briefly, bacterial cells were grown overnight in blood agar medium at 37°C, harvested by centrifugation, and suspended in 60 μl of lysis buffer (40 mM Tris acetate, 2 mM EDTA [pH 12.6]). The cells were then incubated for 30 min at 55°C and extracted with 100 μl of phenol-chloroform (1:1 [vol/vol]). After centrifugation, the supernatant was subjected to agarose gel electrophoresis for the detection and sizing of plasmid DNA. Plasmid electrophoresis conditions included 1% agarose gels run at 0.6 V cm^{-2} for 4 h in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Plasmid sizes were estimated relative to the BAC-Tracker Supercoiled DNA ladder (Epicentre Biotechnologies, Madison, WI) using BioNumerics (Applied Maths) software.

Plasmid transformation and PCR. Plasmid DNA was isolated from donor cells using a plasmid minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Electroporation was performed to transfer plasmids carrying the bla_{CMY-2} gene into competent (*E. coli* DH10B; GeneHog; Invitrogen, Carlsbad, CA) cells in 2-mm cuvettes using a GenePulser (Bio-Rad) based on a previously described method (14). Electroporants were immediately placed into SOC (superoptimal broth with catabolite repression) medium (2% tryptone, 0.5% yeast extract, 8.56 mM Nacl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM, glucose; 200 μl) and incubated for 1 h at 37°C with agitation (300 rpm). Transformants were then plated onto selective medium containing 8 μg/ml ceftazidime chosen based on the resistance phenotype of the donor cells. Antimicrobial susceptibility profiles of transformants were determined using a disk diffusion assay as described above. Plasmid profiles of the transformants were obtained as described above, except that electrophoresis conditions included 0.95% (wt/vol) agarose gels run at 100 V for 3 h in 1% TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The presence of the bla_{CMY-2} gene was confirmed by PCR using bla_{CMY-2} primers (33). Among the transformants with positive PCR results for bla_{CMY-2}, seven were chosen for sequencing of the bla_{CMY} gene to confirm that they carried bla_{CMY-2} (33).

Multilocus variable-number tandem-repeat typing. To examine genetic relationships within the PFGE-defined clade, four VNTR loci (STTR9, STTR6, STTR5, and STTR10pl) were used as described previously (4, 18). For each isolate a boiled cell lysate from a single colony was used to prepare a PCR template. Multiplex PCR was performed with a multiplex PCR kit (Qiagen, Hilden, Germany) in a total reaction volume of 25 μl and 2.50 pmol each of primers STTR9-F, STTR9-R, STTR6-F, and STTR6-R and 1.25 pmol each of primers STTR5-F, STTR5-R, STTR10pl-F, and STTR10pl-R. Amplification was performed with an iCycler thermocycler (Bio-Rad, Hercules, CA) as follows: one cycle of 15 min at 94°C, followed by 25 cycles of 30 s at 94°C, 1 min at 60°C, and 1.5 min at 72°C and finally an extension step of 10 min at 72°C. Then, 19.357 μl of Hi-Di formamide (Applied Biosystems, Foster City, CA), 0.125 μl Liz600 size standard (Applied Biosystems), and 0.5 μl DNA template were mixed for capillary electrophoresis. The final PCR products were separated by capillary electrophoresis using an ABI-3730 DNA analyzer (Applied Biosystems) at the Laboratory for Biotechnology and Bioanalysis at Washington State University. Data were processed with GeneMarker (SoftGenetics, State College, PA) software.

Data analysis. Allele size results from MLVA were analyzed in BioNumerics (Applied Maths) as character values. Each distinct allele string that differed from the others at one or more loci was assigned an MLVA type number (see Table S1 in the supplemental material). MLVA was carried out on bovine- and human-source isolates to determine whether humans and cattle shared genotypes. To identify genetic subgroups within the WA-TYP035/187 clade and their association with bla_{CMY-2} plasmids, MLVA profiles from bovine-source isolates (n = 112) were included in the cluster analysis. The cluster analysis of bovine-source isolates was limited to independent isolates: only the first in each serotype and resistance type within each herd and calendar year was included in the analysis. This was carried out by clustering categorical similarity coefficients using the unweighted pair group method with arithmetic means algorithm (UPGMA) with BioNumerics (Applied Maths) software. A 75.0% similarity threshold was chosen to define clusters in the UPGMA dendrogram (Fig. 1). The chi-square test for linear trend in ceftazidime resistance was calculated using EpiInfo 6.0 (13).

RESULTS

Characteristics of the WA-TYP035/187 clade. One hundred thirty isolates of *Salmonella* Typhimurium PFGE type WA-TYP035/187 from bovine and human sources (98 of TYP035 and 32 of TYP187) were characterized as to their antimicrobial resistance phenotype and MLVA genotype (see Table S1 in the supplemental material). Results from the disk diffusion assay were concordant with results from broth microdilution MIC results for 44 clinical isolates obtained from WADDL (data not shown). Forty-nine of 112 (43.8%) bovine isolates and 12 of 18 (66.7%) human isolates were resistant to ceftazidime. The earliest WA-TYP035 PFGE patterns detected were from two 1999 ceftazidime-susceptible isolates from cattle, and the earliest ceftazidime-resistant WA-TYP035 isolate was obtained in 2000. The WA-TYP187 pattern was first detected in 2004, and all WA-TYP187 isolates were resistant to ceftazidime. Among bovine WA-TYP035/187 isolates, resistance to ceftazidime increased from 0% in 1999 (0/2) to 77.8% (28/36) in 2006. This increment was significant (chi-square test for linear trend, $P < 0.001$) (Table 1). Ceftazidime-resistant isolates from both bovine and human sources exhibited resistance to three to nine of the antimicrobials tested. Among ceftazidime-resistant isolates, the most frequent pattern was resistance to ampicillin, kanamycin, streptomycin, tetracycline, amoxicillin-clavulanic acid, triple-sulfa, and ceftazidime (AKSTAmcSuCaz), exhibited by 57.1% and 25.0% of bovine- and human-source isolates, respectively (Table 2).

Genotyping results. The four-locus MLVA protocol differentiated isolates within the WA-TYP035/187 clade into 35 different types (Table 3; see Table S1 in the supplemental material). Among 18 human-source isolates analyzed by MLVA, 10 had MLVA types identical to those of bovine isolates. An UPGMA dendrogram was constructed to visualize relationships between the various genotypes among the bovine WA-TYP035/187 isolates. A similarity threshold of 75.0% resulted in 11 clusters containing two or more isolates and two single-isolate "clusters" (Fig. 1). Seven of 11 clusters included at least one ceftazidime-resistant isolate. Eighteen of 29 bovine WA-TYP187 isolates (62.1%) were in cluster 1, and the remaining 11 TYP187 isolates were distributed among clusters 2, 3, 4, and 9. Cluster 1 contained the largest number of isolates,

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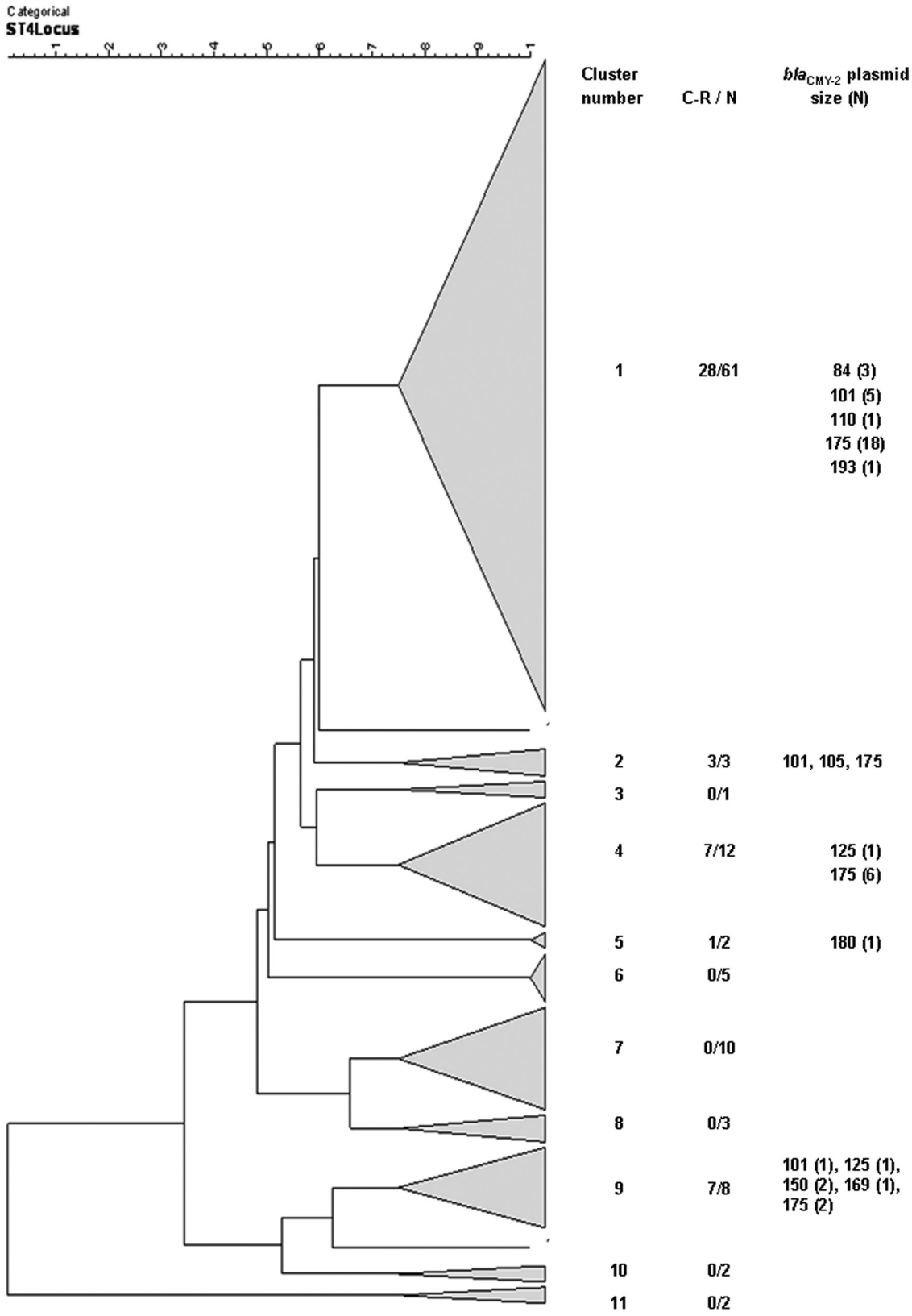

FIG. 1. Dendrogram illustrating genetic relationships among WA-TYP035/187 isolates from bovine sources in Washington State. The dendrogram was generated using the UPGMA algorithm to cluster categorical coefficients from VNTR allele data. Isolates with 75.0% or greater similarity to each other were assigned to a cluster. C-R/N, the number of ceftazidime-resistant isolates divided by the total number of isolates in the cluster. Plasmid sizes are given in kb.

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TABLE 1. Ceftazidime resistance in bovine isolates of *Salmonella* Typhimurium PFGE type WA-TYP035/187

Yr	Total no. of bovine-source WA-TYP035/187 isolates	No. (%) of ceftazidime-resistant isolates[a]
1999	2	0 (0)
2000	13	1 (7.7)
2001	14	0 (0)
2002	9	1 (11.1)
2003	4	2 (50.0)
2004	12	5 (41.7)
2005	12	8 (66.7)
2006	36	28 (77.8)
2007	10	4 (40.0)
Total	112	49 (43.8)

[a] Chi-square test for linear trend; $P < 0.001$.

including 28 ceftazidime-resistant and 33 ceftazidime-susceptible isolates.

***bla*$_{CMY-2}$ plasmids.** Plasmid profiles were determined for the 49 bovine WA-TYP035/187 isolates that were resistant to ceftazidime. These plasmids were isolated after transformations into *E. coli* competent cells on selective medium to determine which were associated with carriage of *bla*$_{CMY-2}$. PCR amplification of plasmid DNA using *bla*$_{CMY-2}$-specific primers revealed that the gene was present in all ceftazidime-resistant isolates and was carried on plasmids ranging between approximately 80 kb and 200 kb in size. *bla*$_{CMY-2}$ plasmids of diverse sizes were found within UPGMA clusters defined using the 75.0% similarity cutoff, as well as within specific MLVA types (Fig. 1; Table 3). A subset of seven ceftazidime-resistant plasmid transformants was tested for the specific *bla*$_{CMY-2}$ gene by sequencing the PCR amplification products. All seven sequences had 100% homology to the accession sequence for *bla*$_{CMY-2}$ (GenBank number Y16784) and had 99% or lower homology to other *bla*$_{CMY}$ gene sequences (data not shown).

TABLE 3. Number of bovine-origin WA-TYP035/187 isolates within UPGMA cluster and MLVA genotype

UPGMA cluster[a]	MLVA type[b]	No. of isolates with *bla*$_{CMY-2}$ plasmid size (kb):										
		84	101	110	125	150	169	175	180	193	None	Total
1	4	3		1				17			3	24
	8										1	1
	12		4									4
	19		1					1		1	18	21
	27										8	8
	32										3	3
2	5							1				1
	20		1			1						2
3	2							1				1
	17										1	1
4	7							6			1	7
	15										1	1
	22				1						1	2
	29										1	1
	33										1	1
5	30								1		1	2
6	11										5	5
7	18										9	9
	21										1	1
8	26										2	2
	28										1	1
9	10					1	1	1				3
	16					1						1
	23		1		1			1			1	4
10	13										1	1
	14										1	1
11	34										1	1
	35										1	1
Unique	6							1				1
	24						1					1
Total		3	7	1	2	3	2	29	1	1	63	112

[a] Cluster results are from UPGMA cluster analysis of categorical similarity coefficients determined by VNTR alleles. A cluster was defined by using a 75.0% similarity cutoff.

[b] Each set of alleles that differed at one or more loci was assigned a distinct MLVA type number (see Table S1 in the supplemental material).

TABLE 2. Antimicrobial resistance patterns from disk diffusion testing of ceftazidime-resistant *Salmonella* Typhimurium WA-TYP035/187 isolates from cattle and humans from 2000 through early 2007

Resistance profile[a]	No. of isolates by species and yr								
	Bovine								Human (2004–2006)
	2000	2002	2003	2004	2005	2006	2007	Total	
AAmcCaz						2		2	
ACSTSuCaz				1				1	
AKSTSuCaz				2		1		3	2
ACKSTSuCaz			2					2	3
AKTAmcSuCaz					1			1	
ACSTAmcSuCaz							1	1	1
AKSTAmcSuCaz					2	23	3	28	3
ACKSTAmcSuCaz					3	1		4	
ACKSxtSTSuCaz	1	1		2	2			6	2
ACKSxtSTAmcSuCaz									1
AGKSxtSTAmcSuCaz						1		1	
Total	1	1	2	5	8	28	4	49	12

[a] A, ampicillin; C, chloramphenicol; K, kanamycin; S, streptomycin; Su, triple-sulfa; T, tetracycline; Sxt, trimethoprim-sulfamethoxazole; Caz, ceftazidime; Amc, amoxicillin-clavulanic acid.

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DISCUSSION

In this study, MLVA differentiated a highly clonal group of *Salmonella* Typhimurium, WA-TYP035/187, into 35 distinct types, indicating that the MLVA technique provides further discrimination within this clonal group. This result is consistent with observations by other investigators that the MLVA method is more discriminatory than PFGE (3, 6, 20, 25), and the method has been proposed as an alternative to PFGE for genotyping highly clonal groups of bacteria (7, 16–18, 21).

Isolates within the specific MLVA types (MLVA types 4, 10, 19, 20, and 23) as well as within the closely related UPGMA clusters 1, 2, 4, and 9 had bla_{CMY-2}-carrying plasmids that were highly diverse in size (Fig. 1; Table 3). The observation of bla_{CMY-2} plasmid size diversity within UPGMA-defined MLVA clusters and even within the more specific MLVA genotypes suggests that resistance to ceftazidime within a lineage was probably acquired as a result of multiple independent genetic events in the form of plasmid transfers. An alternative explanation for this observation could be that an original WA-TYP035/187 bla_{CMY-2} plasmid diversified more rapidly than its host chromosome due to mutational events such as deletions, insertions, or higher rates of recombination at sites on the plasmid. The former hypothesis, however, is consistent with the occurrence of frequent and/or intense selection pressure exerted by ceftiofur use in dairy cattle. Ceftiofur is an expanded-spectrum cephalosporin and is one of the most frequently used therapeutic antimicrobials in dairy cattle (27, 34), a finding that has also been documented in Washington State (22). It is also consistent with the significant increase in prevalence of ceftazidime resistance among *S.* Typhimurium WA-TYP035/187 isolates from bovine sources that occurred between 1999 and 2006 and a similar increase in ceftazidime resistance among cattle-adapted *Salmonella* serovar Dublin isolates in the same region and during the same time period (12). The parallel increases in ceftazidime resistance in the *S.* Typhimurium WA-TYP035/187 clade and the cattle host-adapted *S.* Dublin strongly support a cattle-specific selection mechanism, such as therapeutic ceftiofur use. Studies that examined fecal flora of cattle following ceftiofur administration have failed to find more than a transient increase in the proportion of resistant flora (10, 19, 28), have found an association between resistance and ceftiofur use at the herd level rather than a direct effect on the individual animal (30), or have failed to detect a herd-level association with the magnitude of ceftiofur use (10). These findings are inconsistent with our observations and suggest that the mechanism underlying the increase in ceftazidime resistance in *Salmonella* from cattle is not a simple selection against susceptible organisms. Studies that examine trends in *Salmonella* rather than *E. coli* after reduction in ceftiofur use may provide insights into causes of resistance in pathogenic *Salmonella*.

ACKNOWLEDGMENTS

This work was supported in whole or in part with federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract no. N01-AI-30055.

The skillful technical assistance of Katherine Kaya Baker is gratefully acknowledged. We thank Lindsay Tippett; Lisa Jones and the staff of the FDIU, WSU; Kaye Eckmann, Kathryn MacDonald, and David Boyle of the Washington State Department of Public Health; and Derek Pouchnik of the WSU School of Molecular Biosciences for their technical assistance.

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