Animal and Human Multidrug-Resistant, Cephalosporin-Resistant Salmonella Isolates Expressing a Plasmid-Mediated CMY-2 AmpC β-Lactamase


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Salmonella spp. are important food-borne pathogens that are demonstrating increasing antimicrobial resistance rates in isolates obtained from food animals and humans. In this study, 10 multidrug-resistant, cephalosporin-resistant Salmonella isolates from bovine, porcine, and human sources from a single geographic region were identified. All isolates demonstrated resistance to cephemycins and extended-spectrum cephalosporins as well as tetracycline, chloramphenicol, streptomycin, and sulfisoxazole. Molecular epidemiological analyses revealed eight distinct chromosomal DNA patterns, suggesting that clonal spread could not entirely explain the distribution of this antimicrobial resistance phenotype. However, all isolates encoded an AmpC-like β-lactamase, CMY-2. Eight isolates contained a large nonconjugative plasmid that could transform Escherichia coli. Transformants coexpressed cephalosporin, tetracycline, chloramphenicol, streptomycin, and sulfisoxazole resistances. Plasmid DNA revealed highly related restriction fragments though plasmids appeared to have undergone some evolution over time. Multidrug-resistant, cephalosporin-resistant Salmonella spp. present significant therapeutic problems in animal and human health care and raise further questions about the association between antimicrobial resistance, antibiotic use in animals, and transfer of multidrug-resistant Salmonella spp. between animals and man.

Salmonella spp. are important zoonotic pathogens in humans and animals. In the United States it is estimated that over 1.4 million cases of salmonellosis occur each year, 95% of which are the result of food-borne transmission (28). Large outbreaks have been associated with ingestion of poultry, meat, and milk and other dairy products (6). Although the majority of infections result in asymptomatic or self-limited diarrheal illness, severe, life-threatening bacteremias and other deep-seated infections do occur, particularly in immunocompromised hosts, neonates, and the elderly (7, 14).

Increasing rates of antimicrobial resistance in Salmonella isolates have been reported from a number of developing and developed countries. In the United States, resistance to tetracycline increased from 9% in 1980 to 24% in 1990 and resistance to ampicillin increased from 10 to 14% (25). In Britain, rates of antimicrobial resistance for Salmonella enterica serovar Typhimurium were higher, with 45% of isolates resistant to tetracyclines, 40% of isolates resistant to sulfonamides, and 17% of isolates resistant to ampicillin (42). Much of this multidrug resistance has been linked to the spread of a single strain of Salmonella serovar Typhimurium, definitive phage type 104 (DT104), through food animals and humans (16). Most of these multidrug-resistant DT104 isolates have a chromosomal gene cluster that codes for resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (11, 37). Of increasing concern is the fact that animal- and human-associated multidrug-resistant DT104 isolates resistant to quinolones have been reported (29, 41).

In this study, we report the identification and molecular characterization of bovine, porcine, and human multidrug-resistant Salmonella isolates that are resistant to extended-spectrum cephalosporins and cephemycins. A plasmid-mediated CMY-2 ampC-like gene was identified in all animal and human isolates. Plasmid-mediated AmpC-type β-lactamases have been identified in Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis, and Enterobacter aerogenes clinical isolates from humans in the United States, Europe, and other regions (5, 9, 44). Reports of human Salmonella isolates expressing an AmpC-like β-lactamase have been quite rare (15, 23), though the prevalence may be increasing (E. F. Dunne, P. F. Fey, P. Shillam, P. Kludt, W. Keene, E. Harvey, K. Stamey, T. Barrett, N. Marano, and F. J. Angulo, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 716, 1999). Additionally, animal Salmonella isolates expressing an AmpC-like enzyme have not been reported. In this study, the molecular characterization of cephalosporin-resistant Salmonella isolates from food animals and humans residing in a single geographic region is reported. These results underscore concern for increasing antimicrobial resistance in Salmonella spp. and suggest that careful epidemiological and antimicrobial surveillance studies are needed to assess the selective conditions associated with cephalosporin resistance among Salmonella isolates from farm animals and the association of these isolates with human disease.

MATERIALS AND METHODS

Organisms. A total of 158 isolates of Salmonella spp. were recovered from various animals between November 1998 and May 1999 at the Iowa State University Veterinary Diagnostic Microbiology Laboratory. Organisms were obtained from liver, stool, intestine, lung, and lymph node samples of diseased animals. A total of 320 human isolates of Salmonella spp. were analyzed. These isolates had been referred to the Iowa State Hygienic Laboratory from numerous microbiology laboratories throughout the state of Iowa. All isolates were transferred to the Medical Microbiology Division of the Department of Pathology at the University of Iowa College of Medicine for further characterization. Isolates were stored at −70°C on porous beads (ProLab, Austin, Tex.) until further use.
Antimicrobial susceptibility testing. MICs of selected antimicrobials were determined by broth microdilution as described by the National Committee for Clinical Laboratory Standards. Custom-designed, cation-adjusted Mueller-Hinton broth microdilution trays purchased from TREK Diagnostic Systems Inc., Westlake, Ohio, were inoculated and incubated at 35°C for 16 to 20 h (21, 31). Sulfadiazine and sulfonamide susceptibility tests were performed by disk diffusion (21, 31). Cefotinin and streptomycin MICs were determined using the E test methodology as described by the manufacturer (AB Biodisk, Solna, Sweden). Briefly, organisms were diluted to a McFarland standard of 0.5 and streaked onto Mueller-Hinton agar plates. After E-test strip application, the plates were incubated at 35°C for 16 to 20 h.

Isoelectric focus analysis. Crude β-lactamase extracts were prepared by freeze-thaw lysis of bacterial cultures grown to exponential-growth stage in tryptic soy broth as previously described (8). Analytical isoelectricfocusing was performed using a Multiphor II electrophoresis system with commercially prepared ampholine-polyacrylamide plates (pI 3.5 to 9.5; Amersham Pharmacia Biotech, Piscataway, N.J.). β-Lactamase activity was determined with 0.5 μg of nitrocephin (Becton-Dickinson, Franklin Lakes, N.J.) per ml. TEM-1, TEM-4, and TEM-5 β-lactamases expressed in E. coli were used as isoelectric focus standards. These enzymes are known to migrate at a pI of 5.4 (21, 31). Gels were stained with ethidium bromide and photographed using a Gel Doc 1000 system (Bio-Rad Laboratories). Strains which contained restriction fragment patterns that differed by more than three bands were considered unique (2).

Molecular techniques. Plasmid DNA was isolated using the Concert Mini-prep system (Gibco BRL). Alternatively, large plasmids were isolated using a protocol described previously for isolation of large bacterial artificial chromosome plasmid DNA (36). Large plasmid DNA was then digested using PlasmidSafe DNase (Epipenic Technologies, Madison, Wis.) according to the manufacturer’s recommendations. Conjugation experiments were performed as previously described (35). Briefly, cultures of the E. coli recipient HB101 containing pEm7-Zeo (a Zeocin resistance plasmid; Invitrogen, Carlsbad, Calif.) and the donor Salmonella isolate were grown overnight in Luria broth (LB). A 10-1 suspension of donor-recipient culture was diluted in fresh LB. Aliquots were spotted onto sterile filters placed on LB plates and incubated overnight. Filters were eluted in sterile saline, and serial dilutions were plated on LB plus Zeocin (50 μg/ml) and cefotinin (50 μg/ml). Transformation of large plasmid DNA from the Salmonella isolates was performed using standard electroporation techniques with DH10B electrocompetent E. coli (Gibco BRL, Grand Island, N.Y.). Transformsants were selected on LB agar containing 50 μg of cefotinin (Sigma, St. Louis, Mo.) per ml. Plasmid DNA restriction fragment length polymorphisms were analyzed by agarose gel electrophoresis of plasmid DNA cleaved with various restriction endonucleases (New England Biolabs).

PCR analysis was performed on total DNA as prepared using the CTAB protocol described previously (4) or plasmid DNA treated with PlasmidSafe DNase. Amplification was performed with consensus primers for the βla genes encoding BL1-1, LAT-1, LAT-2, and CMY-2 and the ampC gene of Citrobacter freundii OS60 (ampC1, 5’-AGATATGAAAAACCTTATGC-3’; ampC2, 5’-TGCACTTTCGGAAGAATGCAC-3’ [23]) or TEM-1 (5’-CCGGATCTCCGAGATATGATTCAC-3’ and 5’-CCCCGATCCCCCAATACATTCAATGCC-3’). PCR fragments were isolated using Quick PCR cleanup columns (Qiagen, Valencia, Calif.). DNA sequence analysis was performed using Big Dye terminator cycle sequencing chemistry with AmpliTaq polymerase FS enzyme (Applied Biosystems, Foster City, Calif.). The reactions were performed and analyzed with an Applied Biosystems model 373A stretch fluorescent automated sequencer at the University of Iowa DNA Core Facility.

RESULTS

Characterization of animal and human Salmonella isolates. Eight of 158 (5.1%) Salmonella isolates recovered from symptomatic large animals were determined to be resistant to extended-spectrum cephalosporins (ceftizidime, cefotaxime, and ceftiofur), a monobactam (aztreonam), and a cephamycin (cefoxitin), as well as to ticarcillin and piperacillin (Tables 1 and 2). The β-lactamase inhibitor clavulanic acid (fixed concentration of 2 μg/ml) had no effect on ticarcillin or ampicillin MICs (data not shown). As seen in other CMY studies, tazobactam reduced piperacillin MICs by fourfold or more in most isolates, though in this study the majority of isolates remained resistant to this antimicrobial combination (23, 44). All eight isolates were resistant to ticarcillin, sulfamethoxazole, streptomycin, and chloramphenicol. In addition, six of eight isolates were resistant to gentamicin and three of eight isolates were resistant to trimethoprim-sulfamethoxazole. One isolate (isolate 613) was intermediate to ciprofloxacin and resistant to nalidixic acid.

All eight isolates had been obtained from bovine or porcine sources in geographically distinct areas of Iowa. The majority of isolates were obtained from deep-seated sites, including lung, intestine, and liver or lymph node, with one isolate recovered from stool. Organisms represented a variety of serotypes though five isolates were Salmonella serovar Typhimurium.
or Salmonella serovar Typhimurium subsp. copenhagen. None of these isolates were DT104.

Two of 320 (0.6%) human isolates of Salmonella spp. submitted to the Iowa State Hygienic Laboratory during 1998 were found to have antibiograms similar to the animal isolates. Both had been recovered from stool specimens in two geographically distinct cities in Iowa. The serotypes were Salmonella serovar Typhimurium and Salmonella serovar Newport. The Salmonella serovar Typhimurium isolate, 1339, was DT104. Both human isolates were resistant to cephalosporins as well as tetracycline, streptomycin, chloramphenicol, and sulfamethoxazole.

Molecular epidemiology. The clonal relatedness of the 10 multidrug-resistant isolates of Salmonella was assessed by pulsed-field gel electrophoresis (PFGE) analysis of restriction endonuclease-digested chromosomal DNA (Fig. 1 and Table 1). Overall, eight distinct PFGE patterns were identified in the 10 organisms. Two bovine isolates showed identical chromosomal DNA patterns, and two porcine isolates shared a different restriction fragment pattern. There was no obvious geographic link between isolates demonstrating similar PFGE patterns. Additionally, the PFGE patterns of the human isolates differed from those of the animal isolates.

Molecular analysis of cefepime resistance. The antibiogram demonstrated by these organisms was consistent with the expression of a cefepime-resistant strain similar to the chromosomally encoded inducible AmpC enzymes found in many members of Enterobacteriaceae. However, Salmonella species are not known to encode an inducible chromosomal AmpC enzyme (27). To determine whether these organisms expressed a β-lactamase, crude bacterial isolates were analyzed by isoelectric gel electrophoresis. A β-lactamase with a pI of ñ8.7 was detected in all 10 isolates. Additionally, five isolates demonstrated a second β-lactamase that comigrated with the TEM-1 control, with a pI of 5.4.

Conjugation studies were performed to determine whether cefepime resistance could be transferred to E. coli. Despite multiple attempts, resistance could not be transferred by conjugation to a recipient E. coli strain carrying a Zeocin resistance plasmid (HB101pE7zeo). Cephalosporin resistance, however, could be transferred through bacterial transformation. DH10B electrocompetent bacteria were transformed with Salmonella plasmid DNA. Eight of 10 Salmonella isolates

Table 2. Antimicrobial susceptibilities

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*E. coli* transformants are indicated by designations beginning DH10B.

Amp, ampicillin; Pip, piperacillin; Pip-tazo, piperacillin combined with a fixed concentration of tazobactam (4 μg/ml); CTAX, ceftazidime; CTX, ceftriaxone; CFTI, cefotior; CFOX, cefoxitin; CPIM, cepime; Tet, tetracycline; T/S, trimethoprim/sulfamethoxazole; Gent, gentamicin; Strep, streptomycin; Chlor, chloramphenicol; Sulf, sulfisoxazole; Cipro, ciprofloxacin. Values are MICs (in micrograms per milliliter), except that sulfisoxazole disk results are zone diameters (in nanometers).

*Antimicrobial susceptibility determined by E-test methodology.

**NT:** not tested.
transferred cephalosporin resistance. Additionally, resistances to tetracycline, sulfamethoxazole, and chloramphenicol were transferred to *E. coli*. Since DH10B *E. coli* is streptomycin resistant, this experiment was unable to determine whether streptomycin resistance was also present on plasmid DNA. Transfer of gentamicin resistance was less consistent. Only two of four *Salmonella* isolates carrying gentamicin resistance transferred this resistance to *E. coli*. Trimethoprim-sulfamethoxazole resistance cosegregated in one of two isolates while quinolone resistance did not cotransfer from the one isolate that expressed this resistance. All transformants expressed plasmid DNA that migrated at approximately 75 kb on agarose gels. Additionally, all transformants demonstrated β-lactamase with a pl of ≥8.7. Two of three *Salmonella* isolates that originally expressed a β-lactamase with a pl of 5.4 were able to transfer expression of this enzyme to *E. coli* (data not shown).

The sequences, hydrolysis patterns, and isoelectric points of a number of β-lactamases have been identified over the past decade (12). Review of the literature revealed a chromosomal AmpC enzyme identified in *C. freundii* that migrated at a pl of approximately 8.6 to 8.7 in isoelectric focus analysis (26, 39). Additionally, a single *Salmonella* serovar Seftenberg clinical isolate has been reported to carry a plasmid-mediated cephalosporinase that showed significant homology to the *C. freundii* ampC gene (23). PCR analysis of total bacterial DNA was performed using consensus primers for the *Citrobacter* family of ampC genes (23). A 1,143-bp fragment was amplified from all 10 *Salmonella* isolates while an unrelated *Enterobacter cloacae* isolate known to express a different AmpC enzyme and a wild-type *Salmonella* isolate remained negative (data not shown).

Each PCR fragment was isolated and the entire nucleotide sequence of the ampC-like gene was determined from both strands. All animal and human isolates contained an identical DNA sequence that has been designated CMY-2, an enzyme identified in a *K. pneumoniae* isolate found in Greece (5). A highly related though distinct enzyme, which differs only by two amino acids in the signal peptide sequence, has been identified in a single *Salmonella* serovar Seftenberg organism isolated from an Algerian child (23). TEM-1 PCR primers amplified the TEM gene from isolates 263, 272, 274, 370, 532, and 613, all of which expressed a β-lactamase with a pl of 5.4.

**Plasmid restriction fragment length polymorphisms.** Plasmid DNA from the *E. coli* transformants was isolated and digested with *Pst* I, *Eco* RI (Fig. 2) or *Bam* HI (data not shown). Complete identity between any plasmids was not observed. However, many fragments were shared among the transformants, suggesting that the plasmids may share a highly related plasmid backbone.

**DISCUSSION**

Antibiotic resistance in *Salmonella* has intensified substantially worldwide (19, 25, 33, 42, 46, 47). For years, ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol were the recommended antimicrobial agents for severe *Salmonella* infections. Rising rates of resistance to these agents have significantly reduced the efficacy of these agents. Consequently,
fluoroquinolones and expanded-spectrum cephalosporins have become the recommended antimicrobial agents for invasive *Salmonella* infections. Multidrug-resistant, quinolone-resistant *Salmonella* strains are now being reported (29). The present study describes multidrug-resistant, cephalosporin-resistant *Salmonella* isolates from both food animals and humans.

*Salmonella* isolates resistant to broad-spectrum cephalosporins were first reported in the 1980s, and since that time additional isolates have been identified (10, 30). The majority of cephaparin-resistant *Salmonella* isolates express an extended-spectrum β-lactamase able to hydrolyze oxyimino cephalosporins and monobactams but not the cephaparins. Recently, a second mechanism of cephalosporin resistance in *Salmonella* has been observed (4, 23). These isolates express plasmid-mediated AmpC-like β-lactamas that hydrolyze the cephalosporins as well as the extended-spectrum cephalosporins and monobactams. The isolates described in the present study express a plasmid-mediated CMY-2 AmpC-like enzyme that has been identified previously in a single *Salmonella* isolate from Algeria (23). This enzyme belongs to a small family of plasmid-mediated AmpC-like enzymes (LAT-1, LAT-2, BIL-1, CMY-2 and -2b, CMY-3, CMY-4, and CMY-5) that share homology with the chromosomal *ampC* from *C. freundii* (5, 23, 44, 45). Further analysis has shown that the plasmid-encoded CMY-5 gene is followed by the *bic* and *sugE* genes of *C. freundii* (5, 23, 44, 45). A nonconjugative but transferable plasmid encoding the CMY-2 gene was identified in 80% of isolates in this study. All *E. coli* transformants demonstrated a plasmid of approximately 75 kb. Though the plasmids were not identical, in that cotransfer of gentamicin or trimethoprim-sulfamethoxazole occurred in only a subset of isolates and RFLP patterns were different, the cotransfer of CMY-2, chloramphenicol, sulfamethoxazole, tetracycline, and possibly streptomycin in all isolates suggests that a highly related gene cluster may reside on each plasmid from the human and animal isolates. Additionally, the RFLP patterns identified many highly conserved restriction fragments among each of the plasmids, suggesting that the plasmids may be genetically similar but that they may have evolved over time. Previous studies have shown that plasmid DNA can change rapidly (24, 40).

DT104 isolates of *Salmonella* typically carry a chromosomal integron that encodes all or a subset of the antimicrobial resistance genes (11, 37). More recently, multidrug-resistant *Salmonella* serovar Typhimurium isolates that contain a similar integron-associated gene cluster encoded on a transferable plasmid have been identified (43). Transformants in this study were analyzed for type 1 integrons (34). All animal transformants demonstrated one or more integrons though preliminary evidence did not find the CMY-2 gene to be encoded within an integron (P. L. Winokur, unpublished data). It is tempting to speculate that acquisition of cephalosporin resistance may relate to therapeutic ceftiofur use. However, persistence of these multidrug-resistant strains of *Salmonella* spp. in farm animals may be further encouraged by the use of other antimicrobials as growth promotants, a common practice in the veterinary industry.

The results of this study do not definitively prove spread of multidrug-resistant *Salmonella* from an animal source to humans. However, the genetic relatedness of the plasmids identified and their prevalence in the animal isolates is suggestive. Additional studies will be required to further explore the association of resistance with various antibiotic use practices in food animals carrying a cephalosporin-resistant *Salmonella* and the possible transfer of multidrug-resistant, cephaparin-resistant *Salmonella* spp. between animals and humans.
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ADDITION

In a recent study, Fey et al. (14a) identified two multidrug-resistant, cephalosporin-resistant Salmonella isolates that each carry a 160-kb plasmid encoding the CMY-2 gene. These isolates, one human and one bovine, were epidemiologically linked and shared similar PFGE patterns. These data suggest that ceftriaxone-resistant Salmonella has been transmitted from food animals to humans.

REFERENCES