JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2002, p. 4334–4336 Vol. 40, No. 11
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.11.4334–4336.2002

Expanded-Spectrum Cephalosporin-Resistant *Salmonella* Strains in Romania

Vivi Miriagou,[1]* Roxana Filip,[2] Gabriela Coman,[2] and Leonidas S. Tzouvelekis[3]

Laboratory of Bacteriology, Hellenic Pasteur Institute,[1] and Department of Microbiology, Medical School, University of Athens,[3] Athens, Greece, and Department of Microbiology, University of Medicine and Pharmacy "Gr. T. Popa," University of Iasi, Iasi, Romania[2]

Received 28 May 2002/Returned for modification 14 July 2002/Accepted 5 August 2002

Thirteen *Salmonella enterica* serotype Typhimurium and one *Salmonella enterica* serotype Heidelberg strain resistant to expanded-spectrum cephalosporins were isolated from October 2000 to February 2001 from infants with gastroenteritis in Iasi, Romania. In all but one serotype Typhimurium isolate, resistance was due to the production of a CMY-2 cephalosporinase encoded by a nonconjugative plasmid. The remaining isolate produced an SHV-5-type β-lactamase. Typing by pulsed-field gel electrophoresis indicated that the CMY-2-producing serotype Typhimurium isolates were related.

There is an increasing number of studies reporting the emergence of nontyphoid *Salmonella enterica* serotype Typhimurium strains that are resistant to expanded-spectrum cephalosporins (ESC). This resistance is mostly due to the acquisition of plasmids that encode various β-lactamases, including class A extended-spectrum β-lactamases (ESBLs) (2, 8, 11, 13–16; E. Cardinale, P. Colbachini, J. D. Perrier-Gros-Claude, A. Gassama, and A. Aïdara-Kane, Letter, J. Clin. Microbiol. **39:**2373-2374; B. P. Cherian, N. Singh, W. Charles, and P. Prabhakar, Letter, Emerg. Infect. Dis. **5:**181-182) and class C cephalosporinases (3, 4, 6, 18, 20, 21). The spread of such strains may have serious clinical consequences, since ESC are the antibiotics of choice for invasive salmonella infections in children. They may also facilitate the community spread of *bla* genes. In the present study we describe ESC-resistant *Salmonella* strains that were recently isolated from a pediatric population in Iasi, Romania.

Fourteen ESC-resistant, nontyphoid *Salmonella* isolates were studied (see Table 1). These isolates were derived from stool specimens of children with diarrhea from October 2000 to February 2001 in the pediatric hospital in Iasi. This hospital is the major pediatric tertiary-care institution (640 beds, 40,000 admissions per year) in northeastern Romania (population of the Iasi area and the eight neighboring districts, 4.9 million). Species identification was performed by means of the API 20E system (bioMerieux). Serotyping was performed with commercially available anti-O and anti-H antisera (bioMerieux).

Selected isolates were typed by pulsed-field gel electrophoresis (PFGE) of *Xba*I-restricted genomic DNA as described previously (5). Restriction fragments were separated in a 1.2% agarose gel with a CHEF DRIII apparatus (Bio-Rad).

Susceptibility to antibiotics, including ESC, was assessed by a disk diffusion method (9). Strains were examined for production of ESBLs by using Etest strips containing ceftazidime and clavulanic acid (AB Biodisk). This test was used for all *Salmonella* isolates exhibiting resistance or decreased susceptibility to ESC.

Escherichia coli K-12 strain 14R525 (Nal^r) was used as the recipient in conjugation experiments; *E. coli* DH5α was used in transformation. Conjugation was carried out in mixed broth cultures as described previously (17). Transconjugants were selected on Mueller-Hinton agar containing ampicillin (50 μg/ml) plus nalidixic acid (200 μg/ml). Plasmid DNA preparations were obtained by an alkaline lysis technique (12). Plasmids purified from low-melting-point agarose (0.8%) were used to transform *E. coli* competent cells.

β-Lactamases were extracted by ultrasonic treatment of overnight bacterial cultures in Mueller-Hinton broth. Isoelectric focusing was performed according to the method of Matthew et al. (7), with polyacrylamide gels containing ampholytes (pH range, 3.5 to 9.5; APBiotech).

PCR assays specific for bla_{TEM} and bla_{SHV} genes were performed as described previously (1). Detection of *bla* genes related to the *Citrobacter freundii ampC* was carried out as described by Koeck et al. (6). Nucleotide sequences of the PCR products were determined with an ABI Prism 377 DNA sequencer (Perkin-Elmer).

From October 2000 to February 2001, a total of 40 *Salmonella* isolates were derived from cases of gastroenteritis in the pediatric hospital of Iasi. Hospital records indicated that this *Salmonella* isolation rate was not significantly different from the rates of previous years. Notably, 14 (35%) of these isolates were resistant to ESC (ceftriaxone, cefotaxime, and ceftazidime), while before and after the study period such isolates occurred only sporadically.

Thirteen isolates (ST1 to ST13) belonged to the *Salmonella* serotype Typhimurium. One isolate (SH14) was *Salmonella* serotype Heidelberg. Eleven of the isolates were community acquired. The remaining three were most likely acquired in two district hospitals (ST1 and ST7) and a nursing home (ST11). All but one serotype Typhimurium isolate exhibited a cephalosporinase phenotype, i.e., resistance to penicillins, penicillin-clavulanate combinations, cefoxitin, and ESC. The remaining isolate (ST5) probably produced an ESBL, given that it was resistant to the tested ESC but susceptible to cefoxitin

* Corresponding author. Mailing address: Laboratory of Bacteriology, Hellenic Pasteur Institute, Vass. Sofias 127, Athens 11521, Greece. Phone: 03010-6478810. Fax: 03010-6423498. E-mail: miriagou@mail.pasteur.gr.

TABLE 1. Characteristics of 14 ESC-resistant *Salmonella* strains

Strain	Serotype	Patient data[a]		Likely place of acquisition	Isolation date	β-Lactams to which strain is resistant	β-Lactamase(s) produced	Other antibiotics to which strain is resistant
		Gender	Age (mo)					
ST1	Typhimurium	M	4	District hospital A	October 2000	AMP, AMC, TIM, FOX, ESC[b]	CMY-2, TEM-1	GEN, TOB, S, TET, CHL[c]
ST2	Typhimurium	M	1	Community	October 2000	AMP, AMC, TIM, FOX, ESC	CMY-2, TEM-1	GEN, TOB, S, TET, CHL
ST3	Typhimurium	F	1	Community	November 2000	AMP, AMC, TIM, FOX, ESC	CMY-2, TEM-1	GEN, TOB, S, TET, CHL
ST4	Typhimurium	M	2	Community	November 2000	AMP, AMC, TIM, FOX, ESC	CMY-2, TEM-1	GEN, TOB, S, TET, CHL
ST5	Typhimurium	M	4	Community	November 2000	AMP, ESC	SHV-5, TEM-1	GEN, TOB, S, TET, CHL
ST6	Typhimurium	F	3	Community	November 2000	AMP, AMC, TIM, FOX, ESC	CMY-2, TEM-1	GEN, TOB, S, TET, CHL
ST7	Typhimurium	F	2	District hospital B	December 2000	AMP, AMC, TIM, FOX, ESC	CMY-2	S, TET, CHL
ST8	Typhimurium	M	6	Community	December 2000	AMP, AMC, TIM, FOX, ESC	CMY-2, TEM-1	GEN, TOB, S, TET, CHL
ST9	Typhimurium	M	1	Community	December 2000	AMP, AMC, TIM, FOX, ESC	CMY-2, TEM-1	GEN, TOB, S, TET, CHL
ST10	Typhimurium	M	10	Community	December 2000	AMP, AMC, TIM, FOX, ESC	CMY-2, TEM-1	GEN, TOB, S, TET, CHL
ST11	Typhimurium	F	10	Nursing home	December 2000	AMP, AMC, TIM, FOX, ESC	CMY-2, TEM-1	GEN, TOB, S, TET, CHL
ST12	Typhimurium	F	5	Community	February 2001	AMP, AMC, TIM, FOX, ESC	CMY-2, TEM-1	GEN, TOB, S, TET, CHL
ST13	Typhimurium	M	2	Community	February 2001	AMP, AMC, TIM, FOX, ESC	CMY-2, TEM-1	GEN, TOB, S, TET, CHL
SH14	Heidelberg	M	2	Community	February 2001	AMP, AMC, TIM, FOX, ESC	CMY-2, TEM-1	GEN, TOB, S, TET, CHL

[a] M, male; F, female.
[b] AMP, ampicillin; AMC, amoxicillin-clavulanic acid; TIM, ticarcillin-clavulanic acid; FOX, cefoxitin.
[c] GEN, gentamicin; TOB, tobramycin; S, sulfonamides; TET, tetracycline; CHL, chloramphenicol.

and penicillin-clavulanate combinations. Production of an ESBL was corroborated by a positive result with the Etest. The isolates were also resistant to chloramphenicol, sulfonamides, and tetracycline. Resistance to aminoglycosides was also observed in all but one serotype Typhimurium isolate (ST7) (Table 1).

Isoelectric focusing showed production of a β-lactamase with an isoelectric point (pI) of 9.0 by all 13 isolates exhibiting a cephalosporinase phenotype. Twelve of these also produced a β-lactamase with a pI of 5.4 (presumably a TEM-1, which was also indicated by bla_{TEM}-specific PCR assays). None of the 13 isolates could transfer β-lactam resistance to *E. coli* by conjugation. However, transformation of *E. coli* with plasmid DNA preparations from these isolates yielded clones that were resistant to β-lactams and produced an enzyme with a pI equal to 9.0. Clinical isolates and transformants were positive in a *cmy*-specific PCR assay. The sequencing of PCR products showed a β-lactamase gene identical to *cmy-2*. The gene was located in an approximately 60-MDa plasmid that was common to all 13 isolates, as indicated by restriction endonuclease analysis and hybridization experiments (data not shown). Transformants did not produce TEM-1 yet were susceptible to non-β-lactam antibiotics, indicating that *cmy-2* was probably the sole antibiotic resistance gene carried by this plasmid. The CMY-2-producing isolates exhibited similar PFGE patterns, indicating a genetic relatedness (Fig. 1).

The ESBL-producing serotype Typhimurium isolate (ST5) was able to transfer ESC resistance to *E. coli* by conjugation. This isolate produced a clavulanate-sensitive β-lactamase with a pI equal to 8.2. PCR analysis identified the respective *bla* gene as an *shv*. The sequence of a segment (623 bp) that included codons 238 and 240 (Ambler's numbering) was 100% homologous to the respective segment of the *shv-5* gene. The SHV-5-encoding plasmid (approximately 90 MDa in size) also mediated resistance to gentamicin, tobramycin, sulfonamides, tetracycline, and chloramphenicol.

The emergence of ESC-resistant salmonellae in Romania has been recognized since 1997 (R. Filip et al., Abstr. 10th Eur. Congr. Clin. Microbiol. Infect. Dis., abstr. MoP105, 2000). A compilation of the recent data regarding the epidemiology of salmonella infections in Romania has not yet been performed. Also, data on the susceptibility status of salmonellae to ESC are not available from every hospital. Therefore, the current prevalence of resistance to ESC cannot be estimated. However, of the 40 *Salmonella* isolates recovered in the pediatric hospital, 14 were resistant to ESC and most were community acquired, factors that suggest a widespread resistance, at least during the study period.

Thirteen (12 serotype Typhimurium and 1 serotype Heidelberg) of the 14 isolates produced a CMY-2 cephalosporinase that is similar to the chromosomal β-lactamase of *C. freundii*. The 12 serotype Typhimurium isolates may constitute a single clone, as indicated by the similarity of the PFGE patterns. The time clustering of the respective infections also suggested an

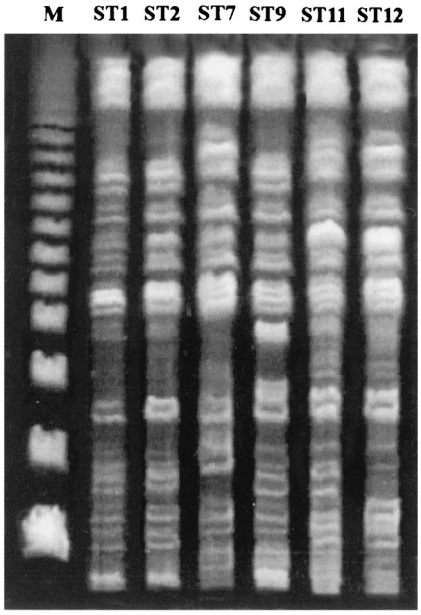

FIG. 1. PFGE of *Xba*I-digested chromosomal DNA of six CMY-2-producing serotype Typhimurium isolates. Lane M contains a molecular size marker (1-kb ladder; GIBCO-BRL).

outbreak, though it was not possible to trace epidemiological associations from the patients' records.

CMY-2 confers resistance to various ESC, including ceftriaxone, which is the antibiotic of choice for invasive salmonella infections in children. Plasmid-mediated AmpC β-lactamases of the CMY type have been found worldwide in nosocomial enterobacteria, particularly *Klebsiella pneumoniae* (10). Salmonellae could have acquired the *ampC* gene from such microorganisms. This hypothesis could also account for the emergence of the SHV-5-producing serotype Typhimurium isolate ST5, which exhibited a multiresistance phenotype similar to that of many ESBL-producing *K. pneumoniae* isolates in Romanian hospitals. Accordingly, previous studies indicated that *Salmonella* strains producing SHV ESBLs might have acquired the respective *bla* genes from bacteria of the hospital flora (15, 19). The emergence of ESC resistance in salmonellae may also have been facilitated by the use of oxyimino-β-lactams in the animal industry. Recent studies from the United States have shown that CMY-2-producing salmonellae have been spread in livestock and that exchange of CMY-2-encoding plasmids between enterobacteria from food animals and humans has occurred (3, 21). Plasmid-mediated CMY-2 has also been described for strains of *Salmonella enterica* serotype Senftenberg in Algeria (6) and *Salmonella enterica* serotype Enteritidis in Italy (18). The fact that salmonellae-producing CMY-2 have also emerged in Romania is interesting. A comparison of the CMY-2-producing salmonellae and the respective plasmids would provide useful clues regarding the evolution of and the mode by which this type of resistance spreads. Finally, the present study indicates that ESC-resistant salmonellae have been established in Romania, rendering the inclusion of ESC in routine susceptibility testing for this microorganism necessary.

We thank Charitini Magana for technical assistance.

REFERENCES

1. **Arlet, G., and A. Philippon.** 1991. Construction by polymerase chain reaction and intragenic DNA probes for three main transferable β-lactamases (TEM, SHV, CARB). FEMS Microbiol. Lett. **82:**19–26.
2. **Bradford, P. A., Y. Yang, D. Sahm, I. Grope, D. Gardovska, and G. Storch.** 1998. CTX-M-5, a novel cefotaxime-hydrolyzing β-lactamase from an outbreak of *Salmonella typhimurium* in Latvia. Antimicrob. Agents Chemother. **42:**1980–1984.
3. **Fey, P., P. Safranek, M. E. Rupp, E. F. Dunne, E. Ribot, P. C. Iwen, P. A. Bradford, F. J. Angulo, and S. H. Hinrichs.** 2000. Ceftriaxone-resistant *Salmonella* infection acquired by a child from cattle. N. Engl. J. Med. **342:** 1242–1249.
4. **Gaillot, O., C. Clement, M. Simonet, and A. Philippon.** 1997. Novel transferable β-lactam resistance with cephalosporinase characteristics in *Salmonella enteritidis*. J. Antimicrob. Chemother. **39:**85–87.
5. **Gautom, R. K.** 1997. Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other gram-negative organisms in 1 day. J. Clin. Microbiol. **35:**2977–2980.
6. **Koeck, J. L., G. Arlet, A. Philippon, S. Basmaciogullari, H. V. Thien, Y. Buisson, and J. D. Cavallo.** 1997. A plasmid-mediated CMY-2 beta-lactamase from an Algerian clinical isolate of *Salmonella* senftenberg. FEMS Microbiol. Lett. **152:**255–260.
7. **Matthew, M., A. M. Harris, M. J. Marshall, and G. W. Ross.** 1975. The use of analytical isoelectric focusing for the detection and identification of β-lactamases. J. Gen. Microbiol. **88:**169–178.
8. **Morosini, M. I., J. Blazquez, M. C. Negri, R. Canton, E. Loza, and F. Baquero.** 1996. Characterization of a nosocomial outbreak involving an epidemic plasmid encoding for TEM-27 in *Salmonella enterica* subspecies *enterica* serotype Othmarschen. J. Infect. Dis. **174:**1015–1020.
9. **National Committee for Clinical Laboratory Standards.** 1997. Performance standards for antimicrobial disk susceptibility tests, 6th ed. Approved standard M2-A6 (M100-S7). National Committee for Clinical Laboratory Standards, Wayne, Pa.
10. **Philippon, A., G. Arlet, and G. A. Jacoby.** 2002. Plasmid-determined AmpC-type β-lactamases. Antimicrob. Agents Chemother. **46:**1–11.
11. **Revathi, G., K. P. Shannon, P. D. Stapleton, B. K. Jain, and G. L. French.** 1998. An outbreak of extended-spectrum beta-lactamase-producing *Salmonella* senftenberg in a burns ward. J. Hosp. Infect. **40:**295–302.
12. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
13. **Simarro, E., F. Navarro, J. Ruiz, E. Miró, J. Gómez, and B. Mirelis.** 2000. *Salmonella enterica* serovar Virchow with CTX-M-like β-lactamase in Spain. J. Clin. Microbiol. **38:**4676–4678.
14. **Tassios, P. T., M. Gazouli, E. Tzelepi, H. Milch, N. Kozlova, S. Sidorenko, N. J. Legakis, and L. S. Tzouvelekis.** 1999. Spread of a *Salmonella typhimurium* clone resistant to expanded-spectrum cephalosporins in three European countries. J. Clin. Microbiol. **37:**3774–3777.
15. **Tosini, F., P. Visca, I. Luzzi, A. M. Dionisi, C. Pezzella, A. Petrucca, and A. Carattoli.** 1998. Class 1 integron-borne multiple-antibiotic resistance carried by IncFI and IncL/M plasmids in *Salmonella enterica* serotype Typhimurium. Antimicrob. Agents Chemother. **42:**3053–3058.
16. **Vahaboglu, H., L. M. Hall, L. Mulazimoglu, S. Dodanli, I. Yildirim, and D. M. Livermore.** 1995. Resistance to extended-spectrum cephalosporins, caused by PER-1 beta-lactamase, in *Salmonella typhimurium* from Istanbul, Turkey. J. Med. Microbiol. **43:**294–299.
17. **Vatopoulos, A. C., A. Philippon, L. S. Tzouvelekis, Z. Komninou, and N. J. Legakis.** 1990. Prevalence of a transferable SHV-5 type β-lactamase in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Greece. J. Antimicrob. Chemother. **26:**635–648.
18. **Villa, L., C. Mammina, V. Miriagou, L. S. Tzouvelekis, P. T. Tassios, A. Nastasi, and A. Carattoli.** 2002. Multidrug and broad-spectrum cephalosporin resistance among *Salmonella enterica* serotype Enteritidis clinical isolates in Southern Italy. J. Clin. Microbiol. **40:**2662–2665.
19. **Villa, L., C. Pezzella, F. Tosini, P. Visca, A. Petrucca, and A. Carattoli.** 2000. Multiple-antibiotic resistance mediated by structurally related IncL/M plasmids carrying an extended-spectrum β-lactamase gene and a class 1 integron. Antimicrob. Agents Chemother. **44:**2911–2914.
20. **Winokur, P. L., A. Brueggemann, D. L. DeSalvo, L. Hoffman, M. D. Apley, E. K. Uhlenhopp, M. A. Pfaller, and G. V. Doern.** 2000. Animal and human multidrug-resistant, cephalosporin-resistant *Salmonella* isolates expressing a plasmid-mediated CMY-2 AmpC β-lactamase. Antimicrob. Agents Chemother. **44:**2777–2783.
21. **Winokur, P. L., D. L. Vonstein, L. J. Hoffman, E. K. Uhlenhopp, and G. V. Doern.** 2001. Evidence for transfer of CMY-2 AmpC β-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. Antimicrob. Agents Chemother. **45:**2716–2722.