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Two Different Extended-Spectrum β-Lactamases (ESBLs) in One of the First ESBL-Producing Salmonella Isolates in Poland

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Two extended-spectrum β-lactamase (ESBL)-producing salmonella isolates, *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium, were analyzed. Both isolates produced the CTX-M-3 ESBL; however, their $bla_{CTX-M-3}$ genes were located on different plasmids. The serovar Typhimurium isolate also expressed another ESBL, SHV-2a, and probably the two ESBL genes had been acquired independently by the strain.

Various types of extended-spectrum β-lactamases (ESBLs) constitute one of the major mechanisms of resistance of gram-negative bacteria to oxyimino-β-lactam antibiotics (10, 13, 17). In general they are most frequently identified in *Klebsiella pneumoniae* and *Escherichia coli*; however, more and more papers in recent years have documented their growing incidence in other organisms of the family *Enterobacteriaceae*, including *Salmonella* spp. (10, 17). Sporadic infections or nosocomial outbreaks caused by ESBL-producing salmonellae have been reported in numerous countries of Latin America, Africa, Asia, and Europe (4, 8, 18, 19). Salmonellae have been found to express a wide variety of ESBL types, including TEM, SHV, PER, and CTX-M enzymes (10, 13, 18). The situation is of particular concern because it demonstrates the danger of ESBL spread among pathogens circulating in livestock and the community (17).

Although frequently found in many enterobacterial species, ESBL had not been reported in *Salmonella* spp. in Poland until 1999. The two cefotaxime-resistant isolates analyzed in this work belong to the first ESBL-producing salmonellae identified in the country. They were recovered in 1999 and 2000 in two regional hospitals located in geographically distant cities (Table 1). Both strains were cultured from the stools of patients with diarrhea. Biochemical identification of the isolates was performed with the ATB ID32E test (bioMérieux sa, Marcy l'Etoile, France). Serogroups and serotypes with respect to somatic (O) and flagellar (H) antigens were determined in the Public Health Sanitary-Epidemiological Station in Warsaw. Isolate 9197 from Grajewo was classified as *Salmonella enterica* serovar Typhimurium, and isolate 33/01 from Koszalin was identified as *Salmonella enterica* serovar Enteritidis. ESBL production was detected by the double disk synergy test (12).

The two salmonella isolates were subjected to the cefotaxime resistance transfer experiment as described previously (3), with *E. coli* A15, resistant to nalidixic acid, as the recipient strain. Transconjugants were selected on MacConkey agar (Oxoid, Basingstoke, United Kingdom) supplemented with cefotaxime (2 μg/ml; Polfa Tarchomin, Warsaw, Poland) and nalidixic acid (64 μg/ml; Sigma Chemical Company, St. Louis, Mo.). Both isolates produced transconjugants with an efficiency of around 10^{-4} per donor cell. MICs of various antibiotics were evaluated for the isolates and the transconjugants by the agar dilution method according to the NCCLS guidelines (14). Antimicrobial standards were supplied by the corresponding manufacturers. Both clinical isolates and their transconjugants revealed MIC patterns that are typical for ESBL-producing strains (13) (Table 2). They were characterized by raised MICs of a variety of β-lactams, except for cefoxitin and imipenem, and β-lactamase inhibitors reduced effectively MICs of β-lactams in all combinations tested. Cefotaxime MICs were significantly higher than those of ceftazidime. Apart from β-lactams the clinical isolates were also resistant to aminoglycosides and cotrimoxazole, and resistance to these drugs was cotransferred with β-lactam resistance to the transconjugants.

β-Lactamases of the clinical isolates and their transconjugants were visualized by isoelectric focusing (IEF), and their cefotaxime-hydrolyzing activity was detected by the bioassay approach. IEF and the bioassay were performed as described by Bauernfeind et al. (3), with the use of a Model 111 Mini Cell (Bio-Rad, Hercules, Calif.) for IEF. The concentration of cefotaxime in the bioassay was 2 μg/ml. Results of the analyses are shown in Table 1. Both isolates produced β-lactamases with isoelectric points (pIs) of 8.4 and 5.4, and these two enzymes were also found in extracts of the transconjugants. The serovar Typhimurium isolate, 9197, but not its transconjugant, expressed an additional β-lactamase with a pI of 7.6. Only enzymes with pIs of 8.4 and 7.6 demonstrated the cefotaxime-hydrolyzing activity under the conditions used in the bioassay (the β-lactamase with a pI of 5.4 was probably TEM-1).

Genes coding for the cefotaxime-hydrolyzing β-lactamases were detected by PCR in DNA of the clinical isolates. Both isolates were tested for the presence of bla_{CTX-M} genes (supposed to encode the pI 8.4 enzymes), and DNA of serovar Typhimurium 9197 was also checked by PCR specific for a bla_{SHV} gene (likely coding for the β-lactamase with a pI of 7.6). PCRs were run as reported previously (6, 11). Amplicons of the expected size of around 1 kb were obtained in all three

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TABLE 1. The salmonella isolates: date and place of isolation, β-lactamase profiles, β-lactamases expressed by transconjugants, cefotaxime-hydrolyzing activity assigned by the bioassay, ESBL sequences deduced from gene sequences, and plasmid fingerprints

Isolate	Date of isolation (day mo yr)	Location of hospital	β-Lactamases (pI values)[a]	β-Lactamases in transconjugants (pI values)[a]	ESBL(s)	*Pst*I plasmid fingerprint[b] (kb)
Serovar Typhimurium 9197	04 11 1999	Grajewo	**8.4**, **7.6**, 5.4	**8.4**, 5.4	CTX-M-3, SHV-2a	E (~110)
Serovar Enteritidis 33/01	14 11 2000	Koszalin	**8.4**, 5.4	**8.4**, 5.4	CTX-M-3	A1 (~90)

[a] pI values in bold indicate β-lactamases which were positive in the bioassay with cefotaxime as a substrate.
[b] Plasmid fingerprint designations were applied according to reference 1.

cases and subjected to sequencing using an ABI PRISM 310 sequencer (PE Biosystems, Foster City, Calif.). Sequencing was performed as previously described (1, 6, 11). The results are shown in Table 1. Coding regions of the two $bla_{\text{CTX-M}}$ genes were identical to that of the $bla_{\text{CTX-M-3}}$ gene (11), which indicated that the β-lactamase with a pI of 8.4 was CTX-M-3. The bla_{SHV} gene coding region was of the same nucleotide sequence as in the originally reported $bla_{\text{SHV-2a}}$ gene (16), and this revealed that the ESBL with a pI of 7.6 was SHV-2a.

Plasmid DNA was purified from the transconjugants with the use of the QIAGEN Plasmid Midi Kit (QIAGEN, Hilden, Germany) and subjected to the fingerprinting analysis along with all the variants of $bla_{\text{CTX-M-3}}$ gene-carrying plasmids that had been identified before in *Enterobacteriaceae* isolates in Poland (1). The *Pst*I restrictase (MBI Fermentas, Vilnius, Lithuania) fingerprinting was performed as reported previously (11). Both transconjugants contained large plasmids (of around 90 to 110 kb) that produced different fingerprints (Table 1 and Fig. 1). The plasmid specific for serovar Enteritidis 33/01 had a fingerprint identical to that of the widely spread $bla_{\text{CTX-M-3}}$ gene-carrying plasmid in Poland (variant A1) (1), whereas the molecule present in serovar Typhimurium 9197 demonstrated a unique *Pst*I restriction pattern.

With the two multiresistant serovar Typhimurium and serovar Enteritidis isolates analyzed here and an *S. enterica* serovar Mbandaka isolate reported elsewhere (21), Poland has joined the group of countries in which ESBL-producing salmonellae have been described. Both strains produced the CTX-M-3 enzyme, identified originally in 1996 in *Citrobacter freundii* isolates from a hospital in Warsaw (11). CTX-M β-lactamases have been relatively frequently found in ESBL-producing salmonellae, including CTX-M-2 in Argentina (2), CTX-M-4 in Russia (9), CTX-M-5 in Latvia (4), CTX-M-6 and -7 in Greece (8), and probably CTX-M-9 in Spain (19). The CTX-M-4 and -5-producing serovar Typhimurium strains caused clonal outbreaks in hospitals in St. Petersburg and Riga, respectively (4, 7, 22), and serovar Typhimurium strains related to those from St. Petersburg have been also observed in Hungary and Greece (22).

The CTX-M-3 β-lactamase is widely spread in Poland, having been identified in numerous strains of seven *Enterobacteriaceae* species in 16 medical centers throughout the country (1, 11, 15). The isolation of CTX-M-3-producing salmonella strains in hospitals in Grajewo and Koszalin has extended the list by two more institutions. The $bla_{\text{CTX-M-3}}$ gene-carrying plasmids of both isolates demonstrated a high transmission potential; moreover, one of these had been observed before in nine hospitals, which suggests its nosocomial origin (1, 11, 15). These data support the hypothesis that plasmid dissemination has been the major mechanism of the CTX-M-3 spread in Poland (1, 15). What is noteworthy is that these plasmids also contained a gene coding for another β-lactamase (likely TEM-1) and genes responsible for resistance to aminoglycosides and cotrimoxazole. One of the salmonella isolates, apart from CTX-M-3, also produced another ESBL variant, SHV-2a, which had previously never been identified in Poland. Genes coding for the two ESBLs have probably been acquired independently by the strain, as they were located on separate

TABLE 2. MICs of various antimicrobial agents obtained for the clinical isolates and their transconjugants

Strain[a]	MIC (μg/ml) of:															
	Ampicillin	Piperacillin	Piperacillin + tazobactam	Ceftazidime	Ceftazidime + clavulanate	Cefotaxime	Cefotaxime + clavulanate	Aztreonam	Cefoxitin	Imipenem	Gentamicin	Tobramycin	Ciprofloxacin	Cotrimoxazole	Chloramphenicol
Salmonella Typhim. 9197	>512	512	1	4	0.25	64	0.125	16	2	0.125	>256	>256	0.016	>64	4
Salmonella Enterit. 33/01	>512	>512	1	8	0.5	128	0.25	32	2	0.125	>256	>256	0.016	>64	4
R^+ (*Salmonella* Typhim. 9197)	>512	128	1	1	0.125	8	≤0.03	4	2	0.125	>256	128	0.25	16	4
R^+ (*Salmonella* Enterit. 33/01)	>512	128	1	1	0.125	16	≤0.03	4	2	0.125	>256	128	0.25	16	4
E. coli A15 nal	2	1	1	0.06	0.06	≤0.03	≤0.03	≤0.03	2	0.125	0.5	≤0.125	0.25	0.06	4
E. coli ATCC 25922	4	2	1	0.125	0.125	0.06	≤0.03	0.125	2	0.06	1	0.5	0.008	0.125	4
S. choleraesuis ATCC 14028	≤1	2	1	0.125	0.125	≤0.03	≤0.03	0.06	2	0.125	1	0.5	0.03	0.06	4

[a] R^+, transconjugant. Typhim., serovar Typhimurium; Enterit., serovar Enteritidis.

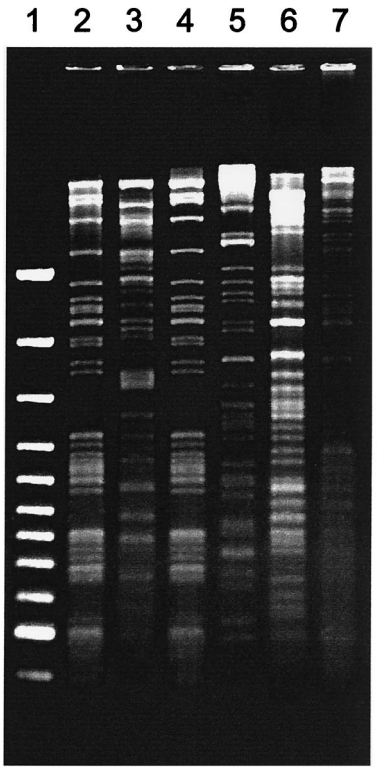

FIG. 1. Plasmid fingerprinting analysis performed with the use of the *Pst*I restriction enzyme (MBI Fermentas). Lanes: 1, GeneRuler 100-bp DNA Ladder Plus (MBI Fermentas); 2, Serovar Enteritidis 33/01, pattern A1; 3, Serovar Typhimurium 9197, pattern E; 4, *C. freundii* 2526/96, pattern A1 (1, 11, 15); 5, *E. coli* 279, pattern B (15); 6, *E. coli* SU 8350, pattern C (1); 7, *E. coli* BB 1775, pattern D (1).

DNA replicons. Such double-ESBL-producer strains, although occasionally reported (1, 5, 20), are rare, and to our knowledge they have not been observed in salmonella to date.

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