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CTX-M-2–Producing *Salmonella* Typhimurium Isolated from Pediatric Patients and Poultry in Brazil

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Ten *Salmonella enterica* serovar Typhimurium isolates producing CTX-M-2 extended-spectrum β-lactamase were identified from clinical and poultry sources in two distant cities in Brazil between 2003 and 2004. They included two isolates from pediatric patients and eight isolates from poultry or its environment. All isolates exhibited coresistance to non-β-lactam antimicrobials including tetracycline and trimethoprim/sulfamethoxazole. The CTX-M-2 gene was located on transferable plasmids with sizes between 90 and 170 kb that also carried other resistance determinants in some isolates. By pulsed-field gel electrophoresis, the genetic similarity of the isolates including clinical and poultry ones ranged from 89% to 100%.

Introduction

Salmonella enterica is a common cause of human gastroenteritis worldwide, and a large variety of animals, particularly food animals, have been identified as reservoirs for nontyphoidal *Salmonella* spp. Among more than 2,500 serotypes of the genus *Salmonella* described, two of them, Enteritidis and Typhimurium, are the most common causes of human salmonellosis in many countries, including Brazil.[7,10]

Salmonella infections that cause severe diarrhea as well as systemic infections, such as bacteremia and meningitis, require antimicrobial treatment. Fluoroquinolones and expanded-spectrum cephalosporins are essential drugs that are often used for treating patients with complicated *Salmonella* infections. Resistance to different β-lactams, primarily caused by the production of acquired extended-spectrum β-lactamases (ESBLs), has emerged worldwide during the last two decades, mostly associated with the *Enterobacteriaceae*, and in particular *Klebsiella pneumoniae* and *Escherichia coli*.[16] ESBL production in *Salmonella* has been considered relatively rare. However, the number of reported cases in various ESBL-producing *Salmonella* serotypes has been increasing worldwide in recent years, with the CTX-M group being predominant.[2,14] ESBL-producing *Salmonella enterica* serotypes, especially of CTX-M-type, have recently been detected in poultry and poultry products in different countries.[1,11,20,23] Growing evidence indicates that ESBL-producing organisms, and CTX-M producers in particular, represent an emerging problem in the community.[18] There is a legitimate concern that food-producing animals may serve as a reservoir for ESBL-producing human pathogens and that resistance genes may be transferred to humans through the food supply and ultimately cause treatment failure in patients receiving cephalosporin therapy for serious infections.[2,14] In the present study, we report 10 isolates of ESBL-producing *S. enterica* serotype Typhimurium identified from clinical and poultry sources in Brazil.

Materials and Methods

Bacterial strains

A total of 153 isolates of *Salmonella* Typhimurium were identified from specimens referred to Instituto Adolfo Lutz in São Paulo, Brazil, between 2003 and 2004. Of the 153 isolates, 73 and 80 were from human and nonhuman sources, respectively. The human isolates were collected from six public hospitals and originated from stool (52), blood (15), cerebrospinal fluid (3), and urine (3). The nonhuman isolates were from poultry and its environment (47), swine and its environment (25), and foodstuffs (8). Of these, 10 displayed an ESBL phenotype, as determined by double-disk diffusion testing. These isolates originated in São Paulo and Porto Alegre, Brazil. The two cities are approximately 1100 km apart. In São Paulo, two isolates were identified, one from the stool of a hospitalized child with gastroenteritis and the other from poultry. In Porto Alegre, one isolate was identified from a blood culture of a hospitalized child and the

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Table 1. Antimicrobial Susceptibility, Pulsed-Field Gel Electrophoresis Types and Extended-Spectrum β-Lactamase in *Salmonella* Typhimurium isolates

			MICs (μg/ml) of:							
Isolate	*Origin*	*City*	*AMP*	*CAZ*	*CPM*	*CTX*	*CRO*	*Additional resistance*	*PFGE*	*ESBL*
581/03	Patient	Porto Alegre	≥256	24	≥32	≥32	≥32	AMC[a] ATM CO SF SFT TT	A	CTX-M2
390/04	Patient	São Paulo	≥256	8	≥32	≥32	≥32	AMC[a] ATM ET SF SFT TT	B	CTX-M2
804/04	Poultry	São Paulo	≥256	8	≥32	≥32	≥32	ATM ET SF SFT TT	C	CTX-M2
1523/04	Drag swab	Porto Alegre	≥256	12	≥32	≥32	≥32	AMC ATM ET SF SFT TT	C	CTX-M2
1524/04	Drag swab	Porto Alegre	≥256	8	≥32	≥32	≥32	ATM ET SF SFT TT	D	CTX-M2
1526/04	Drag swab	Porto Alegre	≥256	16	≥32	≥32	≥32	AMC ATM ET GN SF SFT TT	B	CTX-M2
1527/04	Drag swab	Porto Alegre	≥256	12	≥32	≥32	≥32	AMC ATM ET SF SFT TT	B	CTX-M2
1528/04	Drag swab	Porto Alegre	≥256	12	≥32	≥32	≥32	AMC[a] ATM ET SF SFT TT	B	CTX-M2
1612/04	Drag swab	Porto Alegre	≥256	12	≥32	≥32	≥32	AMC[a] ATM ET SF SFT TT	B	CTX-M2
1613/04	Drag swab	Porto Alegre	≥256	12	≥32	≥32	≥32	AMC[a] ATM ET SF SFT TT	B	CTX-M2

[a]Intermediate resistance.
AMC, amoxicillin/clavulanic acid; AMP, ampicillin; ATM, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; CPM, cefepime; CO, chloramphenicol; ET, streptomycin; GN, gentamicin; SFT, trimethoprim/sulfamethoxazole; SF, sulfonamide; TT, tetracycline; MIC, minimum inhibitory concentration; PFGE, pulsed-field gel electrophoresis; ESBL, extended-spectrum β-lactamase.

remaining seven were identified from drag swabs collected from the floors of poultry environment (Table 1).

Serotyping

The isolates were serotyped on the basis of somatic O and phase 1 and phase 2 of H flagellar antigens by agglutination tests with antisera (prepared in the Laboratory of Enteric Pathogens, Instituto Adolfo Lutz, São Paulo), as specified in the Kauffmann–White scheme for *Salmonella* serotyping.[19]

Susceptibility testing

Antimicrobial susceptibility was determined by the disk diffusion method using Mueller–Hinton agar plates (Merck KGaA, Darmstadt, Germany) according to the guidelines of the Clinical and Laboratory Standards Institute.[3,4] The following antimicrobials disks (Oxoid, Hampshire, United Kingdom) were used: nalidixic acid, amoxicillin/clavulanic acid, ampicillin, aztreonam, ceftazidime, ceftazidime/clavulanic acid, cefotaxime, cefotaxime/clavulanic acid, ceftriaxone, cefepime, ciprofloxacin, chloramphenicol, streptomycin, gentamicin, imipenem, kanamycin, trimethoprim/sulfamethoxazole, sulfonamide, and tetracycline. Minimum inhibitory concentrations (MICs) were determined for ampicillin, ceftazidime, cefotaxime, and ceftriaxone by Etest (AB Biodisk, Solna, Sweden) according to the manufacturer's recommendations.

ESBL production was confirmed by double-disk diffusion testing when the key-hole effect was observed between the cephalosporin and amoxicillin/clavulanate disks, indicating partial or total restoration of the activity of ceftazidime or cefotaxime by clavulanic acid.[12] *E. coli* ATCC25922, *E. coli* ATCC35218, and *K. pneumoniae* ATCC700603 were used as reference strains for antimicrobial susceptibility testing.

Conjugation assays and plasmid analysis

Conjugal transfer was carried out in mixed broth cultures by using *E. coli* K-12 ($Lac^{-}Thr^{-}Leu^{-}Thi^{-}Str^{r}$) as the recipient strain. Transconjugants were selected on Mueller–Hinton agar plates containing cefotaxime (10 μg/ml) and nalidixic acid (50 μg/ml). Plasmid DNA was extracted from *E. coli* transconjugant cells[13] and were analyzed by electrophoresis in 0.7% agarose gel (Tris–acetate buffer), by using plasmids of known sizes as standards.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) analysis was performed according to the Centers for Disease Control and Prevention (CDC) PulseNet protocol (www.cdc.gov/pulsenet/protocols.htm). Briefly, cell lysis was followed by proteinase K treatment and DNA restriction with *Xba*I (New England Biolabs, Ipswich, MA). PFGE was performed with a CHEF DRII system (Bio-Rad, Hercules, CA) and the following run parameters: a switch time of 2.2 to 63.8 seconds and a run time of 20 hours. *S. enterica* Braenderup H9812 was used as molecular size marker. The macrorestriction patterns were compared by using the Gel Compar II software (Applied Maths, Sint-Martens-Latem, Belgium). Dice coefficient of 1.5 was used to calculate the similarity by using the unweighted pair group method with arithmetic averages. A difference of at least one restriction fragment in the patterns was considered the criterion for distinguishing between different profiles.

PCR analysis and DNA sequencing

PCR analysis to detect various ESBL genes was carried out as previously described.[5] PCR products were resolved on 1% agarose gels, stained with ethidium bromide, and photographed with ultraviolet illumination. DNA sequencing of the PCR products was performed with an ABI 3130 instrument (Applied Biosystems, Foster City, CA). Specifically, the entire coding region of the CTX-M-2 gene was sequenced using external primers.[5]

Results

Antimicrobial susceptibility

All 10 *Salmonella* Typhimurium isolates that displayed an ESBL phenotype, as determined by double-disk diffusion testing, were resistant to ampicillin, aztreonam, cefotaxime, ceftriaxone, cefepime, trimethoprim/sulfamethoxazole,

Table 2. Antimicrobial Resistance and Plasmid Characterization of *Escherichia coli* Transconjugants

Escherichia coli *transconjugants*	*Origin*	*City*	*Cotransferred resistance*	*Plasmid size (kb)*
581/03	Patient	Porto Alegre	AMP ATM CAZ CPM CTX CRO	90
390/04	Patient	São Paulo	AMP ATM CAZ CPM CTX CRO SF SFT TT	90
804/04	Poultry	São Paulo	AMP ATM CAZ CPM CTX CRO SF SFT TT	170
1523/04	Drag swab	Porto Alegre	AMP ATM CAZ CPM CTX CRO SF SFT TT	170
1524/04	Drag swab	Porto Alegre	AMP ATM CAZ CPM CTX CRO SF SFT TT	170
1526/04	Drag swab	Porto Alegre	AMP ATM CAZ CPM CTX CRO	90
1527/04	Drag swab	Porto Alegre	AMP ATM CAZ CPM CTX CRO	90
1528/04	Drag swab	Porto Alegre	AMP ATM CAZ CPM CTX CRO	90
1612/04	Drag swab	Porto Alegre	AMP ATM CAZ CPM CTX CRO	90
1613/04	Drag swab	Porto Alegre	AMP ATM CAZ CPM CTX CRO	90

AMP, ampicillin; ATM, aztreonam; CAZ, ceftazidime; CPM, cefepime; CTX, cefotaxime; CRO, ceftriaxone; SF, sulfonamide; SFT, trimethoprim–sulfamethoxazole; TT, tetracycline.

sulfonamide, and tetracycline. Nine were resistant to streptomycin, one to chloramphenicol, and another to gentamicin, as shown in Table 1. Six isolates were susceptible to ceftazidime, whereas four showed intermediate resistance. Three isolates were resistant to amoxicillin/clavulanic acid, five intermediately resistant, and two susceptible. All 10 isolates were susceptible to nalidixic acid, ciprofloxacin, and imipenem. The MICs of β-lactams are also shown in Table 1. Most isolates exhibited a higher level of resistance to cefotaxime, cefepime, ceftriaxone (MICs $\geq 32\,\mu g/ml$) than to ceftazidime (MICs 8–16 μg/ml).

Conjugation experiments

Cefotaxime-resistant *E. coli* transconjugants were readily obtained from each *Salmonella* Typhimurium donor at a frequency of approximately 10^{-4} transconjugants/recipient. The *E. coli* transconjugants showed cross resistance to multiple β-lactams. Except for one isolate, all of them also showed additional resistance to sulfonamide, trimethoprim/sulfamethoxazole, and tetracycline (Table 2). The plasmid sizes of *E. coli* tranconjugants were estimated to be between 90 and 170 kb (Table 2).

PFGE

The PFGE patterns of the *Salmonella* Typhimurium isolates are shown in Fig. 1. Four PFGE patterns (A, B, C, and D) were identified. The genetic relatedness of these strains ranged from 89% to 100%. All PFGE types were identified among the strains isolated in Porto Alegre. The two isolates from São Paulo belonged to groups B and C. The clinical isolate from São Paulo and an avian isolate from Porto Alegre shared an identical pattern in group C.

PCR for detection of ESBL resistance genes and sequencing results

All 10 isolates had positive PCR with primers specific for the CTX-M-type β-lactamase genes. DNA sequencing revealed the genes to encode CTX-M-2.

Discussion

In this study, we identified CTX-M-2–producing *Salmonella* Typhimurium isolates from humans and poultry sources in two distant cities in Brazil. These isolates appeared genetically related based on PFGE using *Xba*I as the restriction enzyme. While not performed in our study, the use of *Bln*I as the second enzyme may have further increased the discriminatory power of PFGE.[24] CTX-M-2 has commonly been identified in *K. pneumoniae* and *E. coli* in Brazil and thus appears to be already widely disseminated in this country.[6,9,21] Various ESBLs have been identified in *Salmonella* spp. in Brazil, including OXA-53, CTX-M-8, and CTX-M-9.[8,15,17] Our findings add to the growing list of ESBLs that are

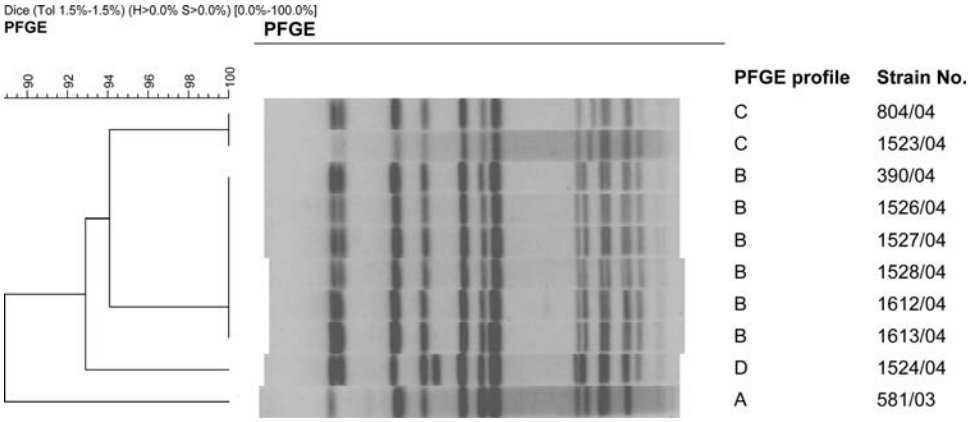

FIG. 1. Dendrogram for *Salmonella* Typhimurium isolates generated by pulsed-field gel electrophoresis (PFGE).

produced by *Salmonella* in Brazil and also suggest possible interspecies transfer of CTX-M-2 into this species from other species in the family *Enterobacteriaceae*, where this ESBL is already established.

The ESBL-producing *Salmonella* Typhimurium in our study were resistant not only to β-lactam antimicrobials but also to a variety of antimicrobials including chloramphenicol, streptomycin, gentamicin, trimethoprim/sulfamethoxazole, sulfonamide, and tetracycline. Over the years, an increasing proportion of *Salmonella* isolates have acquired resistance to various antimicrobials, including *Salmonella* Typhimurium strains isolated in Brazil.[10] Other reports have also highlighted the emergence of ESBL-producing *Salmonella* strains endowed with an extremely wide spectrum of antimicrobials, as also observed in this study.[11,20,22] The ESBL gene responsible for cephalosporin resistance is frequently cotransferred with other resistance determinants, contributing to multidrug resistance phenotype. In the present study, some of the isolates were found to mobilize resistance to tetracycline and trimethoprim/sulfamethoxazole along with the CTX-M-2 gene.

The use of antimicrobial drugs for therapeutic purposes in veterinary medicine and as growth promoters in food-producing animals is speculated to be a major cause of development of resistance in *Salmonella*, thereby presenting a potential risk to public health from zoonotic infections.[2,14] These animals and the products derived from them are then frequently transported regionally as well as internationally, providing ample opportunities for dissemination of multidrug-resistant isolates that possess highly mobile and clinically important resistance mechanisms into the community in a large geographic area.[21] Among the isolates studied, two were isolated from hospitalized children, and the others were isolated from poultry and its environment. While the investigation was limited to poultry and swine in our present study, other animals such as cattle are also potential carrier of ESBL-producing *Salmonella* spp. In summary, our findings of genetically related, ESBL-producing *Salmonella* isolates identified from patients as well as poultry and its environment underscore the importance of implementing control measures to minimize development of antimicrobial resistance in zoonotic organisms such as *Salmonella* spp.

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Disclosure Statement

No competing financial interests exist.

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