High Prevalence of Extended-Spectrum Beta Lactamases among Salmonella enterica Typhimurium Isolates from Pediatric Patients with Diarrhea in China

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Abstract

We investigated the extended-spectrum beta lactamases among 62 Salmonella enterica Typhimurium isolates recovered from children with diarrhea in a Chinese pediatric hospital. A large proportion of S. enterica Typhimurium isolates were resistant to multiple antimicrobial agents, including ampicillin (90.3%), tetracycline (80.6%), trimethoprim/sulfamethoxazole (74.2%), chloramphenicol (66.1%), cefotaxime (27.4%). Forty-nine (79.0%) of S. enterica Typhimurium isolates were positive for blaTEM-1b and resistant to ampicillin. Thirteen S. enterica Typhimurium isolates (21.0%) were positive for blaCTX-M-1-group and blaCTX-M-9-group, and all isolates harboring blaCTX-M genes were positive for IS3Cep1. Two main clones (PFGE type A and D) accounted for nearly 70% of S. enterica Typhimurium isolates, and 7 CTX-M-producing isolates belonged to PFGE type D. Collectively, our data reveal multi-drug resistance and a high prevalence of extended spectrum beta lactamases among S. enterica Typhimurium isolates from children in China. In addition, we report the first identification of blaCTX-M-55 within Salmonella spp. Our data also suggest that clonal spread is responsible for the dissemination of S. enterica Typhimurium isolates.

Introduction

Nontyphoidal Salmonella species, particularly Salmonella enterica Typhimurium, are the most common bacterial pathogens causing enteric infections among pediatric patients [1]. Antimicrobial agents are required for treating invasive infections caused by Salmonella spp., and third-generation cephalosporins are commonly used to treat invasive infections or severe diarrhea because of their pharmacodynamic properties and the low prevalence of resistance [2]. However, increasing resistance to cephalosporins has been reported worldwide for Salmonella spp., particularly S. enterica Typhimurium [3,4,5,6,7]. Resistance to broad-spectrum cephalosporin is often due to production of various plasmid-mediated β-lactamases, especially CTX-M-type extended-spectrum β-lactamases (ESBLs) [4,7,8,9]. Cefotaximases (CTX-M), comprised of five major CTX-M groups (1, 2, 8, 9, and 25), are associated with higher levels of hydrolytic activity against cefotaxime relative to cefazidime and are distributed among a wide range of bacteria with clinical significance over a wide geographic area [10].

In China, a low prevalence of resistance to third-generation cephalosporins has been reported among Salmonella spp., especially those isolated from pediatric patients [11,12]. The aim of the present study was to investigate the frequency of antimicrobial resistance and cephalosporin resistance genes within S. enterica isolates from pediatric inpatients with diarrhea in a Chinese pediatric hospital. However, high prevalence of broad spectrum cephalosporin resistance and ESBLs were found among S. enterica Typhimurium isolates from children in China.
years old, 10 patients; 2–3 years old, 5 patients; 3–4 years old, 6 patients and 4–5 years old, 4 patients. The antimicrobial agents used for treating the infections by *S. enterica* Typhimurium included penicillin, azithromycin, amoxicillin/sulbactam, latamoxef, cefoperazone/sulbactam, cefazidime, ampicillin/sulbactam, ceftriaxone, cefazolin, cefixime, and imipenem. Of 62 patients with diarrhea, 7, 4, 4 and 2 patients suffered bronchitis, pharyngitis, acute tonsillitis and meningitis, respectively. After treated by antimicrobial agents, all patients were cured and left hospital. Persistence of diarrhea ranged from 7 to 40 days. Addition to *S. enterica* Typhimurium, *Campylobacter spp.*, *enteropathogenic Escherichia coli* and *Shigella spp.* were not isolated from the stools of the patients included. This study focused on bacterial, so ethics approval was not needed according to the Ethics Committee of Wenzhou Medical College’s regulations.

**Antimicrobial susceptibility**

Antimicrobial susceptibility was determined by the disk diffusion method with 18 antimicrobial agents according to the criteria recommended by the CLSI [13], including including cefotaxime (30 μg), ceftazidime (30 μg), ampicillin (10 μg), aztreonam (30 μg), cefaclor (30 μg), cefoxitin (30 μg), piperacillin plus tazobactam (100/10 μg), imipenem (10 μg), meropenem (10 μg), chloramphenicol (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), tobramycin (10 μg), gentamicin (10 μg), amikacin (30 μg), ticarcillin (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), and levofloxacin (5 μg). MICs for cefotaxime and ceftazidime, were further determined by the agar dilution method in accordance with CLSI guidelines [13]. *Escherichia coli* ATCC 25922 was used as quality control strain for antimicrobial susceptibility testing.

**Detection of resistance genes**

Total DNA was extracted by boiling. Briefly, a fresh bacterial colony was suspended in 150 μL of sterile distilled water and boiled at 100°C for 10 minutes. After centrifugation at 15000 rpm for 15 minutes at 4°C, the supernatant was removed and stored at −20°C for PCR assays. PCR and DNA sequencing were performed for the detection of ESBL genes in all ampicillin-resistant isolates with oligonucleotide primers previously described, including those for bla*TEM*, *bla*SHV, *bla*CTX-M, *bla*VIM and *bla*PER genes [14,15]. All amplicon sequences were compared with those in the GenBank nucleotide database (www.ncbi.nlm.nih.gov/blast/). PCR mapping experiments using combinations of the *ISeCP1* forward primers and the reverse primers were performed to detect the flanking regions of *bla*CTX-M-1-group and *bla*CTX-M-9 group reverse primers were performed to detect the flanking regions of *bla*CTX-M-1-group and *bla*CTX-M-9-group genes.

**Transfer of resistance genes**

In order to determine whether cephalosporin resistance was transferable in *S. enterica* Typhimurium isolates, a conjugation experiment was carried out in Luria-Bertani broth with *E. coli* J53 as the recipient as previously described [16]. Transconjugants were selected on tryptic soy agar plates containing sodium azide (100 μg/mL) for counterselection and cefotaxime (30 μg/mL) for plasmid-mediated cephalosporin resistance selection.

**Pulsed-field gel electrophoresis (PFGE)**

Chromosomal DNA was prepared from all *S. enterica* Typhimurium isolates and cleaved with 10 U *XbaI*. Electrophoresis was performed on 1% agarose gels in 0.5 M Tris/borate/EDTA buffer on a CHEF-Mapper YA PFGE system (Bio-Rad, Hercules, CA) for 22 h at 14°C, with run conditions of 6 V/cm, a pulse angle of 120° and pulse times from 5 to 20 s. A λDNA ladder (Amersham Biosciences) was used to confirm molecular mass and bands stained with ethidium bromide (0.5 μg/mL) prior to their identification through photography under UV light. Comparison of the PFGE patterns was performed with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) using the Dice Similarity coefficient. Clusters were defined as DNA patterns sharing more than 85% similarity.

**Results**

**Antimicrobial susceptibility**

Of the 62 *S. enterica* Typhimurium isolates, 59 (95.2%) were resistant to at least three antimicrobial agents, while only 2 (3.2%) were susceptible to all antimicrobial agents tested. *S. enterica* Typhimurium isolates tested were most commonly resistant to ampicillin (90.3%, 56/62), followed by tetracycline (80.6%, 50/62), trimethoprim/sulfamethoxazole (74.2%, 46/62) and chloramphenicol (66.1%, 41/62). Seventeen (27.4%, 17/62) and 8 (8/62, 12.9%) isolates were resistant to cefotaxime (MIC range from 64 to >256 μg/mL) and ceftazidime (MIC range from 32 to >256 μg/mL), respectively. All cefazidime-resistant isolates were also resistant to cefotaxime. Twelve isolates (19.4%, 12/62) were highly resistant to ciprofloxacin (MIC>4 μg/mL). The frequencies of resistance and intermediate to other antimicrobial agents among *S. enterica* Typhimurium isolates were as follows: cefoxitin, 8.1% (5/62) and 1.6% (1/62); aztreonam, 16.1% (10/62) and 12.9% (8/62); cefaclor, 35.5% (22/62) and 0; ampicillin/sulbactam, 51.6% (32/62) and 25.8% (16/62); piperacillin/tazobactam, 19.4% (12/62) and 51.6% (32/62); amikacin, 17.2% (11/62) and 41.9% (26/62); gentamicin, 51.6% (32/62) and 6.5% (4/62); and tobramycin, 56.5% (35/62) and 17.7% (11/62). All isolates were susceptible to imipenem and meropenem.

**β-lactam resistance genes**

Forty-nine (79.0%) of 62 *S. enterica* Typhimurium isolates were positive for *bla*TEM and resistant to ampicillin. All *bla*TEM ampicillins were confirmed by *bla*TEM-b by DNA sequencing. Thirteen *S. enterica* Typhimurium isolates (21.0%, 13/62) were positive for *bla*CTX-M-1-group and *bla*CTX-M-9-group genes (8 for *bla*CTX-M-14, 3 for *bla*CTX-M-15, 1 for *bla*CTX-M-55, and 1 for both *bla*CTX-M-14 and *bla*CTX-M-55). Characteristics of *S. enterica* Typhimurium isolates producing ESBLs were showed in Figure 1. Among the 8 CTX-M-14-producing isolates with MICs ranging from 128 to 256 μg/mL for cefotaxime, only one was highly resistant to cefazidime (128 μg/mL) whereas the other 7 exhibited reduced susceptibility to cefazidime (2–8 μg/mL). Three CTX-M-15-producing isolates with MICs ranging from 32 to 128 μg/mL for cefazidime were highly resistant to cefotaxime (MIC>256 μg/mL). Two isolates harboring *bla*CTX-M-55 were highly resistant to cefotaxime and cefazidime (MIC>256 μg/mL). All isolates harboring *bla*CTX-M-55 were positive for *ISeCP1*. Analysis of the flanking regions of *bla*CTX-M genes showed that the insertion sequence *ISeCP1* was located 48 bp, 45 bp or 42 bp upstream from *bla*CTX-M-15 (accession number GQ330540), *bla*CTX-M-55 (accession number GQ456157) or *bla*CTX-M-14 (accession number GQ353235), respectively.

**Transfer of antimicrobial resistance genes**

Extended-spectrum cephalosporin resistance could be transferred by conjugation from seven ESBL-producing donors (two isolates harboring *bla*CTX-M-14, six harboring *bla*CTX-M-15 and one harboring both *bla*CTX-M-14 and *bla*CTX-M-55). Seven cephalosporin resistance transconjugants were resistant to chloramphenicol and trimethoprim/sulfamethoxazole and harbored *bla*TEM-b.
PFGE

Ten different PFGE clusters were identified among the 62 S. enterica Typhimurium isolates. PFGE types A, B, and D accounted for 19.4% (12/62), 9.7% (6/62) and 50% (31/62) of these isolates, respectively. Thirteen ESBL-producing isolates distributed in six PFGE clusters listed in Figure 1. Of the 8 CTX-M-14-producing S. enterica Typhimurium isolates, 5, 2 and 1 belonged to PFGE type D, B and I, respectively. Three CTX-M-15-producing isolates belonged to three different PFGE types (E, G and H). One CTX-M-55-producing isolate and both CTX-M-14 and CTX-M-55-producing isolates belonged to PFGE type D.

Discussion

The majority of S. enterica Typhimurium isolates in our study were resistant to multiple antimicrobial agents, indicating that fewer antibiotics may be useful for treating S. enterica Typhimurium infections. Carbenapencens exhibit high antimicrobial activity against S. enterica Typhimurium isolates in vitro in our studies. The CLSI recommends that for fecal isolates of Salmonella and Shigella spp. only ampicillin, fluoroquinolone, and trimethoprim/sulfamethoxazole sensitivities should be reported routinely, whereas for extraintestinal isolates of Salmonella spp. only sensitivities to chloramphenicol and third-generation cephalosporins should be reported [13]. However, S. enterica Typhimurium resistance rates for ampicillin, trimethoprim/sulfamethoxazole and chloramphenicol were very high (more than 60%) in our studies. In addition, although no patient had received fluoroquinolones prior to isolation of Salmonella spp. from their stool, 48% and 19% of S. Typhimurium isolates exhibited low- or high-level resistance to ciprofloxacin, respectively. Therefore, our data suggest that ampicillin, trimethoprim/sulfamethoxazole, chloramphenicol and fluoroquinolones should be used with caution for the treatment of S. enterica Typhimurium infections in the pediatric population. ESBL- and AmpC-mediated resistance has been identified in non-typhoidal Salmonella isolates in many geographic areas [5,17,18,19]. However, reported resistance of non-typhoidal Salmonella isolates to broad-spectrum cephalosporins remains low [12,20,21]. In the study conducted in Wuhan, China using clinical samples from children ages 0–3 years, only 7 of 221 (3.2%) S. enterica isolates were resistant to ceftriaxone (10). In another study from the Henan province, only 2.1% of S. enterica Typhimurium patient isolates were resistant to ceftriaxone over a 2-year timeframe [11,12]. Relative to these studies, the rates of resistance to broad-spectrum cephalosporins for S. enterica Typhimurium isolates in our study was very high.

Broad-spectrum cephalosporins are commonly used to treat serious Salmonella infections. ESBLs are the predominant cause of resistance to broad-spectrum cephalosporins in Enterobacteriaceae, particularly E. coli and Klebsiella spp. Therefore, understanding patterns and mechanisms for interspecies and intraspecies transfer of ESBLs is of great interest. The TEM-, SHV- and CTX-M-type ESBLs are the most widely distributed worldwide. Published reports indicate that CTX-M-type ESBLs are the most prevalent in China [22,23]. blaCTX-M-14 and blaCTX-M-15 genes have been identified in S. enterica Typhimurium isolates from many areas including China [4,7,11,12,24]. In the present study, CTX-M-type ESBLs, mainly CTX-M-14, were detected in S. enterica Typhimurium isolates, indicating that CTX-M-type ESBLs were the predominant cause of resistance to broad-spectrum cephalosporins for S. enterica Typhimurium isolates in our study. The mobile genetic element IS\text{Ec1}, a single copy insertion sequence responsible for mobilization of \text{bla} genes and identified upstream of several \text{bla}_{CTX-M} genes carried by E. coli and Klebsiella spp isolates, was found in association with all of our isolates. We speculate that the S. enterica Typhimurium isolates in China could have acquired the \text{bla}_{CTX-M} genes from E. coli and Klebsiella spp in the community, and that subsequent spread of these genes among S. enterica Typhimurium isolates may have occurred. Future epidemiologic studies may confirm whether these genes were transmitted in this or perhaps other sequences, and the potential clinical implications of ESBL-mediated resistance for gastrointestinal and/or invasive infections caused by S. Typhimurium.

\text{bla}_{CTX-M-55}, which increases the catalytic efficiency against cefazidime, is a variant of the \text{bla}_{CTX-M-15} gene and previously found only in E. coli and K. pneumoniae spp. in China and Thailand [25,26,27]. Interestingly, we found that two S. enterica Typhimurium isolates with resistance to both cefotaxime and ceftazidime harbored \text{bla}_{CTX-M-55}, and one of these harbored both \text{bla}_{CTX-M-14} and \text{bla}_{CTX-M-55}. To our knowledge, this is the first report of \text{bla}_{CTX-M-55} in Salmonella spp. Recently, 24 (24.5%) of 98 E. coli isolates recovered from pets in South China were found to harbor \text{bla}_{CTX-M-55}, among which 6 isolates harbored both \text{bla}_{CTX-M-14} and \text{bla}_{CTX-M-55} [25]. The partial DNA sequence of \text{IS\text{Ec1}} was 45 bp upstream of \text{bla}_{CTX-M-55}, in the single isolate in our study harboring both \text{bla}_{CTX-M-14} and \text{bla}_{CTX-M-55} was 100% identical to
the corresponding sequences of blaCTX-M-55 genes harbored by *E. coli* isolates from the pets in this study. We speculate that *E. coli* which either colonize or cause clinically apparent infection in pets may serve as a reservoir for blaCTX-M-14 and blaCTX-M-55 genes and, ultimately, cause clinically apparent infections in pediatric patients. Confirmation of this hypothesis, and the development of subsequent clinical strategies for limited these infections, would require additional comprehensive surveillance efforts among communities in China and elsewhere.

In our study, two main clusters (PFGE types A and D) were found, indicating that gastrointestinal infections in children were caused mainly by clonally related *S. enterica* Typhimurium isolates and clonal spread was responsible for the dissemination of *S. enterica* Typhimurium. Because 5 isolates harboring blaCTX-M-14 genes and two isolates harboring blaCTX-M-55 genes belonged to the same PFGE cluster (D), the spread of blaCTX-M-14 and blaCTX-M-55 genes was also associated with clonal spread.

In conclusion, the present study demonstrates significant multidrug resistance among *Senterica* Typhimurium within a pediatric population in China, including a higher prevalence of broad-spectrum cephalosporin resistance and expression of blaCTX-M-55 genes among *S. enterica* Typhimurium isolates than what has previously been reported. Moreover, the present study is the first report of the presence of blaCTX-M-55 genes in Salmonella spp. Finally, our data suggest that clonal spread is responsible for the dissemination of *S. enterica* Typhimurium isolates and blaCTX-M-14 genes in this population.

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**Author Contributions**

Conceived and designed the experiments: FY QC LW JP. Performed the experiments: XY QL LY BD CC. Analyzed the data: ZQ CP. Contributed reagents/materials/analysis tools: XZ JH. Wrote the manuscript: FY. Assisted with dealing with figures: YL.

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