Characterization of extended-spectrum ß-lactamase-producing Salmonella isolates in a children's hospital in Ankara- first report of SHV-2a and SHV-9 in Salmonella spp. from Turkey

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The rate of in vitro resistance to various antimicrobials in 179 consecutive isolates of Salmonella spp., which included serogroups D (109), B (52), C_1 (10) and C_2 (8) isolated from children, was investigated. Production of extended-spectrum ß-lactamase (ESBL) was studied in ampicillin-resistant isolates. Antimicrobial susceptibilities were determined by disk diffusion tests and by BIOMIC video reader system. Overall resistance rates to ampicillin and amoxicillin/clavulanate were 26.3% and 10.6%, respectively. Resistance to ceftriaxone and ceftazidime was 3.3%. Resistance rates for chloramphenicol, trimethoprim-sulfamethoxazole, ciprofloxacin, amikacin and gentamicin were 40.7%, 31.3%, 2.2%, 2.2% and 6.1%, respectively. ß-lactamase production was detected in 42 isolates. Mating out experiments, isoelectric focusing, dot blot hybridization, polymerase chain reaction (PCR) and sequencing were performed on two *S. paratyphi* B isolates that produced ESBLs. One isolate produced SHV-2 and TEM-1 and the other produced SHV-2a, SHV-5a (SHV-9) and TEM-1. This is the first report of SHV-2a and SHV-5a (SHV-9) in *S. paratyphi* B in Turkey.

Key words: salmonella, antibiotic resistance, ß-lactamases, SHV, TEM.

Salmonella spp. resistant to cephalosporins due to the production of different extended-spectrum ß-lactamases (ESBLs) such as TEM, SHV, PER and CTX-M are increasing worldwide[1-8]. Some of these ESBLs have been reported exclusively from Turkey, especially PER-1[9,10].

Spread of ESBL-producing Salmonella spp. has an important impact on public health since these organisms are mostly responsible for community-acquired infections and these plasmid- mediated ß-lactamases render them resistant to ß-lactam antibiotics, including third- and fourth-generation cephalosporins.

In this study, in vitro antimicrobial resistance rates and ESBL production in Salmonella spp. were investigated in a children's hospital. Two isolates that were shown to produce ESBLs were further analyzed and types of ß-lactamases were identified.

Material and Methods

Bacterial Strains

Salmonella spp. that were isolated consecutively between October 1998-January 2001 in Hacettepe University Children's Hospital, Clinical Microbiology Laboratory (Ankara, Turkey) were included in this study. A total of 179 Salmonella spp. were isolated from clinically significant samples, which were stools (176), urine[2] and blood[1]. A single isolate per patient was retained in the study. All isolates were identified by conventional

methods and serotyped using specific antisera (Bacto, poly A-I and Vi Difco, Detroit, MI, USA). Identifications were confirmed using Crystal test (Crystal Enteric Panel, Becton Dickinson). All isolates were stored at –70°C until studied.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the disk diffusion test methodology in Mueller Hinton agar with ampicillin, amoxicillin, amoxicillin-clavulanic acid, ceftriaxone, ceftazidime, trimethoprim-sulfamethoxazole, ciprofloxacin, chloramphenicol, amikacin, and gentamicin disks (Oxoid Ltd.; Basingstoke, UK). Clinical Laboratory Standards Institute (CSLI) guidelines were followed in the tests[11]. The minimum inhibitory concentrations (MICs) of antibiotics were then determined by BIOMIC video reader system (Giles Scientific Inc.; Santa Barbara, CA, USA).

Screening of ESBL Production

Ampicillin-resistant isolates were screened for ß-lactamase production by nitrocefin test (Oxoid). Disk approximation test was used in these isolates as a screening test for ESBL production using disks of amoxicillin-clavulanic acid, ceftriaxone and ceftazidime[12].

Mating Out Experiments

Transfer of ß-lactam resistance from ampicillin-resistant isolates to nalidixic acid-resistant, ampicillin-susceptible recipient *Escherichia coli* K-12 J53-1 was attempted by standard mating technique. Cultures of the donor (0.5 ml) and recipient (0.1 ml) strains were inoculated in 5 ml of Mueller Hinton broth and incubated overnight at 37°C. The suspension was spread on EMB plates containing 25 µg/ml nalidixic acid and the ß-lactam(s). After an overnight incubation, transconjugant colonies were picked off from the medium and retested for production of ß-lactamase and in vitro antimicrobial susceptibility testing[13].

Genomic DNA Preparations in ESBL-Positive Isolates

Plasmid DNA of two *Salmonella paratyphi* B isolates was tentatively extracted with the Nucleobond AX kit (Macharey-Nagel; Hoerdt, France)[13]. The putative extracted plasmid DNA suspension was electroporated into *E. coli* JM 109, and recombinant bacteria were selected on Trypticase Soy Agar (TSA) plates containing amoxicillin (100 µg/ml). Genomic DNAs from two isolates were extracted as described previously[14].

Isoelectric Focusing and ß-Lactamase Assays

Cultures of the ampicillin-resistant isolates were grown overnight at 35°C in 10 ml Trypticase Soy Broth (TSB) containing amoxicillin at 100 µg/ml. One milliliter of each overnight culture was then grown for 3 h at 35°C in 10 ml of TSB without antibiotic. Bacterial suspensions were disrupted by sonification (twice for 30 s each time at 20 Hz [phospholyzer Vibra Cell 300, Bioblock; Illkirch, France]) and were centrifuged (48,000 x g, 1 h, 4°C). The supernatant containing the enzyme extracts was subjected to analytical isoelectric focusing (IEF) with a mini IEF 111 apparatus (Bio-Rad) with a polyacrylamide gel containing a gradient made up of ampholytes with a pH range from 3 to 10 (Bio-Rad). Migration was performed with three consecutive voltages (100V for 15 min, 200 V for 15 min, and 450 V for 1 h). The focused ß-lactamases were detected by overlaying the gel with 1 mM nitrocefin (Oxoid; Paris, France) in a 50 mM phosphate buffer (pH 7.0). The pI values were determined and compared to those for ß-lactamases with known pI values.

ß-lactamase-specific activities of ESBL-positive isolates were determined as described previously[15]. One unit of enzyme activity was defined as the activity that hydrolyzed 1 nmol of cephalothin per min per mg of protein. The total protein content was measured with the Bio Rad DC Protein assay kit.

Dot Blot Hybridization Experiments

DNA-DNA hybridizations were performed as described by Sambrook et al.[13]. Three microliters of total heat-denaturated DNA from a culture of each isolate was placed on nylon membrane (Hybond N^+; Amersham, Les Ullis, France) and the DNA was UV cross-linked for 2 min (UV cross-linker, Stratagene). Dot blot hybridizations were performed with the 354-bp ScaI fragment internal to bla $_{PSE-1}$[16], the 450-bpPstI-NotI fragment from recombinant plasmid pHUC37 for bla $_{SHV-3}$[17] or the 560-bp SspI-PstI fragment internal to bla $_{TEM-1}$ from recombinant plasmid pBR322[18].

PCR for bla_{TEM}, bla_{SHV}, bla_{CARB}, bla_{VEB}, bla_{CTX-M} and Sequencing

For each reaction, 2 µg of genomic DNA from each of the 2 S. paratyphi B isolates was used. The polymerase chain reaction (PCR) amplification for bla_{TEM}, bla_{SHV}, bla_{CARB}, bla_{VEB-1}, bla_{CTX-M} detection was performed with laboratory designed primers: bla_{TEM}, F(5'-ATA AAA TTC TTG AAG ACG AA-3' and R(5'-GAC AGT TAC CAA TGC TTA ATC-3'[19], bla_{SHV} F(TTA TCT CCC TGT TAG CCA CC-3' and R(5'-GAT TTG CTG ATT TCG CTC GG-3'[20], bla_{CARB} F(5'-CCA TCT GTA GTT TTT GCA AGC AG-3') and R(5'-CAA CGC GAC TGT GAT GTA TAA AC-3'[21], bla_{VEB-1} F(5'-CGA CTT CCA TTT CCC GAT GC-3') and R(5'-GGA CTC TGC AAC AAA TAC GC-3'[22], and bla_{CTX-M} universal F(5'-CGA TGT GCA GTA CCA GTA A-3') and R (5'TTA GTG ACC AGA ATC AGC GG-3')[23]. The PCR product for TEM and SHV for the two strains was sequenced with Applied Biosystems sequencer (ABI 311).

Results

Bacterial Isolates

Among 179 Salmonella spp., the most frequent serotype was serogroup D (n=109), followed by serogroup B (n=52), C1 (n=10) and C2 (n=8).

Antimicrobial Susceptibility Testing

Eighty isolates (45%) were susceptible to all the antimicrobial agents tested. The antibiotic resistance rates of 179 isolates are shown in Table I. Isolates with resistance to at least two different groups of antibiotics were defined as "multidrug resistant". There were 61 (34%) multidrug resistant isolates with various resistance patterns (Table II). The most frequent multidrug resistance phenotype was ampicillin, chloramphenicol and trimethoprim/sulfamethoxazole (TMP/SMX) resistance, which was observed in 11 isolates. Six isolates were resistant to ceftazidime and ceftriaxone (5 were serotype B and 1 was serotype D). All isolates were also resistant to ampicillin and chloramphenicol.

ESBL Production

Out of 47 isolates that were resistant to ampicillin, 42 were shown to produce ß-lactamase with the nitrocefin test. Six isolates were resistant to ceftazidime and ceftriaxone and in two of these, ESBL production was suspected with the double disk technique. Both isolates were serotyped as *S. paratyphi* B and were multidrug resistant, showing the same phenotype (ampicillin, amoxicillin-clavulanic acid, ceftazidime, ceftriaxone, chloramphenicol, gentamicin and TMP/SMX resistant), as shown in Table III.

Mating Out Assays and Isoelectric Focusing

There were six isolates that were ceftazidime-resistant and cefotaxime-resistant; two of these were ESBL-positive. In one of them, resistance could be transferred to the transconjugant.

ESBL-producing strain 859479 had three bands with pIs 5.4, 7.4, 7.6, and strain 2444316 had only two bands with pIs 5.4, 7.4. Although

Table I. Resistance Rates to Antibiotics in Salmonella Serogroups

Antibiotics	Serogroup B n (%)	Serogroup D n (%)	Serogroup C_1 n*	Serogroup C_2 n*	Total n (%)
Ampicilin	31 (59.6)	13 (11.9)	2	1	47 (26.3)
Amoxicillin-clavulanic acid	12 (23.0)	7 (6.4)	0	0	19 (10.6)
Ceftriaxone	5 (9.6)	1 (0.9)	0	0	6 (3.3)
Ceftazidime	5 (9.6)	1 (0.9)	0	0	6 (3.3)
Chloramphenicol	36 (69.2)	29 (26.6)	6	2	73 (40.7)
TMP/SMX	25 (48.0)	25 (22.9)	3	3	56 (31.3)
Ciprofloxacin	1 (1.9)	1 (0.9)	1	1	4 (2.2)
Amikacin	3 (5.8)	0 (0)	0	1	4 (2.2)
Gentamicin	6 (11.5)	3 (2.7)	0	2	11 (6.1)

*Numbers are not sufficent to give percentages.
TMP/SMX: Trimethoprim/sulfamethoxazole.

Table II. Multiple Resistance Phenotypes Among Antibiotic-Resistant Salmonellae

	Serogroups			
Resistance phenotype[a]	B n (%)	D n (%)	C1 n*	C2 n*
Ak, C	1 (1.9)			
Ak, C, T/S, Cp			1	1
Ak, C, T/S, G	1 (1.9)			
Amp	4 (7.7)	2 (1.8)		1
Amp, A/C		1 (0.9)		
Amp, C	3 (5.8)	4 (3.7)		
Amp, C, A/C	3 (5.8)	3 (2.8)		
Amp, C, T/S	10 (19.2)		1	
Amp, C, T/S, Cp			1	
Amp, C, G, T/S	1 (1.9)			
Amp, C, T/S, A/C	4 (7.7)	1 (0.9)		
Amp, C, A/C, Ctrx, Caz		1 (0.9)		
Amp, C, G, Ctrx, Caz	1 (1.9)			
Amp, C, G, T/S, A/C		1 (0i9)		
Amp, Ak, C, T/S, A/C, Cp	1 (1.9)			
Amp, C, G, A/C, Ctrx, Caz	1 (1.9)			
Amp, C, G, T/S, A/C, Ctrx, Caz	2 (3.8)			
Amp, C, T/S, A/C, Ctrx, Caz	1 (1.9)			
C	4 (7.7)	8 (7.3)	3	
C, G		1 (0.9)		
C, T/S	3 (5.8)	9 (8.2)	1	1
C, T/S, Cp		1 (0.9)		
G				2
G, T/S		1 (0.9)		
T/S	2 (3.8)	12 (11.0)		1
TOTAL	52 (100)	109 (100)	10	8

*Percent is not given because of the low number[a].
Ak: Amikacin. A/C: Amoxicillin/clavulanic acid. Amp: Ampicillin. C: Chloramphenicol. Caz: Ceftazidime. Ctrx: Ceftriaxone. Cp: Ciprofloxacin. G: Gentamicin. T/S: Trimethoprim/sulfamethoxazole.

Table III. Minimum Inhibitory Concentrations and pI Values of ESBL-Producing Salmonella Isolates

	Strain 859479	Strain 2444316
Ampicillin	64	64
Amikacin	<0.5	4
Chloramphenicol	>64	>64
Ciprofloxacin	<0.12	<0.12
Gentamicin	8	16
Trimethoprim-sulfamethoxazole	>256	>256
Amoxicillin-clavulanic acid	16	16
Ceftriaxone	8	>128
Ceftazidime	32	64
pI values	5.3, 7.4, 7.6	5.4, 7.4

these two isolates had the same resistance pattern, strain 2444316 had higher MICs than 859479 (Table III).

Dot Blot Hybridizations

Dot blot hybridization results for total DNAs from two salmonella strains were positive with bla $_{TEM-1}$ probe. Two isolates were also positive with the SHV probe.

PCR for bla_{TEM}, bla_{SHV}, bla_{CARB}, bla_{VEB}, bla_{CTX-M} and Sequencing

By using bla_{TEM} specific primers, PCR fragment was obtained from genomic DNA from both of the salmonella isolates. Direct sequencing of the PCR product for TEM and SHV from 2 S. paratyphi B revealed 100% identity with $bla_{TEM.}$ These results together with the pI data suggested that both strains produced TEM-1.

Sequencing of PCR products of ESBL producers and the transconjugant identified an SHV-2 enzyme in strain 859479 and two coexisting enzymes: SHV-2a and SHV-5a (SHV-9) in strain 2444316.

Discussion

The emergence and spread of resistance in enterobacteriaceae is complicating the treatment of serious nosocomial infections and threatening to create species resistant to all currently available agents. Salmonella and other enterobacteriaceae that cause gastroenteritis may also be ESBL producers, which is of relevance when children require treatment for invasive infections[24]. In this study, we aimed to determine the rate of resistance to several antimicrobial agents in Salmonella spp. isolated from children, with special emphasis on ESBLs.

According to our results, among 179 salmonella isolates, 47 (26.3%) were resistant to ampicillin. Ampicillin resistance was higher in Salmonella B (59.6%) and Salmonella D (11.9%) serogroups. Out of 10 C1 serotypes, two were resistant to ampicillin and none of the eight C2 serotypes were resistant.

In 42 of the ampicillin-resistant isolates, resistance was due to the production of a ß-lactamase, and isoelectric focusing results suggest that this enzyme has pI of 5.4 and is consistent with TEM-1 enzyme. TEM-1 enzyme has been reported as the most frequent ß-lactamase found in ampicillin-resistant salmonella in Europe, South Africa and South America[25-28]. In this study, 18 of the ampicillin-resistant isolates were also resistant to amoxicillin/clavulanic acid, which cannot be explained by the presence of TEM-1 enzyme alone and may be due to inhibitor-resistant ß-lactamases, some of which have the same pI as the TEM-1 enzyme; however, these isolates were not further examined[27].

In a study from Romania, two major different patterns of ß-lactamases were identified in *S. typhimurium*: a group with pIs of 5.4 and 8.2 and another group with pIs of 5.4. The blaTEM ß-lactamase was identified in 14/16 of the clinical isolates and the blaSHV-5 gene in one strain[28]. In South Africa, 15.6% of isolates produced SHV or TEM type ESBLs[29]. In the Netherlands, the bla SHV-12 gene was found alone in S. concord and together with the bla TEM-52 gene in *S. typhimurium*[30].

Various CTX-M enzymes are prevalent in Salmonella spp. in different regions of the world[20,31-35]. In a national multicenter study in Turkey, among 620 salmonella isolates, 6 (1%) were shown to produce ESBLs. These isolates were *S. typhimurium* (n=2), serogroup C1 (n=2) and S. enteriditis (n=2). All the ESBL producers harbored CTX-M type enzymes; in three isolates, a TEM-type enzyme was also present (10). CTX-M-3 has also been reported from a strain of Salmonella enterica serovar Virchow in Turkey[36].

PER-1 enzyme in *S. typhimurium* has been reported in Turkey[6] and PER-2 has been reported in a variety of serotypes in Argentina[37]. In the present study, two salmonella isolates, serotyped as *S. paratyphi* B, were shown to be ESBL producers. However, neither PER-1 nor CTX-M was found in our isolates. SHV-2 enzyme in strain 859479 and SHV-2a and SHV-5a in strain 2444316 were confirmed by sequencing. The first SHV-2a in *S. typhimurium* was recently reported in an isolate from Poland[38], and from Canada where *S. typhimurium* isolate was harboring two plasmids: one containing a bla $_{\text{TEM-1}}$ gene and the other containing a bla$_{\text{SHV-2a}}$ gene[39]. A variety of SHV-type ESBL-producing salmonella strains have also been reported from the United Kingdom, Romania and Italy[40-42].

SHV-2 has been reported in Gram-negative bacteria from Turkey[43]. In İzmir, Turkey, TEM-1, CTX-M-3 and SHV-12 were detected in ESBL-producing clinical isolates of *S. typhimurium*[44]. In our study, SHV-2a and SHV 5a were found in *S. paratyphi* B isolates. This is the first report of SHV-2a and SHV-5a (SHV-9) in *S. paratyphi* B from Turkey.

The findings of this study show that resistance rates to ampicillin, chloramphenicol, and TMP/SMX are quite high in Salmonella spp., especially in serogroup B isolates. ESBLs are not frequent in Salmonella spp., but emerging and multidrug resistant phenotypes of these isolates are especially disturbing and require awareness.

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