PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF
SALMONELLA TYPHIMURIUM STRAINS PRODUCING
EXTENDED-SPECTRUM BETA-LACTAMASES (ESBLs)
ISOLATED FROM CHILDREN (UNDER 4 YEARS OF AGE) WITH
DIARRHEA IN ROMANIA

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Abstract
The purpose of this study was to screen the genotype of antibiotic resistance markers of a
collection of 18 Salmonella Typhimurium strains isolated from children with gastrointestinal disorders
admitted to a county hospital pediatrics. 16 of the 18 clinical isolates of S. Typhimurium (88.89%) showed the phenomenon of multiresistance to antibiotics (MDR), at the same time being resistant to beta-
lactams, aminoglycosides, nalidixic acid, tetracycline and sulfonamides.

Extended-spectrum beta-lactamases (ESBLs) production was detected by the double disk synergy
test (DDST). The 16 strains showed ESBLs phenotype associated with phenotypes of resistance to
aminoglycosides, nalidixic acid, cotrimoxazole (trimethoprim/sulfamethoxazole) and tetracycline, and of these, seven showed ESBLs phenotype associated with chloramphenicol resistance phenotype.

Molecular analysis of the 18 strains of S. Typhimurium with multiple resistance to antibiotics
revealed three PCR resistance profiles: blaSHV+, blaSHV+ blaTEM+, and blaSHV+ blaTEM+ blaCTX-M+. Out of
the 18 strains of S. Typhimurium, 88.89% were blaTEM gene carriers, 100% blaSHV gene, and the 16
strains were positive for both genes (88.89%). A single strain introduced blaCTX-M gene in combination
with the other two genes (5.56%).

Keywords: Salmonella Typhimurium, extended-spectrum β-lactamases (ESBLs), PCR amplification,
PCR-RFLP method;

1. Introduction
Given that intestinal infections themselves are a public health problem, etiological
involvement of microorganisms resistant to antibiotics increases their severity. In this context,
the increasing prevalence of *Salmonella enterica* serovar Typhimurium strains resistant to antibiotics, and mainly those resistant beta-lactams is worrying for the treatment of human salmonellosis. Given the rising incidence of gastroenteritis and *Enterobacteriaceae* continuous decrease sensitivity to a range of antibiotics, we need to underline the prime importance of the choice of optimal anti-infective chemotherapy in order to prevent the selection of multidrug-resistant strains (MDR) [1-5].

2. Material and Methods

**Microbial strains**

We have studied a total of 18 strains of *Salmonella* Typhimurium isolated from children (between 2 months to 3.9 years of age) hospitalized for gastrointestinal disorders (infectious diarrhea) in a pediatric hospital district and sent for further investigation to National Institute of Research-Development for Microbiology and Immunology “Cantacuzino” (NIRDMIC) Bucharest.

**Antibiotic susceptibility testing**

Antibiotic sensitivity was tested by disc diffusion antibiogram method (Kirby-Bauer) according to the recommendations of the the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008) [6] using microtablets supplied by Oxoid Ltd. (Basingstoke, UK) or Mast Diagnostics (Bootle, UK), an inoculum turbidity of 0.5 McFarland scale and sowing was done on Mueller-Hinton medium. *E. coli* ATCC 25922 and *E. coli* ATCC 35218 (for the combination of beta-lactam antibiotics with beta-lactamase inhibitors) strains have served as a control performed pathogen susceptibility (ref. no. 0335P, MicroBioLogics).

The following antibiotics were tested: ampicillin (AMP 10 µg), amoxicillin (AML 10 µg), amoxicillin/clavulanic acid (AMC 20/10 µg), cefoxitin (FOX 30 µg), cefotaxime (CTX 30 µg), ceftazidime (CAZ 30 µg), imipenem (IPM 10 µg), nalidixic acid (NA 30 µg), ciprofloxacin (CIP 5 µg), gentamicin (CN 10 µg), kanamycin (K 30 µg), streptomycin (S 10 µg), sulfonamide/sulfadiazine (S3 300 µg), trimethoprim (W 5 µg), co-trimoxazole (trimethoprim/sulfamethoxazole, SXT 1.25/23.75 µg), tetracycline (TE 30 µg), and chloramphenicol (C 30 µg) [7-14].

**Phenotypic test for the confirmation of beta-lactamase production - DDST (double disk synergy test)**

The identification of the production of extended spectrum beta-lactamases (ESBLs) was carried out using the double disc [15].

Phenotypic confirmation of *S. serovar Typhimurium* strains suspected producing ESBLs was achieved through simultaneous testing by Kirby-Bauer disc diffusion method, the synergy between disks ceftazidime, cefotaxime and amoxicillin with clavulanic acid disc. Oxoid discs were used containing the combination of amoxicillin / clavulanic acid (20 µg/10 µg), ceftazidime (30 µg) and cefotaxime (30 µg), placed at a distance of 2 cm (measured between the centers of the discs) in the Mueller-Hinton agar. The plates were incubated for 18-20 hours at 37°C.

**Genotypic methods for the analysis of the profile of beta-lactam antibodies resistance**

a) **PCR detection of blaTEM, blaSHV and blaCTX-M** (Bio-Rad) [16-18].

DNA was extracted from the culture broth, brain heart infusion or by boiling of bacteria in the distilled water which resulted in cell lysis with the release of DNA or by using a commercial DNA extraction kit (Qiagen). In the latter case the total genomic DNA extraction was performed in the 25-30 µL cellular sediment (a bacterial culture obtained by centrifugation for 10 minutes at 8000 rpm and then the supernatant was aspirated with an
automatic pipette) with QIAamp DNA Mini Kit (cat. no. 51304, Qiagen, Germany) according to manufacturer's recommendations.

Determination of DNA purity and concentration were carried out spectrophotometrically (A260/280 nm), using the device Biochrom model Libra S32.

PCR was performed in a final volume of 50 µL: 5 µL of DNA suspension test and 45 µL PCR premix containing 5 µL buffer (Tp) PCR 1X (10X stock concentration ) and 2.5 mM MgCl₂, 0.3 µL Taq Polymerase (1,5 × U) (Invitrogen), 0.25 µL of each dNTP (10 mM) - dATP, dGTP, dCTP, dTTP (Promega)-, 1.5 µL of each TEM primer (15 pmol ×) (TEM 1 - TCGGGGAAATGTGCCGC, TEM 2 - TGCTTAATCAGTGAGGCACC), SHV primers (SHV 1 - TGGTTATGCGTTATATTCGCC, SHV 2 - GGTTAGCGTTGCCAGTGCT, and CTX-M primer (group 1: CTX M7 - GCGTGATACCACTGCTCTC, CTX M8 - TGAAGTAAAGTGACCAGAATC, group 2: CTX M17 - TGATACCACACGCGCTCT, CTX M18 - TATTCGATCAAAACCGTGGGG, group 8 and 25/26: CTX M19 - CAATCTGACGGGACCTGACATT, CTX M20 - ATXAGCGCGCTGACATT, group 9: CTX M11 - ATXAGCGCGCTGACATT, CTX M12 - GAAATGCGACGCAACGCGTCTG), and double distilled H₂O ad 45 µL.

The amplification was carried out on MyCycler (Bio-Rad), and programs used were as followed: for blaTEM gene an initial denaturation at 94°C for 5 minutes, followed by 34 cycles of denaturation at 94°C for 30 seconds, aligning at 50°C for 1 minute, elongation at 72°C for 1 minute, and final extension was performed at 72°C for 5 minutes; for blaSHV gene an initial denaturation at 94°C for 5 minutes followed by 35 cycles - denaturation at 94°C for 30 seconds, aligning at 58°C for 1 minute, elongation at 72°C for 1 minute, and final extension at 72°C for 10 minutes; for blaCTX-M genes an initial denaturation at 94°C for 4 min followed by 30 cycles - an initial denaturation at 94°C for 1 min, aligning for 1 minute at 54°C for CTX-M of group 1, or 67°C for CTX-M of group 2, or 58°C for CTX-M group 8 and 25/26, and 62°C for the group 9, elongation at 72°C for 1 min., and final extension at 72°C for 10 minutes.

The sequences of primers used in PCR reactions and the molecular weight of the amplification products are presented in table 1.

**Table 1.** Sequences of the primers used in PCR reactions for blaTEM, blaSHV and blaCTX-M genes in this study

<table>
<thead>
<tr>
<th>Detected genes</th>
<th>Primer</th>
<th>Primer sequence (5’ → 3’)</th>
<th>Amplicons size (bp)</th>
<th>Position of primers</th>
<th>Annealing temperature (°C)</th>
<th>Source of primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>blaTEM</strong></td>
<td>TEM-1F</td>
<td>TCGGGGAAATGTGCCGC</td>
<td>972</td>
<td>90-105</td>
<td>50</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td>TEM-2R</td>
<td>TGGTTATGCGTTATATTCGCC</td>
<td>868</td>
<td>120-140</td>
<td>58</td>
<td>[17]</td>
</tr>
<tr>
<td><strong>blaSHV</strong></td>
<td>SHV-1F</td>
<td>GGTTAGCGTTGCCAGTGCT</td>
<td>341</td>
<td>543–561</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHV-2R</td>
<td>GCGTGATACCACTCACGCTCT</td>
<td>260</td>
<td>540–559</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td><strong>blaCTX-M-1</strong></td>
<td>CTX-M7</td>
<td>GCGTGATACCACTCACGCTCT</td>
<td>207</td>
<td>582–601</td>
<td>62</td>
<td>[18]</td>
</tr>
<tr>
<td><strong>blaCTX-M-2</strong></td>
<td>CTX-M17</td>
<td>TGATACCACACGCGCTCT</td>
<td>293</td>
<td>298–319</td>
<td>62</td>
<td>[18]</td>
</tr>
<tr>
<td><strong>blaCTX-M-8</strong></td>
<td>CTX-M19</td>
<td>CAATCTGACGGGCAATG</td>
<td>207</td>
<td>582–601</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>and</td>
<td>CTX-M20</td>
<td>ATXAGCGCGCTGACATT</td>
<td>293</td>
<td>298–319</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td><strong>blaCTX-M-25/26</strong></td>
<td>CTX-M12</td>
<td>GAAATGCGACGCAACGCGTCTG</td>
<td>293</td>
<td>570–590</td>
<td>62</td>
<td></td>
</tr>
</tbody>
</table>
Phenotypic and genotypic characterization of *salmonella* typhimurium strains producing extended-spectrum beta-lactamases (esbls) isolated from children (under 4 years of age) with diarrhea in Romania

The control of amplicons presence was done by electrophoresis in 1.5% agarose gel prepared in TBE buffer 0.5X (pH 8.3): Tris base (ad 108 g in 1000 mL double-distilled H$_2$O - 890 mM), boric acid (55 g ad in 1000 ml dd H$_2$O - 890 mM), Na$_2$EDTA 2H$_2$O (7.5 g ad in 1000 ml dd H$_2$O - 20 mM), the migration at 100 V for 60 min, followed by staining for 20 min in a solution of bromide ethidium (final concentration 1 mg/mL), UV visualization and finally photographing with a video camera system (Vilbert Lourmat).

The expected size of the amplicons (972bp for *bla*$_{TEM}$, 868bp for *bla*$_{SHV}$ and 260bp, 341bp, 207pb, and 293pb for *bla*$_{CTX-M}$) was estimated using a specific molecular weight control (Promega, 100bp DNA ladde, Southampton, UK, containing 11 ADNc. fragments with sizes of 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000, respectively 1500bp). One positive control (*Salmonella* Typhimurium ATCC 14028), and two negative controls (sterile water and *E. coli* ATCC 11775) were used.

b) Confirmation of the amplicons identity by RFLP (Restriction Fragment Length Polymorphism)

In this genetic analysis amplicons *bla*$_{TEM}$ and *bla*$_{SHV}$ were subjected to restriction enzyme using specific endonucleases (DdeI, and PstI), four hours at 37°C. Restriction mix (20 µL) contained 10 µL of PCR product, 1.5 µL (15 U) DdeI, respectively, PstI (both made by Promega), 0.2 µL BSA (bovine serum albumin), 2 µL specific buffer (Tp) and 6.3 µL double distilled H$_2$O. Digestion fragments were separated by electrophoresis in 3% agarose gel, stained with ethidium bromide (Sigma), detected by UV illumination and photographed with a video camera system (Vilbert Lourmat). Product sizes were estimated using sequenced controls and 50 bp or 0,5 kb DNA ladders (Promega).

3. Miscanthus Results and Discussion

Antibiotic resistance phenotypes

On the basis of the phenotypic expression, 16 of the 18 clinical isolates of *S.* Typhimurium (88.89%) showed the phenomenon of antibiotic multi-resistance (MDR), which is at the same time resistant to beta-lactam antibiotics, aminoglycosides, nalidixic acid, tetracycline and sulfonamides.

Higher resistance rates (> 85%) were recorded to ampicillin, amoxicillin and clavulanic acid combination, cefotaxime, ceftazidime, gentamicin, kanamycin, and streptomycin, sulfonamide, trimethoprim, and nalidixic acid (see Table 1). The highest level of resistance was obtained to tetracycline (95%). However, the analyzed strains showed 100% sensitivity to cefoxitin, imipenem, and ciprofloxacin. Only 11.11% of the strains were sensitive to penicillins and semisynthetic cephalosporins (3rd generation). A few strains showed resistance to chloramphenicol (38.89%).

The same 16 strains were resistant to amoxicillin and clavulanic acid, showing a portion of the substrate specificity of beta-lactamas and, on the other hand, possible adaptation of clavulanic acid in *S.* Typhimurium (a beta-lactamase inhibitor that remains active on ESBLs therefore will enhance the action of cephalosporins), probably due to high selective pressure in the hospital.

Two strains of *S.* Typhimurium isolated from the same serovar outbreak did not raise particular resistance problems to the β-lactam antibiotics tested, these being sensitive to all antibiotics of this class. Instead, one of them showed resistance to streptomycin and tetracycline associated and the other resistance only to streptomycin. The two strains of *Salmonella* Typhimurium were included in the study group for molecular detection of resistance to beta-lactam antibiotics.
Antimicrobial test results by the method of double diffusion synergy test showed that 16 strains tested produced beta-lactamase. The strains belonging to this phenotype (ESBLs) were characterized by cross-resistance to most of the beta-lactam test. The level of expression of the resistance varied depending on the strain. All 16 strains had ESBLs phenotype associated with the phenotypes of resistance to the aminoglycosides, nalidixic acid, tetracycline, and co-trimoxazole, and of these, 7 have shown ESBLs phenotype associated with resistance to chloramphenicol.

Molecular analysis of the 18 strains of S. Typhimurium with multiple resistance to antibiotics PCR profiles revealed three resistance profiles: \( \text{bla}_{\text{SHV}}^+ \), \( \text{bla}_{\text{SHV}}^+ \text{ bla}_{\text{TEM}}^+ \), and \( \text{bla}_{\text{SHV}}^+ \text{ bla}_{\text{TEM}}^+ \text{ bla}_{\text{CTX-M}}^+ \) (see Figure 1).

Out of the 18 strains of S. Typhimurium, 16 strains were \( \text{bla}_{\text{TEM}} \) gene carriers, 100% were positive for \( \text{bla}_{\text{SHV}} \), from which 16 had been shown to carry both genes. A single strain introduced \( \text{bla}_{\text{CTX-M}} \) gene in combination with the other two genes (Table 3).
Phenotypic and genotypic characterization of *salmonella* typhimurium strains producing extended-spectrum beta-lactamases (esbls) isolated from children (under 4 years of age) with diarrhea in Romania

Table 3. Prevalence genes \(\text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}},\) and \(\text{bla}_{\text{CTX-M}}\) of S. Typhimurium strains analyzed

<table>
<thead>
<tr>
<th>No. strains tested</th>
<th>Genes coding for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TEM</td>
</tr>
<tr>
<td>18</td>
<td>16</td>
</tr>
</tbody>
</table>

The interpretation of the restriction enzyme profiles of isolates revealed that fragments with expected molecular weight were obtained (Figure 2).

![Figure 2. Digests of the PCR product.](image)

Fragments were generated by digestion of the PCR product with:

- Lanes 2 and 3: \(\text{bla}_{\text{TEM}}\) positive amplicons / *Dde*I (28bp, 404bp, 540bp);
- Lanes 4 and 5: \(\text{bla}_{\text{TEM}}\) positive amplicons / *Pst*I (313bp, 659bp);
- Lanes 7 and 8: \(\text{bla}_{\text{SHV}}\) positive amplicons / *Dde*I (95bp, 281bp, 492bp);
- Lanes 9 and 10: \(\text{bla}_{\text{SHV}}\) positive amplicons / *Pst*I (254bp, 614bp);
- Lanes 1, 6 and 11: DNA ladder (molecular weight marker - 50bp).

Analysis of the restriction fragments by PCR-RFLP therefore confirmed the presence of \(\text{bla}_{\text{TEM}}\) and \(\text{bla}_{\text{SHV}}\) genes coding for ESBLs in *S. Typhimurium* strains resistant to \(\beta\)-lactam antibiotics (see Table 4).

Table 4. Restriction profiles of \(\text{bla}_{\text{TEM}}\) and \(\text{bla}_{\text{SHV}}\) derived from digested with *Dde*I and *Pst*I

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Dimension (bp)</th>
<th><em>Dde</em>I</th>
<th><em>Pst</em>I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Restriction fragments (bp)</td>
<td>Restriction fragments (bp)</td>
<td></td>
</tr>
<tr>
<td>TEM</td>
<td>972</td>
<td>540</td>
<td>404</td>
</tr>
<tr>
<td>SHV</td>
<td>868</td>
<td>492</td>
<td>281</td>
</tr>
</tbody>
</table>

4. Conclusions

The results show the involvement of multidrug-resistant of *S. Typhimurium* strains in salmonellosis pediatric cases analyzed, which raises a very serious therapeutic problem. All the ESBLs producing isolates were resistant to amoxicillin plus clavulanic acid combination and ceftazidime, and at a rate of about 85% to ampicillin, cefotaxime, nalidixic acid, gentamicin and antibiotics commonly used in the treatment of acute gastrointestinal infections.

Intestinal strains of *S*. Typhimurium exhibited a variety of genes which were shown to be involved in beta-lactams resistance. Consequently, the intestinal microflora may play an important role as acceptor and donor of transmissible antibiotic resistance mechanisms. These \(\text{bla}_{\text{TEM}}\), \(\text{bla}_{\text{SHV}}\), and \(\text{bla}_{\text{CTX-M}}\) genes encoding antibiotic resistance placed on mobile genetic constructs such as plasmids show a horizontal spreading of antibiotic resistance among strains of *Enterobacteriaceae* ESBL-positive.

Outcomes illustrate the dissemination of \(\text{bla}_{\text{TEM}}\) and \(\text{bla}_{\text{SHV}}\) genes among *S*. Typhimurium strains producing ESBLs, which increases the risk of spread of strains highly resistant to beta-lactam antibiotics in the geographic area of Romania.

The study is a warning to healthcare professionals using the prescription drug poliantibiotherapy given the selection of high resistant strains and even multiresistant strains.

5. Acknowledgments

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