EXTENDED SPECTRUM β-LACTAMASES IN ESCHERICHIA COLI ISOLATES FROM PEDIATRIC POPULATION

Branka Bedenić
University Hospital Centre Zagreb
BACKGROUND AND AIM

- The bacteria producing extended-spectrum β-lactamases (ESBL) have been increasingly reported in pediatric population.
- Production of ESBLs is the major mechanism of resistance to oxymino-cephalosporins and aztreonam in Gram-negative bacteria.
- ESBLs are predominantly derivatives of plasmid-mediated TEM or SHV β-lactamases and arise through mutations that alter the configuration of active site, thereby expanding the hydrolytic spectrum of enzyme.
- Both TEM and SHV ESBLs are distributed worldwide with lots of variants described (data are available at http://www.lahey.org/studies/) and are mostly found in Enterobacteriaceae species like Klebsiella pneumoniae and Escherichia coli.
BACKGROUND AND AIM

• Recently a new family of ESBLs with predominant activity against cefotaxime (CTX-M β-lactamases) has been reported.
• In contrast to TEM or SHV-ESBLs CTX-M β-lactamases are native ESBLs and are derived from the chromosomal β-lactamases of the genus Klyvera.
• In particular in Europe, CTX-M ESBLs are now spreading rapidly and are increasingly dominant.
• There are over 50 CTX-M enzymes so far grouped into five main subgroups according to amino acid sequence identity (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25).
• Most CTX-M β-lactamases hydrolyze cefotaxime better than ceftazidime, but some of them such as CTX-M-15 hydrolyze also ceftazidime efficiently.
• In some countries CTX-M $\beta$-lactamases are the most prevalent types of ESBLs, for instance in Russia, Greece, Spain, Switzerland, Japan, Taiwan, China and Argentina.
• In Europe the most common types are CTX-M-3 previously described in Russia, Greece, Poland, France, United Kingdom and CTX-M-15 reported previously in Poland, Austria and Bulgaria.
• CTX-M-15 has been reported worldwide.
• ESBL producing *Enterobacteriaceae* are described with increasing frequency as causative agents of urinary tract infections.
AIM OF THE STUDY

- The aim of the present study was to characterize ESBL-producing *E. coli* isolates collected over a 2-year period at the Split University Hospital, Croatia, with a main focus on uropathogenic strains isolated in neonates and infants.
- We investigated the prevalence of different types of ESBLs among clinical isolates.
- Furthermore, ESBLs were characterized genetically and clonal relatedness of the strains was assessed thereby allowing the molecular epidemiology of the ESBL-producing *E. coli* strains.
MATERIAL AND METHODS

• **Bacteria**

• From January 2002 until December 2003, 661 *E. coli* isolates were recovered at Split University Hospital.

• The overall prevalence of ESBL producing *E. coli* was 17.2 % (114/661).

• *The percentage of the uropathogenic ESBLs was 11.64% (77/661). Seventy seven strains were processed in this study.*
MATERIAL AND METHODS

- **Susceptibility to antibiotics**
- Antibiotic susceptibilities were determined by broth microdilution method according to CLSI.
- MICs were determined by a twofold microdilution technique using microtiter plates and Mueller-Hinton broth inoculated with $10^5$ CFU/ml according to CLSI.
- Clavulanic acid was added to amoxycillin and ceftazidime in the fixed concentration of 4 mg/L.
Detection of extended-spectrum β-lactamases

- ESBL production was determined by double-disk synergy test and confirmed by at least threefold reduction in ceftazidime minimal inhibitory concentration (MIC) by clavulanate.
Transfer of resistance determinants

- *E. coli* isolates were investigated for the transferability of their resistance determinants.
- Conjugation experiments were set up employing *E. coli* A15 R strain free of plasmids and resistant to rifampicin.
- Transconjugants were selected on the combined plates containing ceftazidime (1mg/L) and rifampicin (256 mg/L).
- The frequency of tranconjugation was expressed relatively to the number of donor cells.
Characterization of extended-spectrum β-lactamases

- The presence of *bla*TEM, *bla*SHV and *bla*CTX-M genes was determined by polymerase chain reaction (PCR) using primers and conditions as described previously.
- Bacterial DNA was extracted by boiling
PCR

- 94°C for 5 min,
- then 35 cycles consisting of
  - 95°C for 30 s,
  - 58°C for 30 s, and
  - 72°C for 50s each,
- followed by a final extension at 72°C for 8 min.
PRIMERS

1. SHV-F 5’ CGC CGG GTT ATT CTT ATT TGT CGC-3’
2. SHV-R 5’ TCT TTC CGA TGC CGC CGC CAG TCA-3’,
3. TEM-F: (5’-CGC-CGG-GTT-ATT-CTT-ATT TGT-CGC-3’
4. TEM-R: (5’-TCT-TTC-CGA-TGC-CGC-CGC-CAG-TCA-3’
5. CTX-F: 5’-GCG GTA AAT CGG AGT GAT GAT
6. CTX-R: 5’-ATT CGG CAA GTT TTT GCT GT-3’
7. PER-F:5’ GGG ACA (A/G) TC (G/C) (G/T) ATG AAT GTC A
8. PER-R:5’ gg (C/T) (G/C) GCT TAG ATA GTG CTG AT
Using these primers, all amplicons of the $bla_{ESBL}$ genes spanned the entire open reading frame, respectively.

The PCR product were visualized by agarose gel electrophoresis. Amplicons were then column-purified ($Nucleo Spin Extract II, Machery-Nagel, Germany$) and sequenced directly using and ABI PRISM 377 Genetic Analyser (Applied Biosystems).

Designation of $bla$ genes based on identified mutations was done according to [http://www.lahey.org/studies/](http://www.lahey.org/studies/).

Lysates from reference strains producing TEM-1, TEM-2, SHV-1, SHV-2, SHV-4, SHV-5 and CTX-M-15 were used as positive controls for PCR.
PCR Nhe test

- PCR Nhe test was performed to distinguish between SHV-1 and SHV-ESBL.
- The PCR products of blaSHV-ESBL genes are cleaved in three fragments of 1017, 770 and 247 bp as described previously in contrast to blaSHV-1 which is not digested by NheI restriction endonuclease.
Determination of virulence factors

- Susceptibility to bactericidal serum activity was tested by Shiller and Hatch method.
- Adhesin expression was determined by the method of hemagglutination and hemagglutination inhibition in microtiter plates.
Molecular typing by pulsed-field gel electrophoresis (PFGE) of bacterial DNA

- PFGE of Xba-digested genomic DNA was performed with a CHEF-DRIII system (Bio-rad) as described previously.
- The images were processed using the Gel-Compar software, and a dendrogram was computed after band intensity correlation using global alignment with 2% optimization and UPGMA (unweighted pair-group method using arithmetical averages) clustering.
Antimicrobial susceptibility

- All isolates were resistant to:
  - amoxicillin,
  - 97% to cefazoline,
  - 93% to gentamicin,
  - 89% to ceftazidime,
  - 80% to cefuroxime,
  - 73% to netilmicin,
  - 56% to ceftriaxone,
  - 38% to cefepime,
  - 22% to cefotaxime,
  - 21% to ceftibuten,
  - 13% to co-amoxiclav
- There was no resistance observed to
  - ciprofloxacin,
  - carbapenems and
  - ceftazidime combined with clavulanate
Antimicrobial susceptibility

- The range of antimicrobial potency was: ciprofloxacin=meropenem>imipenem>ceftazidime/clavulanate>cefoxitin,>coamoxiclav>cefotaxime=cefepime>ceftibuten>ceftriazone=cefuroxime>gentamicin>amoxycillin=ceftazime=cefazoline.
- All except three strains displayed CAZ phenotype which means greater resistance to ceftazidime and aztreonam compared to cefotaxime and ceftriazone.
Conjugation

- 18 strains transferred ceftazidime resistance to *E. coli* recipient. Resistance to tetracycline was co-transferred alongside with ceftazidime resistance from 13 strains and to aminoglycosides from 14 strains.
- Resistance to cloramphenicol was co-transferred from only one strain.
Characterization of ESBLs

- All 77 ESBL-producing E. coli strains were tested for the presence of \textit{blaTEM}, \textit{blaSHV} and \textit{blaCTX-M} genes by PCR.
- The \textit{blaTEM} gene was detected in 20 strains (26%), the \textit{blaSHV} gene in 56 strains (72.7%) and the \textit{blaCTX-M} gene was found in only 3 strains isolates (3.9%).
- \textit{BlaPER} genes were not detected in our strains.
Characterization of ESBLs

- **BlaSHV** amplicons were cleaved with NheI enzyme proving the presence of SHV-ESBL.
- SHV-type ESBL were identified by sequencing as SHV-5 in 51 (66.2%) strains, SHV-2 SHV-2a in two strains (2.6%), and SHV-12 in one strain (1.3%).
- Sequencing of TEM-type ESBLs yielded TEM-1 in 15 strains (19.4%), TEM-2 in 3 strains (3.9%), TEM-E1 in one strain (1.3%) and TEM-116 in one strain (1.3%).
- CTX-M-type ESBLs corresponded to CTX-M-3 β-lactamase in three strains (3.9%)
Characterization of virulence factors

- Adhesins were detected in 24 (48%) strains (5 in neonate and 19 in infant group), and difference in adhesin expression between the two groups was statistically significant ($p=0.01$).
- P-fimbriae were detected in only 7 strains, six of them in infant urine samples.
- Hemolysin was produced by 42 (84%) strains (19 in neonate and 23 in infant group), whereas 37 (74%) strains were resistant to serum bactericidal activity (16 in neonate and 21 in infant group); the difference was not statistically significant ($p>0.05$).
PFGE

• The *E. coli* isolates causing urinary tract infections were clonally related as demonstrated by PFGE (Fig. 1). According to the genetic relatedness they were assigned into five clusters.

• There were several outbreaks of infections caused by ESBL producing *E. coli* strains in the University Hospital Split during 2002-2003.
CONCLUSIONS

• The ESBL strains isolated in neonates and infants predominantly produced hemolysin and were resistant to serum bactericidal activity, however, adhesin expression was detected in less than 50% of the strains and was especially rare in the strains isolated from neonatal urine samples.

• The association between the detection of blaESBL genes and expression of adhesins needs additional studies.
CONCLUSIONS

- SHV-5 was found to be the predominant type of extended-spectrum β-lactamase. Some isolates harboured also additional TEM-1 β-lactamase.
- SHV-5 β-lactamase is widespread in middle and East Europe and has been previously described in Austria, Germany, Hungary, Poland, Italy, France, United Kingdom, Greece, Bosnia and Herzegovina, Australia, Mexico, and many other countries in the world.
CONCLUSIONS

• The resistance phenotype is consistent with SHV-5 β-lactamase (high level ceftazidime resistance in most strains).

• The presence of additional TEM-1 β-lactamase in some isolates could be responsible for resistance to co-amoxiclav (amoxicillin /clavulanate).
CONCLUSIONS

• **CTX-M β-lactamases identified in only 3 of 77 strains and were all of the same type (CTX-M-3). This is in contrast with reports from many other countries in which**

• **CTX-M β-lactamases appear to be the most frequent type for instance in Russia, Switzerland, Greece, Spain Japan, Taiwan, China and Argentina.**

• **CTX-M-3 β-lactamase was identified by sequencing of blaCTX-M gene in all three isolates.**

• **This type of ESBL is very frequent in Europe and was previously described in Poland, France, UK, Greece, and Russia.**
CONCLUSIONS

- Our strains producing SHV-5 type of ESBL were frequently clonally related and associated with breakouts in contrast to strains producing SHV-2, SHV-2a, SHV-12 and CTX-M-3 β-lactamases which were usually single isolates without tendency to spread throughout the hospital wards.
- The fact that the strains were clonally related and show similar resistance phenotypes points out that there is endemic and epidemic spread of SHV-5 producing *E. coli* in University hospital Split.
- Clustering of the *E. coli* isolates sustains the hypothesis of either patient to patient transmission of strains or a common source acquisition.
- The fact that genotypically related strains persisted over years raises the possibility that these strains may have persisted unnoticed in the pediatric units on the hospital, which served as a source of patient contamination.