Molecular characterization of extended-spectrum β-lactamase-producing *Enterobacteriaceae* strains

Doktoral (Ph.D.) thesis

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1. Introduction

Enterobacteriaceae have become one of the most important causes of nosocomial and community acquired infections. Beta-lactams (mainly expanded-spectrum cephalosporins and carbapenems) and fluoroquinolones constitute the main therapeutic choices to treat infections caused by these microorganisms. However, resistance to these compounds has been reported more and more frequently in Europe in the past years.

Acquired resistance to expanded-spectrum cephalosporins is mainly mediated by extended-spectrum β-lactamases (ESBLs) that confer bacterial resistance to all β-lactams except carbapenems and cephamycins, which are inhibited by other beta-lactamase inhibitors such as clavulanic acid.

Higher mortality rate and increased treatment costs of infections caused by ESBL-producing *Enterobacteriaceae* is well established, relative to non-ESBL-producing *Enterobacteriaecae* strains.

ESBLs have been increasingly described worldwide since their description in the early 1980s and have risen to prominence among *Enterobacteriaceae* isolates in nearly all European countries, now not only in the nosocomial but also in the community setting.

Until the end of the 1990s: i, most of the ESBLs detected were SHV and TEM types; ii, isolates expressing these enzymes were almost associated with nosocomial outbreaks, mainly in Intensive Care Units, and it was very unusual for them to be associated with community-acquired infections; iii, the prevalence of ESBL producers was higher among *Klebsiella pneumoniae* than among *Escherichia coli* isolates. This situation

has now changed dramatically, with CTX-M enzymes replacing TEM and SHV mutants as the predominant ESBLs in many European countries, with *E. coli* joining *K. pneumoniae* as a major host, and with producers increasingly isolated from community patients.

The spread of mobile genetic elements, mainly conjugative plasmids, and the dispersion of specific clones (e.g. *E. coli* O25-ST131/B2) have been responsible for the increase in ESBL-producing isolates and for the spread of TEM-52, SHV-12 and CTX-M-15 in particular.

Since the ESBL-producing organisms frequently also carry genes encoding resistance to other antibiotic classes, including quinolones, aminoglycosides, tetracyclines and antifolates, the therapeutic options are seriously reduced in these cases.

In Hungary the first study for estimating the incidence of ESBL-producers among *Enterobacteriaceae* isolates was performed in 1996, and the first outbreak caused by ESBL-producing *K. pneumoniae* strain was described in 1999. However, an comprehensive survey for characterization of ESBL-producing *Enterobacteriaceae* in Hungary has not been performed.

In the course of my work PCR based methods and DNA sequencing were introduced for characterization of different ESBL-types produced by *Enterobacteriaceae* isolates submitted to the National Reference Laboratory for ESBL-producing Gram-negative pathogens (ESBL-NRL) at the National Center for Epidemiology (NCE). Using these methods today we are able to detect and characterize the ESBL and other antimicrobial resistance genes, and their genetic environment as well.

Moreover, mating assays and electroporation were introduced for investigation of transferability of resistance determinants.

2. Aims

1. Characterization of the SHV genes carried by ESBL-producing *Enterobacteriaceae* strains isolated between 2002-2003 in Hungary. Establishment of the regional distribution of various SHV-type ESBL enzymes and comparison of the Hungarian data with data reported from neighboring countries

2. Establishment of epidemiological relationship among ESBL-producing *Klebsiella* spp. strains isolated from outbreaks in different Neonatal Intensive Care Units (NICUs) between 2002-2003 and investigation of the common ESBL-types expressed by these strains and the plasmids harbouring the ESBL genes.

3. Characterization of the nosocomial isolates of ciprofloxacin resistant, CTX-M-producing *Klebsiella pneumoniae* submitted to the NCE in 2003 and the comprehensive molecular analysis of such isolates from 2005.

4. Systematic screening and molecular characterisation of the non-typhoid *Salmonella enterica* recovered from human samples received by the National Refence Laboratory of Phage- and Molecular-typing of Enteric Bacteria (NRL-PMTE) for the ESBL producing isolates.

5. Characterization of ESBL-producing *E. coli* isolates from human clinical and animal samples gathered from whole country during 2006 and 2007, and comparison of the genetic background of the two collections.

3. Methods

Bacterial strains

Almost all of isolates included in our investigations were submitted to ESBL-NRL at the NCE for confirmation and further characterization. The *E. coli* isolates of animal origin were collected by National Veterinary Institute, while the *Salmonella enterica* isolates were collected by the NRL-PMTE at NCE.

For characterization of SHV-type ESBLs in Hungary 35 *Enterobacteriaceae* isolates selected from 252 ESBL-producing isolates submitted to the NCE from 25 microbiology laboratories throughout Hungary in 2002-2003 were investigated. The 35 isolates included one to three isolates from 14 counties and Budapest. In addition, a single isolate of each nosocomial outbreak reported during the study period was selected.

For molecular epidemiological investigation of SHV-type ESBL-producing *Klebsiellae* 126 consecutive, non-duplicate isolates were selected from outbreaks reported to the NCE in 2002–2003 and in 1998 from NICUs of five Hungarian hospitals.

Comprehensive molecular and epidemiological analysis of ciprofloxacin resistant, CTX-M-producing *Klebsiella pneumoniae* was

performed on 17 isolates from 2003 and 196 isolates from 2005 collected from 35 Hungarian hospitals.

Screening of ESBL-production in non-typhoid *Salmonella enterica* was performed on 13962 isolates from the collection of the NRL-PMTE, NCE.

During 2006-2007 113 ESBL-producing *E. coli* clinical isolates were submitted to the NCE for further investigation. Of these 45 isolates from 21 centres were selected for molecular typing and genetic characterization. The isolates were compared to the 18 ESBL-producing *E. coli* isolates of animal origin isolated between 2006-2007 from whole country.

Biochemical identification and antibiotic susceptibility testing

Biochemical identification of the isolates was carried out by API20E, ATB ID32E (bioMerieux) and Micronaut E (Genzyme Virotech GmbH). Serotyping of *E. coli* and *S. enterica* isolates was done by standard methods. The antibiotic susceptibility tests were performed by disk diffusion method according to the recommendations of Clinical and Laboratory Standards Institute (CLSI). The minimal inhibitory concentrations (MICs) of antibiotics were determined by the Etest (AB Biodisk) or agar dilution method according to the manufacturer's instruction or CLSI's recommendations, respectively. The putative production of an ESBL was confirmed by combined disk method (MAST), ESBL Etest (AB Biodisk) or modified double disk synergy test.

Molecular methods

PCR (polymerase chain reaction) amplifications were performed on selected isolates for several antibiotic resistance genes: β-lactam resistance encoding-genes (*bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{OXA-1-like}}$), tetracycline resistance encoding-genes (*tet*(A), *tet*(B), *tet*(C)), aminoglycoside resistance encoding-genes (*aac-(3)-IIa*, *aac-(6')-Ib*) and quinolone resistance encoding-genes (*qnrA*, *qnrB*, *qnrS*) as well as insertion elements (IS*26* and IS*Ecp1*). The PCR amplicons were sequenced for deep characterization and confirmation.

The *E. coli* phylogenetic groups were determined by using a multiplex PCR. For identification of international O25-ST131 clone the appropriate multiplex PCR was performed.

Plasmid DNA was extracted and electrophoresed by the method of Kado and Liu.

The mating assays and electroporation were carried out with selected ESBL-producing isolates. For fingerprinting analysis, plasmid DNA from transconjugants or transformants was obtained by using a QIAprep Spin Miniprep Kit (Qiagen), digested with *Pst*I (New England Biolabs) and detected by gel electrophoresis in 0.7% agarose at 110 V for 2 h.

Alterations in the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* encoding subunits of gyrase and topoisomerase IV enzymes in selected CTX-M-15-producing *K. pneumoniae* isolates were determined by sequencing.

The pulsed-field gel electrophoresis (PFGE) was performed in line with the standardized Centers for Disease Control and Prevention (CDC) protocol. Levels of similarity were calculated with the Dice coefficient, and UPGMA ('unweighted pair group method with arithmetic averages') was used for the cluster analysis of the PFGE patterns.

MLST with seven housekeeping genes was performed on selected CTX-M-15-producing *K. pneumoniae* isolates according to published method. Allele sequences and sequence types (STs) were verified at the http://pubmlst.org/kpneumoniae web site.

4. Results

Occurence and regional distribution of SHV-type ESBLs in Hungary, 2002-2003

Of the 252 ESBL-producing isolates, 232 (92.1%) harbored SHV-type ESBL genes. Among the strains carrying the SHV-type ESBL gene, 35 were selected for further study as follow: *K. pneumoniae* (*n*=21), *Klebsiella oxytoca* (*n*=6), *Serratia marcescens* (*n*=3), *E. coli* (*n*=3) and *Enterobacter cloacae* (*n*=2). The two dominant SHV-types of ESBL genes were SHV-5 and SHV-2a produced by 28 and six of the strains, respectively. In addition, we detected one SHV-12 gene carried by a single isolate.

Epidemiology of SHV-type ESBL-producing *Klebsiella* spp. from Hungarian outbreaks, 2002-2003

The 126 ESBL-producing clinical isolates of *Klebsiella spp.* collected in 1998, 2002 and 2003 from seven outbreaks in neonatal intensive care units (NICUs) of five Hungarian hospitals were multidrug resistant but were susceptible to ciprofloxacin. PFGE revealed the existence of 12 distinct genetic clones, 10 of which proved epidemic in the studied NICUs. All isolates harboured plasmids ranging from 2.3 kb to 228 kb, representing 12 diverse plasmid profiles. Sequence analysis detected the $bla_{\text{SHV-2a}}$ gene in three and the $bla_{\text{SHV-5}}$ gene in seven epidemic clones (ECs). In the majority of isolates the bla_{SHV} genes were on transferable plasmids of app. 90 kb. *Pst*I digestion of plasmid DNA from transconjugants revealed identical or closely related restriction patterns in nine $bla_{\text{SHV-5}}$ -harbouring R-plasmids and in two $bla_{\text{SHV-2a}}$ -harbouring R-plasmids carried by strains obtained from geographically distant NICUs.

Molecular epidemiology of Hungarian CTX-M-15-producing *K. pneumoniae*

The first 17 ciprofloxacin resistant, CTX-M-15-producing K. pneumoniae isolates – belonging to the Hungarian epidemic clone (HEC) - were detected in 2003.

In 2005 196 ciprofloxacin-resistant CTX-M-15-producing isolates collected from 35 centres were subjected to comprehensive molecular

analysis. PFGE revealed the existence of three genetic clusters defined as ECs, where 129 isolates belonged to the previously described Hungarian EC (HEC, N pulsotype (PT)), 46 isolates to EC II (R PT) and 21 isolates to EC III (S PT), respectively.

*Pst*I digestion of plasmid DNA from transconjugants/transformants revealed diverse restriction patterns from distinct ECs. Sequencing revealed that plasmids from all three ECs equally harboured $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, *aac(6')-Ib-cr* and *aac-(3)-IIa* genes; the latter gene was not found in the transconjugants from gentamicin-susceptible isolates of HEC. Only the large non-conjugative plasmid of 50 kb—transformed from S PT—carried an additional $bla_{\text{TEM-1}}$ gene and IS*Ecp1* associated with $bla_{\text{CTX-M-15}}$. *tet(A)* and *tet(C)* genes were detected on plasmids of 140 and 90 kb originating from HEC.

Based on the alterations of nucleotide sequences in the QRDRs of GyrA and ParC in the HEC and S PT isolates, two amino acid changes (Ser-83→Phe and Asp-87→Ala) and one amino acid change (Ser-80→Ile) were detected, respectively. The R PT isolates exhibited a single nucleotide mutation in both *gyrA* (resulting in Ser-83→Ile substitution) and *parC* genes (resulting in Ser-80→Ile substitution).

MLST identified three allelic profiles: ST 15 (HEC), ST 11 (S PT) and the novel ST 147 (R PT), which correspond to the PFGE clusters.

ESBL-producing *S. enterica* strains from humans in Hungary, 2000-2004

Of the 13 962 non-typhoid Salmonella isolates tested, 14 *S.* Typhimurium isolates and 1 *S.* Enteritidis isolate were found to be ESBL-producing. There were no ESBL-producing isolates found among the other serotypes tested (*S.* Infantis, *S.* Hadar, *S.* Saintpaul, *S.* Blockley and *S.* Panama). Besides two isolates which originated from urine and haemoculture, all other isolates originated from stool samples. The single *S.* Enteritidis isolate harboured a $bla_{\text{SHV-5}}$ β-lactamase gene. Of the 14 *S.* Typhimurium isolates, 3 possessed a $bla_{\text{SHV-5}}$ β-lactamase gene. These isolates also had a $bla_{\text{TEM-1}}$ gene. The remaining 11 *S.* Typhimurium isolates possessed $bla_{\text{CTX-M}}$, of which 8 carried $bla_{\text{CTX-M-5}}$. Three isolates were proven to be $bla_{\text{CTX-M-15}}$-positive by sequencing, two of which also had a $bla_{\text{TEM-1}}$ gene. All the $bla_{\text{CTX-M}}$ positive isolates possessed the IS*Ecp1* element. The $bla_{\text{CTX-M-5}}$ gene was located on and transferred by an app. 7 kb plasmid, while $bla_{\text{CTX-M-15}}$ was located on an app. 90 kb conjugative plasmid.

PFGE revealed that all the $bla_{\text{CTX-M}}$-carrying Hungarian isolates including the isolates from Ukrainen patients hospitalised in Hungary, were 83% similar to each other and represented one cluster.

Molecular epidemiology of ESBL-producing *E. coli* isolated from human clinical and animal samples in Hungary, 2006-2007

The majority of strains of human origin carried resistance to more than two drugs, while among animal isolates such resistance proved to be 30%.

Among human isolates several ESBLs were detected (SHV-2, -5, -12, CTX-M-1) with CTX-M-15 being the most common. CTX-M-1, CTX-M-32 and SHV-2 ESBL genes were found in the animal strains.

Thirty-six out of 45 human isolates belonged to B2 and D phylogenetic groups, which typically comprise extra-intestinal virulent *E. coli* strains. Only nine human isolates shared commensal phylogenetic groups A (6/45) and B1 (3/45). Conversely, 15 animal isolates belonged to commensal phylogenetic groups A (9/18) and B1 (6/18), and just three isolates from swine, calf and one-day-old chicken represented phylogenetic group D.

Near half of human strains (19/45) from 12 centres belonged to the international O25-ST131/B2 clone, while 9 isolates from 7 centres shared O15 serotype. Sixteen out of 18 animal isolates were nontypeable. One isolate from calf belonged to O162 serotype, while another calf isolate had serotype O8.

Twenty-two different PTs were distinguised among human isolates, with 18 O25-ST131/B2 strains representing EC003 cluster, and 8 O15/D strains representing two closely related clusters (EC026 and EC027). Overall, 11 different PTs were distinguised among 18 animal isolates, and no common PTs were found between two collections.

5. Conclusions

Occurence and regional distribution of SHV-type ESBLs in Hungary, 2002-2003

This was the first study conducted in Hungary to identify ESBL genes by DNA sequencing, yielding unequivocal classification of the β-lactamase genes. The results of this study showed that two of the three most prevalent SHV-types in Europe, SHV-5 and SHV-2a, were also present in Hungary during this period.

The geographical distribution of the SHV-5 and SHV-2a genes seemed to be clearly defined in Hungary during the study period. SHV-5 was widespread, proving to be the only SHV-type ESBL detected in the western and central regions, and it was also present in the southern counties. In contrast, SHV- 2a occurred exclusively in the southern and easternmost parts of the country.

Our results demonstrated that primarily two SHV genes (SHV-5 and SHV-2a) have been responsible for the increasing number of nosocomial infections caused by SHV-type ESBL-producing pathogens in Hungary between 2002-2003.

Epidemiology of SHV-type β-lactamase-producing *Klebsiella* spp. from Hungarian outbreaks, 2002-2003

Our results demonstrated that the seven investigated outbreaks observed in five separate hospitals were caused by 10 epidemiologically

unrelated clones. No interhospital ECs were detected.

These findings suggested that clonal dissemination of SHV-producing *Klebsiella* spp. strains has been confined to one hospital and did not play a prominent role in the wide dissemination of these organisms as suggested in other studies. However, similarities observed in the size and restriction pattern of the ESBL-harbouring plasmids alluded to the multiple transfer of epidemic R-plasmids responsible for a sequence of outbreaks in Hungary.

Molecular epidemiology of Hungarian CTX-M-15-producing Klebsiellae

The emergence of CTX-M-15 in *K. pneumoniae* in Hungary in 2003 is not surprising considering its extended dissemination among different species of the family *Enterobacteriaceae* in Europe. Two years after the first description of a CTX-M-15-producing *K. pneumoniae* EC (HEC) countrywide dissemination of ciprofloxacin resistant, CTX-M -15-producing *K. pneumoniae* was observed. In 2005, 97% of all CTX-M-producing *K. pneumoniae* isolates detected across Hungary and submitted to the NCE were highly ciprofloxacin-resistant CTX-M-15 producers and represented just three stable genetic clones (ST11, ST15, ST147).

ESBL-producing *S. enterica* strains from humans in Hungary, 2000-2004

Our results provide further evidence for the spread of plasmid-borne $bla_{\text{CTX-M}}$-carrying *S.* Typhimurium in Europe. The emergence of the $bla_{\text{CTX-M-5}}$, $bla_{\text{CTX-M-15}}$ and $bla_{\text{SHV-5}}$ genes in *S.* Typhimurium and $bla_{\text{SHV-5}}$ in *S.* Enteritidis in Hungary are reported for the first time.

The sequencing analysis of $bla_{\text{CTX-M-5}}$-harbouring plasmid and PFGE analysis of the Hungarian and the Ukrainian *S.* Typhimurium isolates suggest a relationship of our isolates with the Russian, Belorussian and Latvian isolates also carrying closely related plasmids. However, whether the same CTX-M-producing clone of *S.* Typhimurium was disseminated across Eastern and Central Europe requires further studies.

Molecular epidemiology of ESBL-producing *E. coli* isolated from human clinical and animal samples in Hungary, 2006-2007

This was the first comprehensive study on molecular epidemiology of ESBL-producing *E. coli* clinical and animal isolates in Hungary.

The ciprofloxacin-resistant CTX-M-15-producing O25-ST131/B2 and O15/D strains of human origin proved to be widespread among Hungarian healthcare facilities.

CTX-M-1, CTX-M-32 and SHV-2 ESBL genes were found in the animal strains, which are present in animal strains in the other European countries as well. According to results of PFGE no clonal relationship was found between human and animal strains.

These findings suggest that between 2006-2007 there are no *E. coli* clones of animal origin causing infections in nosocomial settings. However, if identical ESBL-producing *E. coli* genetic clones occured in livestock or food chain and in community requires further studies.

6. List of publications

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