Molecular epidemiological investigation of SHV- and CTX-M-type extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolates in Hungary

Doktoral (PhD) thesis

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Budapest
2011

1. Introduction

Klebsiella pneumoniae (KP) strains are ubiquitous in the natural environment. They are found in surface water, sewage and soil and on plants, furthermore they colonize the mucosal surfaces of mammals such as humans, horses or swine. In humans, KP is present as a saprophyte in the nasopharynx and in the intestinal tract. KP is still one of the leading causes of community acquired pneumonia and pyogenic liver abscess in some countries, and as opportunistic pathogen is responsible for severe nosocomial infections and large nosocomial outbreaks primarily in neonatal and adult intensive care units. KP strains represent an increadibly great epidemic potencial and are one of the major sources of horizontally spreading antimicrobial resistance. Today the extended-spectrum β-lactamase-producing KP (ESBL-KP) constitute one of the most common Gram-negative bacteria showing multiple antibiotic resistance worldwide.

Currently the carbapenems are widely used for treatment of nosocomial infections caused by globally disseminated TEM, SHV or CTX-M ESBL-KP strains. As a result of ascendant consumption of carbapemens the emergence of strains with aquired carbapenemase production (eg. metallo-β-lactamases (MBL), KP carbapenemases (KPC)) was found in KP population. Practically there are no therapeutic options for treatment of infections caused by such multidrug resistant – and additionally colistin resistant – strains and their international dissemination mostly lead to the global health-care crisis of the XXI century as well.

Data collected by the National ESBL Survey (NS-ESBL) initiated by the National Center for Epidemiology (NCE) in 2002, showed that KP is the most frequent ESBL-producing pathogen in Hungary, with an incidence of 65% to

75% of all ESBL-producing *Enterobacteriaceae*. According to National Bacteriological Surveillance (NBS) the proportion of 3rd generation cephalosporin resistant KP from nosocomial bloodstream infections had rose from 9% in 2003 to nearly 50% in 2010 and 80% of these isolates were co-resistant to aminoglycosides and fluoroquinolones.

By the turn of the XXI century dramatic increase of large nosocomial otbreaks caused by ESBL-KP strains was observed in Hungary, mainly in Neonatal Intensive Care Units (NICUs). This alarming situation prompted a comprehensive molecular epidemiological analysis of ESBL-KP strains submitted to the National Reference Laboratory for Typing of Enteric and Nosocomial Bacterial Pathogens (NRL-TENBP).

The main purpose of my work is understanding molecular epidemiology of 3rd generation cephalosporin resistant KP, monitoring of changes within ESBL-KP population and probably explanation of rapid and succesfull nationwide dissemination of multidrug resistant plasmids and epidemic KP clones. Macrorestriction profile analysis by pulsed-field gelelectrophoresis (PFGE) was the main method used for molecular epidemiological investigation of hundreds of KP isolates submitted to the National Reference Laboratory for ESBL-producing Gram-negative pathogens (NRL-ESBL) and to NRL-TENB. Additionally plasmid profil analysis, mating assays and electroporation, sequencing of several antibiotic resistance genes and multilocus sequence typing (MLST) were introduced and applied for in-depth genetic characterization of multidrug resistant KP isolates. We have set up the National *Klebsiella* PFGE Database which currently comprise over 1000 KP macrorestriction profiles. The comprehensive analysis of database help us tracing the spatio-temporal spread of different PFGE types – genetic clones –

in Hungarian healthcare facilities, as well as identifiing the new ones. Results of my work contribute to the knowledge on population dinamycs, molecular epidemiology and antibiotic resistance of one of the most important nosocomial pathogens in Hungary, furthermore could give assistance to clinicians, infectologists and infection control specialists to improve the extremly worrisome Hungarian situation.

2. Aims

1. Establishment of epidemiological relationship among ESBL-producing *Klebsiella* spp. strains isolated from outbreaks in different NICUs between 2002-2003 and 1998 and investigation of the common ESBL types expressed by these strains and the plasmids harbouring the ESBL genes.
2. Molecular epidemiological investigation of first Hungarian ciprofloxacin resistant CTX-M- producing KP isolates submitted to the NCE in 2003 and comprehensive in-depth molecular characterization of such isolates from 2005. Developing and expansion of National *Klebsiella* PFGE Database for continuous monitoring of the incidence and spatio-temporal distribution of multidrug resistant KP epidemic clones.
3. Molecular epidemiological investigation and genetic characterization of first Hungarian SHV-type ESBL- and KPC-producing KP isolates.
4. Molecular epidemiological investigation and genetic characterization of first Hungarian CTX-M-type ESBL- and VIM-type MBL-producing KP isolate.

5. Molecular epidemiology of first plasmid-mediated AmpC-type β-lactamase producing KP isolates in Hungary and monitoring of their dissemination.

3. Methods

Bacterial strains

The Phage typing and molecular epidemiological department and the NRL-TENBP continously received KP isolates from nosocomial outbreaks and sporadic cases since 1960's for epidemiological typing, furthermore since 2002 suspected ESBL-producing isolates were submitted to the ESBL-NRL for confirmation. Investigated isolates were choosed among 1708 3rd generation cephalosporin resistant KP isolates submitted between 2002 and 2010.

Molecular epidemiological investigation of SHV-producing Klebsiella spp. outbreak strains: 126 consecutive, non-duplicate ESBL-producing *Klebsiella spp*. isolates were selected from seven nosocomial outbreaks reported to NCE in 2002-2003 and in 1998 from NICUs in five Hungarian hospitals.

Investigation of CTX-M-producing KP isolates : In 2003 among ESBL-KP isolates submitted to the ESBL-NRL and NRL-TENBP 17 isolates from eight hospitals showed high level resistance to ciprofloxacin. These isolates and additionally 196 ciprofloxacin-resistant CTX-M-type β-lactamase-producing KP isolates collected in 2005 from 35 Hungarian county and teaching hospitals were selected for comprehensive molecular epidemiological analysis and investigation of antibiotic resistance mechanisms.

Investigation of ESBL-KP isolates with elevated ciprofloxacin MIC values: As a result of continuous monitoring of ESBL-KP from January 2006 to December 2008, 27 isolates with elevated ciprofloxacin MIC values, collected from five healthcare facilities and submitted to the National Center for Epidemiology were selected for molecular epidemiological analysis and investigation of antibiotic resistance mechanisms. Of these, 12 isolates originated from adult inpatients in two healthcare facilities and 15 isolates were collected from newborns in four healthcare facilities.

Investigation of KPC-producing KP isolates: From October 2008 to April 2009 nine carbapenem non-susceptible KP isolates from 3 centres in the Northeastern Region of Hungary were submitted to the ESBL-NRL. Molecular typing and genetic characterization were performed on all isolates.

Investigation of a MBL-producing KP isolate: One carbapenem-susceptible KP isolate (KP3686) isolated from bronchoalveolar lavage in Budapest in February 2009 was suspected to be MBL producer and was submitted to the ESBL-NRL for confirmation. Molecular typing and in-depth investigation of antibiotic resistance mechanisms were performed.

Investigation of plasmid-mediated AmpC-type β-lactamase-producing KP isolates: In 2009 and 2010 117 cefoxitin and 3rd gen. cephalosporin resistant KP isolates collected in 15 Hungarian hospitals were submitted to the ESBL-NRL for confirmation of putative plasmid-mediated AmpC-type β-lactamase-production.

Biochemical identification and antibiotic susceptibility tests.

Biochemical identification of the isolates was carried out by API20E, ATB ID32E (bioMerieux) and Micronaut E (Genzyme Virotech GmbH). The antibiotic susceptibility tests were performed by disk diffusion method according to the recommendations of Clinical and Laboratory Standards Institute (CLSI). The putative production of an ESBL was confirmed by combined disk method (MAST), ESBL Etest (AB Biodisk) or modified double disk synergy test. For phenotypic detection of putative carbapenemase production was confirmed by the modified Hodge-test. For differentiation of other β-lactamases disk tests containing imipenem, meropenem or ertapenem either alone or combined with different inhibitors (ethylenediaminetetraacetic acid, dipicolinic acid, 3-amino-phenil-boronic acid or cloxacillin) were used. The minimal inhibitory concentrations (MICs) of antibiotics were determined by the E-test (AB Biodisk), broth microdilution or agar dilution methods according to the manufacturer's instruction or CLSI's recomendatons, respectively.

Molecular methods

Polymerase chain reaction (PCR) method was used for screening of several antibiotic resistance genes on selected isolates: β-lactam resistance encoding genes (*bla*$_{\text{SHV}}$, *bla*$_{\text{TEM,}}$ *bla*$_{\text{OXA-1-like}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{DHA}}$), aminoglycoside resistance encoding genes (*aac-(3)-IIa, aac-(6')-Ib*), quinolone resistance encoding genes (*qnrA, qnrB, qnrS*) and tetracycline resistance encoding genes (*tet*(A), *tet*(B), *tet*(C). Sequencing of PCR amplicons was performed for correct determination of resistance genes.

Plasmid DNA was extracted and electrophoresed by the method of Kado and Liu. The mating assays and electroporation were carried out with selected isolates and *E. coli* K12J5-3Rif and *E. coli* DH5α were used as recipients. For fingerprinting analysis, plasmid DNA from transconjugants (TC) or electroporants (EP) was obtained by using a QIAprep Spin Miniprep Kit (Qiagen), digested with *Pst*I (Biolabs) and detected by gel electrophoresis in 0.7% agarose at 110 V for 2 h.

Alterations in the QRDRs of *gyrA* and *parC* encoding subunits of gyrase and topoisomerase IV enzymes in selected isolates were determined by sequencing. The nucleotide substitutions resulting in amino acid changes were identified on the basis of the previously published sequences.

The pulsed field gel electrophoresis (PFGE) was performed in line with the standardized CDC protocol. Gels were interpreted with Fingerprinting II Informatix Software (Bio-Rad). 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. Pulsotypes (PTs) were defined at 85% similarity between macrorestriction patterns and clonally related isolates were supposed if they belonged to the same PT.

MLST with seven housekeeping genes was performed on selected isolates according to Diancourt et al. Allele sequences and sequence types (STs) were verified at the http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html web site.

4. Results

Molecular epidemiology of SHV-type β-lactamase-producing *Klebsiella* spp. from several outbreaks in neonatal intensive care units in Hungary

Between 1998 and 2003 most nosocomial outbreaks (19 of 25) reported from Hungarian NICUs were caused by *Klebsiella* spp. Of these seven were caused by ESBL-producing strains.

One hundred and twenty-six extended-spectrum β-lactamase-producing clinical isolates of *Klebsiella* spp. were collected in 1998, 2002 and 2003 from seven outbreaks in neonatal intensive care units (NICUs) of five Hungarian county and teaching hospitals. The isolates 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 of SHV-specific polymerase chain reaction products from 13 representative isolates detected the *bla*SHV-2a gene in three and the $bla_{\text{SHV-5}}$ gene in seven outbreak strains, respectively. In the majority of isolates the bla_{SHV} genes were on transferable plasmids of ~90 kb. *Eco*RI and *Pst*I digestion of plasmid DNA from TCs 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 CTX-M-15-producing *K. pneumoniae* isolated between 2003 and 2005 in Hungary

Ciprofloxacin resistant ESBL-KP isolates were detected for the first time in Hungary in 2003. All of the 17 isolates harboured the $bla_{\text{CTX-M-15}}$ ESBL-gene and showed high resistance to 3rd gen. cephalosporins, ciprofloxacin, tetracyclin and with one exeption to gentamicin. The CTX-M-15 ESBL-gene and the aminoglycosides modifying enzyme encoding *aac-(3)-IIa* gene were detected on ~140 kb plasmid from TCs of selected isolates. Plasmids from the transconjugants were digested with the *Pst*I restriction endonuclease and very similar fingerprints were obtained.

PFGE analysis revealed strong clonal relationship between 17 isolates originated from 8 hospitals (Hs) in 5 counties and Budapest city. The isolates were assigned to N PT and we proposed the Hungarian Epidemic Clone designation.

Rapid dissemination of ciprofloxacin resistant CTX-M-15-producing KP isolates was observed in 2005, thus comprehensive molecular epidemiological analysis was performed on 196 such isolates collected in 35 hospitals in 13 Hungarian counties,

Molecular typing by PFGE identified only three distinct PTs (N, R and S) among the 196 highly ciprofloxacin-resistant CTX-M-producing KP isolates. One hundred and twenty-nine isolates from 31 centres were assigned to PT N (the original PT of HEC). Of these strains, 70 ESBL-KP isolates were isolated from three nosocomial outbreaks. Forty-six isolates submitted from

nine centres including those from two parallel nosocomial outbreaks comprised the second cluster designated PT R (Epidemic Clone II, ECII). Twenty-one isolates collected from four centres and including one nosocomial outbreak comprised the third cluster designated PT S (ECIII).

All isolates harboured plasmids ranging from ~2.0 to 230 kb grouped in distinct plasmid profiles. *Pst*I digestion of plasmid DNA from TCs or EPs 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 EC III—carried an additional $bla_{\text{TEM-1}}$ gene and ISEcp1 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 EC III isolates, two amino acid changes (Ser-83→Phe and Asp-87→Ala) and one amino acid change (Ser-80→Ile) were detected, respectively. The EC II isolates exhibited a single nucleotide mutation in both *gyrA* (resulting in Ser-83→Ile substitution) and *parC* genes (resulting in Ser-80→Ile substitution).

Three distinct allelic profiles were obtained by MLST: ST 15 corresponding to HEC including the holotype isolate, ST 11 corresponding to EC III and the novel ST 147 corresponding to EC II

Molecular epidemiology of ESBL-KP **between 2006 and 2010 and the National *Klebsiella* PFGE Database**

The Database has been developed mainly for continuous nation wide monitoring of dissemination of the three ECs identified in 2005. By the end of 2010 153 isolates from 35 Hs belonged to HEC (N/ST15), 76 isolates from 12 Hs belonged to R/ST147 EC and 51 isolates from 12 Hs belonged to S/ST11 EC were identified and are included in the database.

In 2006 the ciprofloxacin resistant CTX–M–15–producing Z/ST525 EC was identified and during the past five years it caused several nosocomial outbreaks across the country. Currently 130 Z/ST525 isolates from 37 Hs are included in the database.

Molecular epidemiological investigation of isolates from numerous outbreaks in NICUs between 2004 and 2010 was performed. The majority of investigated outbreaks were caused by one or two genetic clones (M and O PTs in 2004, T in 2005, W and X in 2006, U, Y and V in 2007, KP025 in 2008 and KP041 in 2009) and all of them harboured SHV-5 or SHV-2a ESBL-genes. The Q, H, P and L PTs were found in more than one hospital and in different years.

The molecular typing of 27 isolates with elevated ciprofloxacin MICs collected from newborn or adult wards of five healthcare facilities between 2006-2008 revealed the existence of only one genetic cluster – the L PT. *Pst*I digestion of plasmid DNA revealed two highly diverse restriction patterns in "adult" and "newborn" isolates corresponding to plasmids from the HEC and plasmids isolated from a neonatal nosocomial outbreak in 1998, respectively. Sequencing revealed that plasmids from "adult" isolates harboured $bla_{\text{CTX-M-}}$

$_{15}$, *aac(6')-Ib-cr* and *aac-(3)-II* genes, while on plasmids from TCs and EPs of "newborn" isolates, *bla*$_{\text{SHV-2a}}$ and *aac-(3)-II* genes were found. MLST established that strains of the PFGE cluster belonged to a novel sequence type ST274.

According to the efflux pump inhibitor test, the ciprofloxacin MIC elevation in only one „newborn" isolate can be explained with overexpression of efflux pump. On the basis of the alterations of nucleotide sequences in the QRDRs of GyrA and ParC in the L/ST274 isolates, one amino acid change (Ser-83→Tyr) was detected in *gyrA.*

Emergence and dissemination of plasmid-mediated DHA-1 AmpC-type β-lactamase-producing KP **isolates**

The first CTX-M-type ESBL and inducible, plasmid-mediated AmpC-producing KP isolates were detected in December 2009 and showed resistance to 3rd gen. cephalosporins, aminoglycosides, fluoroquinolones and trimethoprime/sulphamethoxasole. Thirty per cent of isolates were resistant to at least one carbapenem as well.

Altogether 117 such isolates from 15 Hs were submitted to the reference laboratories by the end of 2010. Among these, 108 isolates proved to be CTX-M-type ESBL-producers as well. The majority (83%) of *bla*$_{\text{DHA-1}}$ and *bla*$_{\text{CTX-M-15}}$ –producing isolates belonged to PT KP053 and ST11. Additionally 20 isolates belonged to further 8 different PTs and the *bla*$_{\text{DHA-1}}$ gene was found in one S/ST11 and one Z/ST525 isolates.

Emergence of KPC-producing KP in Hungary

Nine *K. pneumoniae* isolates showing non-susceptibility to carbapenems collected from 3 centres in the Northeastern Region of Hungary were investigated. All isolates showed extensively drug-resistant (XDR) phenotype, of these eight isolates were highly resistant to colistin. The isolates carried $bla_{\text{KPC-2}}$, $bla_{\text{SHV-12}}$, $bla_{\text{TEM-1}}$ and $bla_{\text{SHV-11}}$ and belonged to ST258 sequence type.

PFGE analysis of the nine KPC-2-producing Hungarian ST258 KP isolates and 33 CTX-M-15 producing ST11 isolates revealed the existence of one genetic cluster at 88% similarity level.

Investigation of the first Hungarian MBL-producing KP **isolate**

Based on screening and confirmatory tests, one carbapenem-susceptible KP isolate (KP3686) isolated from bronchoalveolar lavage (Budapest, February 2009) proved to be MBL producer.

In KP3686, the chromosomally encoded non-ESBL-type $bla_{\text{SHV-11}}$, plasmid carried $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1}}$ (~50 kb plasmid), and the class 1 integron-located $bla_{\text{VIM-4}}$ (~90 kb plasmid) genes were detected.

The ~1800 bp integron carried two resistance gene cassettes, namely an *aac(6′)-Ib* gene in the first position, followed by a $bla_{\text{VIM-4}}$ gene cassette. The results of integron sequencing showed the same VIM-4-containing class 1 integron identical to that previously characterized from *Pseudomonas aeruginosa* strains that originated from southern Hungary and from the first MBL-producing *Aeromonas hydrophila* strain.

Results of both PFGE and MLST proved that KP3686 belonged to the previously described sequence type (ST) 11 clone, detected throughout Hungary as a CTX-M-15-producing epidemic clone S (EC III).

5. Conclusions

Molecular epidemiology of SHV-type β-lactamase-producing *Klebsiella* spp. from several outbreaks in neonatal intensive care units in Hungary

Since nosocomial outbreaks caused by *Klebsiella* spp. at NICUs emerged as a serious problem in 2002–2003, we have investigated the epidemiological relationship between strains collected from seven multifarious outbreaks from geographically separated NICUs.

Our results demonstrated that the seven investigated outbreaks observed in five separate hospitals were caused by 10 epidemiologically unrelated clones. No interhospital epidemic clones were detected and the outbreak clones did not persist for a long time in the same hospital ward. These findings suggest that clonal dissemination of SHV-producing *Klebsiella* spp. strains has been confined to one hospital and did not play a prominent role in the country-wide dissemination of these organisms.

Although the seven different SHV-5 β-lactamase producing outbreak clones carried several plasmids, only a large plasmid of 94 kb was transferred from each one. Fingerprinting analysis of these plasmids showed all of them to have identical or very similar restriction profiles. Results suggested that a similar $bla_{\text{SHV-5}}$-harbouring plasmid has been spread among the outbreak-

causing KP and *K. oxytoca* clones in geographically distant regions of Hungary.

This was the first report in the international literature of such wide and fast country-wide dissemination of a bla_{SHV-5}- harbouring R-plasmid, causing a chain of outbreaks over a period of eight months in five Hungarian counties.

We have observed identical fingerprinting patterns for two SHV-2a-encoding conjugative plasmids as well. These plasmids were transmitted from different outbreak clones in geographically distant NICUs over a 4-year period. It was found that the high number of nosocomial outbreaks experienced by several Hungarian NICUs from 2002 to 2003 was a result of extensive and effective plasmid dissemination. The ESBL situation in Hungarian NICUs between 2002 and 2003 could be characterised by countrywide spread of similar bla_{SHV-5}- and bla_{SHV-2a}-harbouring multidrug resistant epidemic R-plasmids, which are able to transfer within KP and *K. oxytoca* bacterial populations, empowering them as forceful outbreak agents.

The term 'allodemic'(allos = other) is used to define a type of polyclonal epidemic. If the epidemiological situation is equivocal (neither outbreaks nor endemic spread is observed), the term 'allodemic situation' is more appropriate. Our results suggest that an interhospital 'allodemic situation' caused by multiple polyclonal transfer of similar SHV-encoding R-plasmids in *Klebsiella* spp. has emerged in Hungary. We proposed to call these R-plasmids 'allodemic R-plasmids'.

Characterization of CTX-M-15-producing KP **and the National *Klebsiella* PFGE Database**

We identified the first ciprofloxacin resistant CTX-M-15-type ESBL-KP isolates in Hungary during 2003. The 17 isolates, collected at different times in different hospitals across country, belonged to single genetic clone (N/ST15). It was the first description in the international literature of countrywide dissemination of an multidrug resistant KP epidemic clone (EC), and we proposed the Hungarian Epidemic Clone (HEC) designation.

After the description of the HEC in 2003, a continuous monitoring system was implemented covering ciprofloxacin-resistant and CTX-M-producing KP isolates. In 2005, an eruptive and extensive dissemination of ciprofloxacin resistant CTX-M-15-producing KP was observed. PFGE analysis of all isolates revealed the existence of just three different genetic clusters named ECs: the HEC (N/ST15), EC II (R/ST147) and EC III (S/ST11). The HEC could be subdivided into three main subclusters, indicating its relative heterogeneity. In contrast, EC II and EC III showed quite a homogeneous structure. The heterogeneity of HEC could be explained by its long-term and nationwide dissemination since 2003, whereas EC II and EC III were newly detected clones with a relatively small dissemination area.

The MLST technique that is based on indexing of the genetic variation in housekeeping genes was successfully employed for the first time in the international literature for studying the longitudinal epidemiology of multidrug resistant KP. The interpretation of MLST results suggests a stable clonal structure for several KP strains. Interhospital ECs were detected, and the same ECs persisted for a long time in the same hospital.

Our findings proved that the nationwide dissemination of cefotaximases and especially those of CTX-M-15 can be explained by the rapid and efficient expansion of a few KP-ECs with surprisingly similar'resistance equipment'. Six of the seven KP-related nosocomial outbreaks reported to the NCE in 2005 were caused by the three ECs. Such events illustrate the high epidemic potential of these ECs and their exceptional adaptation to the hospital environment. This phenomenal situation, in which 97% of the CTX-M-producing KP isolates detected across Hungary in 2005 were highly ciprofloxacin-resistant and represented just three stable genetic clones that persisted for a long time in the hospital settings, suggests a convergent population evolution in the KP species as in the case of the *Staphylococcus aureus* species (MRSA versus MSSA).

After the publication of our results the researchers from all over the world began to apply the MLST method for studying population genetics of KP, and in the past two years our findings confirmed. Indeed, there are spatially and temporally stable epidemic or pandemic KP clones showed world wide distribution and carried the most important resistance mechanisms. Among these the most commonly identified types are the ST11 (and the closely related ST258 and ST437) and the ST15 (and the closely related ST14).
Since the dissemination of CTX-M-15-producing KP in Hungary proved to be clonal, we are performing continuous monitoring of ESBL-producing KP-ECs in Hungary using the National *Klebsiella* PFGE Database. Today the N/ST15 (Σ152 isolates, 35 Hs), the R/ST147 (Σ76 isolates, 12 Hs) and the S/ST11 (Σ53 isolates, 10 Hs), epidemic clones continuously persist in different medical and intensive care units throughout the country. During the

past five years two additional multidrug resistant CTX-M-15-producing KP-ECs were identificated in many hospitals. The ciprofloxacin resistant Z/ST525 EC was identified from six nosocomial outbreaks and many sporadic cases mainly from intensive care units in 30 hospitals. The emergence and rapid nationwide spread of this EC confirmed our previous findings for monoclonal dissemination of CTX-M-type ESBLs in Hungary.
Between 2006 and 2008 the epidemic clone L/ST274 was isolated and identified from three nosocomial outbreaks (in neonatal and adult intensive care units) in six different hospitals located in four Hungarian counties. Three different mechanisms used by L/ST274 EC for developing resistance to ciprofloxacin were supposed: the basic *gyrA* mutation, an efflux pump and the presence of *aac(6')-Ib-cr* gene. Sequence analysis of β-lactamase genes from plasmids of selected L/ST274 clone isolates detected $bla_{\text{SHV-2a}}$ in strains isolated exclusively from newborns and $bla_{\text{CTX-M-15}}$ in strains isolated exclusively from adult inpatients. We have shown for the first time that there has been a particular antimicrobial resistance strategy used by L/ST274 KP-EC for successful dissemination in quite diverse newborn and adult hospital settings. Such fusions of epidemic clones and longstanding epidemic or allodemic plasmids at the stringent evolutionary pressure of hospital environment probably lead to the evolution and spread of highly resistant clones

Molecular epidemiology of carbapenemase-producing KP **isolates**

We identified and characterized the first KPC-producing and VIM-producing KP isolates in Hungary. The KPC-2-producing ST258 isolates and the CTX-

M-15-producing ST11 Hungarian isolates belonged to the same genetic cluster (S PT). Considering the overall results of the PFGE clustering, MLST and the presence of SHV-11 in both ST11 and ST258 we described in the international literature the first hyperepidemic clonal complex in KP population and proposed CC258/CC340 designation. Today, there is evidence that this hyperepidemic clonal complex spread throughout the world.

Molecular epidemiological typing and genetic characterization of the VIM-4-producing isolate confirmed our observations on structure and dinamycs of KP population. The isolate belonged to the intercontinental S/ST11 pandemic clone and during five years of its countrywide persistence aquired an previously described integron, probably from the environmental genetic pool. We described for the first time in the international literature the existence of an successful hyperepidemic clone - the ST11 - within KP population and its VIM-4-type MBL-production.

6. List of publications

Related to the thesis

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