Molecular epidemiology of multiresistant *Klebsiella pneumoniae* strains isolated at the Clinical Centre University of Pécs

Doctoral (PhD) thesis

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Pécs University
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Pécs
2015

Introduction

Klebsiella pneumoniae is a Gram-negative, rod-shaped, facultative anaerobic bacterium belonging to the family Enterobacteriaceae. It is usually considered as an opportunistic pathogen, mainly infecting hospitalised patients with underlying medical conditions. Common clinical manifestations are urinary tract, pulmonary, surgical site and blood stream infections. Despite the opportunistic nature hypervirulent strains can also occur, which are capable of causing severe disease (primary liver abscess, sometimes with metastatic complications) in otherwise healthy individuals.

Several virulence determinants were identified in *K. pneumoniae* to date. The polysaccharide capsule, the hypermucoviscosity phenotype, the lipopolysaccharide component of the cell wall, resistance to complement mediated killing, type 1 and type 3 fimbriae, biofilm formation and various iron acquisition systems (enterobactin, salmochelin, aerobactin, yersiniabactin and Klebsiella Ferric ion Uptake system) are all considered to contribute to its pathogenicity. In hypervirulent strains the simultaneous expression of different virulence factors are thought to give rise to the higher virulence potential.

Resistance to β-lactam antibiotics (penicillins, cephalosporins, carbapenems and monobactams) demand a particular attention as these agents constitute the most widely used group of antimicrobials. All *K. pneumoniae* isolates are naturally resistant to aminopenicillins due to the production of a chromosomally encoded SHV type β-lactamase. The most important acquired β-lactam resistance mechanism of *K. pneumoniae* are the production of (1) extended spectrum β-lactamases (ESBLs), (2) AmpC enzymes and/or (3) carbapenemases.

The most frequently encountered ESBLs include SHV, TEM and CTX-M enzymes, which are capable of inactivating third generation cephalosporins, and their activity can be inhibited by clavulanic acid. SHV and TEM ESBLs evolved from chromosomally encoded SHV and TEM β-lactamases with narrower substrate spectrums through the accumulation of point mutations.

Plasmid-borne AmpC enzymes, like ACC, ACT, CMY, DHA, FOX, LAT, MIR and MOX, also hydrolyse third generation cephalosporins, and their activity can be inhibited by cloxacillin. Production of AmpC enzymes is less frequently responsible for third generation cephalosporin resistance than ESBL production.

Widespread carbapenemases enclose IPM, KPC, NDM, OXA-48-like and VIM enzymes, they efficiently inactivate carbapenems, and their specific inhibitor varies among groups.

Isolates producing ESBLs, AmpC enzymes and/or carbapenemases should be considered to be multidrug resistant, as all these mechanisms confer cross resistance to various β-lactam compounds, and they often harbour additional resistance mechanisms affecting non-β-lactam antibiotics as well. The worldwide spread of these multiresistant strains is attributed to the dissemination of epidemic resistance plasmids and expansion of successful clones. The exact types of enzymes and their prevalence vary considerable with respect to geographical regions.

During the last two decades a considerable expansion of ESBL production by *K. pneumoniae* was observed in Hungary. In 1996 the first ESBLs in *K. pneumoniae* isolates were identified as *bla*$_{\text{SHV-2}}$ and *bla*$_{\text{SHV-5}}$. Around the turn of the millennium several nosocomial outbreaks due to the dissemination of epidemic resistance plasmids harbouring either *bla*$_{\text{SHV-2a}}$ or *bla*$_{\text{SHV-5}}$ occurred in separate neonatal intensive care units across the country. In 2003 the ciprofloxacin resistant, CTX-M-15 producing Hungarian Epidemic Clone (HEC/ST15) emerged in the adult healthcare setting, and by 2005 it became predominant alongside with two other ciprofloxacin resistant, CTX-M-15 producing clones (EC II/ST147 and EC III/ST11). The fourth epidemic clone (EC IV/ST274) emerged in 2006 and were shown to produce either SHV-2a or CTX-M-15.

Since 2008 carbapenemase producing *K. pneumoniae* isolates have been detected in an increasing number in Hungary. Three distinct classes of carbapenemases in conjunction with the presence of different ESBLs and occurrence in different high risk clones were detected in geographically distant parts of the country. The ST258 isolates from the north-eastern Hungarian outbreak in 2008-2009 presented with an extensively drug resistant phenotype owing to the combination of KPC-2 carbapenemase and SHV-12 ESBL production coupled with resistance to colistin. Acquisition of *bla*$_{\text{VIM-4}}$ by the CTX-M-15 producing EC III/ST11 was observed in the capital city in 2009. The third type of carbapenemase, namely OXA-162, was detected in south-eastern Hungary in 2012. These isolates were proven to belong to ST15, and beyond carbapenemase production they expressed *bla*$_{\text{CTX-M-15}}$ as well.

Aims

As no extensive studies had been performed on multiresistant *K. pneumoniae* isolates from the Clinical Centre University of Pécs before we started our work, the aim of our investigations was to gain comprehensive knowledge on the isolates with acquired β-lactam resistance mechanism(s) in order to (1) estimate the dissemination of specific clones in time and place, (2) look for clonal characteristics in antimicrobial susceptibility pattern and virulence associated factor content, and (3) support an ongoing surveillance by acquiring a well-characterised strain collection. The specific questions raised are listed below.

Trends in β-lactam resistance of K. pneumoniae isolates

- How did the rate of β-lactam resistance in *K. pneumoniae* isolates change over time in the Clinical Centre University of Pécs?
- How do our rates compare to national data?
- Which ESBL genes were the most prevalent?
- Which ESBL producing clones were the most prevalent?
- How were the different ESBL producing clones distributed in time and place?
- What was the molecular background of carbapenemase production?
- Were the carbapenemase producing isolates clonally related?

Antimicrobial susceptibility and virulence associated trait content of K. pneumoniae isolates with acquired β-lactam resistance mechanisms

- What was the distribution of various virulence associated traits among different ESBL producing clones?
- What kind of resistance patterns were characteristic for the different ESBL producing clones?
- What antibiotics were the carbapenemase producing isolates susceptible to?

Materials and methods

Trends in β-lactam resistance of K. pneumoniae isolates

Surveillance of acquired β-lactam resistance mechanisms

- Calculation of annual rate of resistance to third generation cephalosporins and/or demonstration of either ESBL or AmpC production for the time period 2003-2014 for all clinical samples ($ceph_{all}$)
- Calculation of annual rate of resistance to carbapenems and/or demonstration of carbapenemase production for the time period 2003-2014 for all clinical samples ($carb_{all}$)

Isolates

- 102 ESBL producing *K. pneumoniae* isolates from 2004-2008
- 102 carbapenemase producing *K. pneumoniae* isolates from 2009-2011

Molecular typing

- Macrorestriction profile analysis by pulsed-field gel electrophoresis (PFGE)
- Multilocus sequence typing (MLST)

Detection of β-lactamases

- Confirmation of ESBL production by combined disc method
- Screening for carbapenemase production by modified Hodge-test
- Confirmation of carbapenemase production by phenotypic inhibition assay
- Detection of β-lactamase genes (*bla*$_{CMY}$, *bla*$_{CTX-M}$, *bla*$_{DHA}$, *bla*$_{FOX}$, *bla*$_{IMP}$, *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{VIM}$) by PCR
- Restriction fragment length polymorphism analysis of *bla*$_{SHV}$ by *Nhe*I in order to identify most common point mutation associated with the hydrolysis of third generation cephalosporins
- Sequencing of *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$ genes and integron for selected isolates

Virulence associated trait content and antimicrobial susceptibility of K. pneumoniae isolates with acquired β-lactam resistance mechanisms

Virulence associated trait content

- Detection of virulence associated genes (*magA*, *k2a*, *rmpA*, *irp2-1* and *kfuB*) by PCR
- Screening for hypermucoviscosity phenotype by string-test
- Measurement of susceptibility to serum bactericidal activity
- Detection of enterobactin and aerobactin production by cross feeding bioassay
- Detection of type 1 and type 3 fimbriae by agglutination assays
- Estimation of static biofilm forming capacity by microtiter plate assay

Antimicrobial susceptibility testing

- Disc diffusion method
- Broth microdilution
- MIC (minimum inhibitory concentration) gradient test

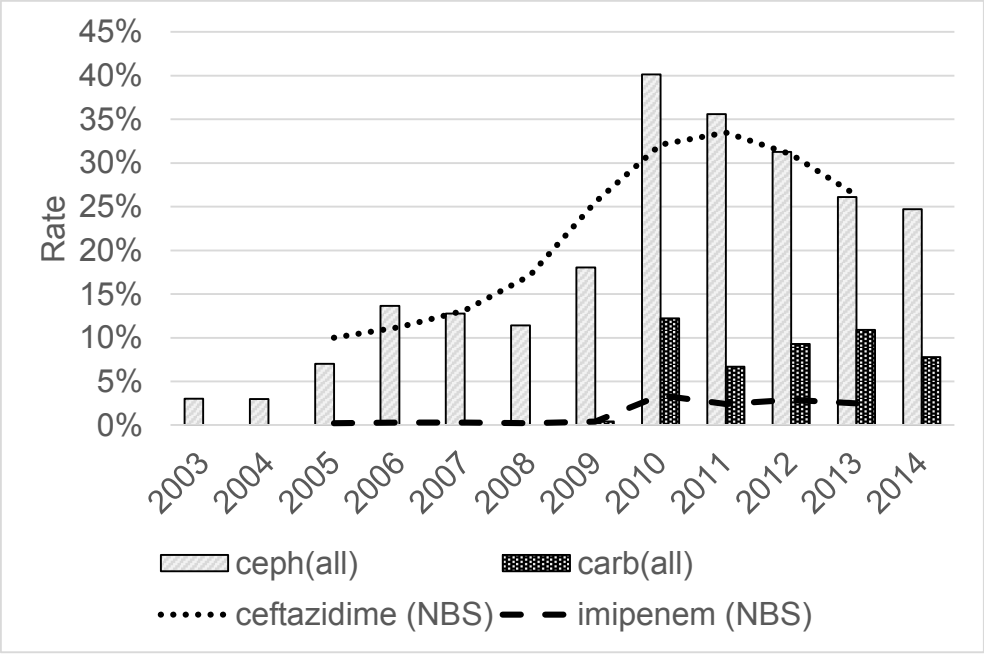
Statistical methods

- Hypothesis testing by likelihood ratio test and Kruskal-Wallis-test

Results and discussion

Trends in β-lactam resistance of K. pneumoniae isolates

In order to estimate trends in the rate of acquired β-lactam resistance mechanisms of *K. pneumoniae* isolates from the Clinical Centre University of Pécs the following data series were calculated and analysed: (1) $ceph_{all}$ to track resistance mechanisms affecting third generation cephalosporin susceptibility, namely ESBL and AmpC; and (2) $carb_{all}$ to monitor resistance mechanism affecting carbapenem susceptibility, namely carbapenemase production and hyper production of ESBL/AmpC in conjunction with porin mutations. The annual rates are presented in Figure 1. The corresponding data on non-susceptibility rates to ceftazidime and imipenem from the National Bacteriological Surveillance (NBS) database is also featured in order to enlighten the comparison.



1. Figure Annual rate of *K. pneumoniae* isolates with acquired β-lactam resistance mechanisms at the Clinical Centre University of Pécs and corresponding data from National Bacteriological Surveillance (NBS)

When looking at Figure 1 two extensive escalations in our rates can be recognised: the first one occurred between 2004 and 2006 ($ceph_{all}$: 3.0-13.7%), and the second happened between 2008 and 2010 ($ceph_{all}$: 11.4%-40.1%; $carb_{all}$: 0.0-12.2%). Both escalations were followed by a slight decrease in resistance rates.

As comparing $ceph_{all}$ and $carb_{all}$ to rates by National Bacteriological Surveillance, it can be concluded that the general trends observed for local and national data were much alike, but two major differences noticed in the scale of the numbers should be pointed out. First, in 2010 $ceph_{all}$ and $carb_{all}$ significantly exceeded the corresponding national rates of non-susceptibility to ceftazidime and imipenem, and second, $carb_{all}$ remained at a substantially higher level even after 2010 (it seemed to fluctuate around 8.7% as compared to national data with an average of 2.6%).

In order to find explanation for the observations described above 102 ESBL producing *K. pneumoniae* isolates from 2004-2008 and 102 putative carbapenemase producing *K. pneumoniae* isolates from 2009-2011 originating from different departments of the Clinical Centre University of Pécs were selected for molecular typing and detection of β-lactamases. The selection was based on the following considerations: (1) acquired AmpC production was not identified in the early period, and it is still rarely detected in *K. pneumoniae* in our institution, and (2) since the first detection of carbapenemase production by *K. pneumoniae* in our institution in November 2009 all *K. pneumoniae* isolates with reduced susceptibility to carbapenems were consistently positive in the modified Hodge-test presuming the production of carbapenemases. The results are summarised in Table 1.

ESBL producing isolates (2004-2008)

The ESBL producing isolates from 2004-2008 were clustered into three major and eleven minor clones based on macrorestriction profile analysis by PFGE (Table 1). The major clones were identified and designated as Hungarian Epidemic Clone (HEC/ST15) for pulsotype PT-01, Epidemic Clone Pécs (ECP/ST101) for pulsotype PT-02 and Epidemic Clone II (EC II/ST147) for pulsotype PT-03. Among minor clones ST34, ST113 and ST323 were identified in addition to a novel sequence type, namely ST1193 (allelic profile: 2-83-2-1-9-4-135), which harboured a unique variant of the *infB* allele (designated as number 83).

1. Table Results of molecular typing and detection of β-lactamase genes

		Pulsotype (PFGE)	Number of isolates	Result of MLST	β-lactamase genes detected
ESBL producing isolates 2004-2008	Major clones	PT-01 (HEC)	69	ST15	*bla*$_{SHV}$ (*Nhe*I: negative) *bla*$_{CTX-M-15}$
		PT-02 (ECP)	10	ST101	*bla*$_{SHV}$ (*Nhe*I: negative) *bla*$_{CTX-M-15}$ *bla*$_{TEM-1}$ (in 6 isolates)
		PT-03 (EC II)	9	ST147	*bla*$_{SHV}$ (*Nhe*I: negative) *bla*$_{CTX-M-15}$
	Minor clones	PT-04	2	NA	*bla*$_{SHV}$ (*Nhe*I: negative) *bla*$_{CTX-M}$
		PT-05	1	NA	*bla*$_{SHV}$ (*Nhe*I: negative) *bla*$_{CTX-M}$
		PT-06	1	NA	*bla*$_{SHV}$ (*Nhe*I: negative) *bla*$_{CTX-M}$
		PT-07	1	NA	*bla*$_{SHV}$ (*Nhe*I: negative) *bla*$_{CTX-M}$
		PT-08	1	NA	*bla*$_{SHV}$ (*Nhe*I: negative) *bla*$_{CTX-M}$
		PT-09	1	NA	*bla*$_{SHV}$ (*Nhe*I: negative) *bla*$_{CTX-M}$
		PT-10	2	ST1193	*bla*$_{SHV-5}$
		PT-11	1	ST34	*bla*$_{SHV-5}$
		PT-12	1	ST113	*bla*$_{SHV-5}$
		PT-13	1	ST323	*bla*$_{SHV-5}$
		PT-14	2	NA	*bla*$_{SHV}$ (*Nhe*I: positive) *bla*$_{CTX-M}$
carbapenemase producing isolates 2009-2011		PT-01 (HEC)	101	ST15	*bla*$_{SHV-28}$ *bla*$_{CTX-M-15}$ (in 99 isolates) *bla*$_{TEM-1}$ *bla*$_{VIM-4}$ (in 100 isolates)
		PT-15	1	NA	*bla*$_{SHV}$ (*Nhe*I: negative) *bla*$_{CTX-M}$ *bla*$_{TEM}$ *bla*$_{VIM}$

NA = not analysed

HEC/ST15, ECP/ST101 and EC II/ST147 were shown to harbour *bla*$_{\text{CTX-M-15}}$; six minor clones contained *bla*$_{\text{CTX-M}}$, four minor clones encompassed *bla*$_{\text{SHV-5}}$ and one minor clone comprised *bla*$_{\text{CTX-M}}$ and ESBL type *bla*$_{\text{SHV}}$ at the same time.

HEC/ST15 and EC II/ST147 were shown to be epidemic clones in Hungary by others. Although ST101 was found to be a prevalent CTX-M-15 producer in several European countries (France, Italy and Greece), its presence in Hungary was first indicated by our investigations.

Considering that 95% (97/102) of the investigated EBSL producing isolates harboured *bla*$_{\text{CTX-M}}$, and the majority of them (91%, 88/97) belonged to one of the three major ESBL producing clones; it can be concluded that the elevated rate of *ceph*$_{all}$ between 2004 and 2008 could mainly be attributed to the dissemination of *bla*$_{\text{CTX-M-15}}$ genes owed to the spread of the three major clones.

Regarding the spatial and timely distribution of the different ESBL producing clones in the Clinical Centre University of Pécs (Table 2 and 3) it can be concluded that:

- ECP/ST101 might have been the dominant clone in 2004-2005 and was mainly related to Internal Medicine 1 and 2;
- HEC/ST15 started to prevail in all departments of the Clinical Centre during the initial period of this study, and continued to be the most prevalent ESBL producing clone of our institution;
- EC II/ST147 might have emerged around 2007 in our institution, and since then it has spread to several departments;
- minor clones were most common at the Department of Anaesthesia and Intensive Therapy and at other smaller departments.

Although we did not have the possibility to investigate every isolate originating from our institution, and for nine minor clone isolates the exact type of ESBL was not identified, local characteristics in the molecular epidemiology of ESBL producing *K. pneumoniae* isolates could be presumed when comparing our findings to national data. Two of the epidemic clones described (EC III/ST11 and EC IV/ST274) were not observed during our study period, and despite widespread dissemination of EC II/ST147 across the country in 2005, it was only detected first in 2007 in our institution possibly due to later importation or low incidence rates. According to our study HEC/ST15 and CTX-M-15 β-lactamases were the dominant clone and ESBL types, moreover SHV-5 was the only SHV-type ESBL identified in our institution, which corresponds to national data.

2. Table Distribution of ESBL producing *K. pneumoniae* isolates among clones and departments

Department	HEC ST15	ECP ST101	EC II ST147	minor clones
Anaesthesia and Intensive Therapy (n=11)	6	0	0	5
Internal Medicine 1 (n=40)	27	9	1	3
Internal Medicine 2 (n=5)	3	1	1	0
Neurology (n=3)	2	0	0	1
Surgery (n=3)	3	0	0	0
Urology (n=35)	27	0	6	2
Other (n=5)	1	0	1	3

3. Table Distribution of ESBL producing *K. pneumoniae* isolates among clones and year of isolation

Year	HEC ST15	ECP ST101	EC II ST147	minor clones
2004 (n=2)	0	1	0	1
2005 (n=9)	4	5	0	0
2006 (n=17)	12	3	0	2
2007 (n=47)	37	1	2	7
2008 (n=27)	16	0	7	4

Carbapenemase producing isolates (2009-2011)

All but one putative carbapenemase producing isolates from 2009-2011 belonged to the formerly characterized HEC according to macrorestriction profile analysis by PFGE (Table 1). HEC was confirmed with MLST to belong to ST15. PCR showed the presence of *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{VIM}}$ genes for 99, 101, 101 and 100 HEC/ST15 isolates respectively. The genes in HEC/ST15 were identified as *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV-28}}$, *bla*$_{\text{TEM-1}}$, and *bla*$_{\text{VIM-4}}$ according to sequencing. The genes *bla*$_{\text{SHV-28}}$ and *bla*$_{\text{TEM-1}}$ identified in ST15 in this study was noted earlier by others, and SHV-28 was proven to be non-ESBL type in another study. The VIM-4 is a carbapenemase belonging to the group of metallo-β-lactamases. The *bla*$_{\text{VIM-4}}$ gene was present on a class 1 integron, which carried an *aac(6')-Ib* in the first gene cassette, followed by

bla$_{VIM-4}$ in the second gene cassette. The integron was designated as In238b according to the Integrall database (integrall.bio.ua.pt).

To our knowledge, this was the first description of VIM-4 carbapenemase production in the nationally disseminated and regionally dominant CTX-M-15 producing *K. pneumoniae* HEC/ST15. To date six different carbapenemases were identified in relation to this sequence type, namely: NDM-1 in Canada, France, Morocco and Thailand; OXA-48 in Finland, France and Spain; OXA-162 in southern Hungary; VIM-1 in Spain; VIM-34 in Portugal and VIM-4 revealed by our study. Considering this distribution two conclusions should be drawn. First, independent acquisition of different carbapenemase genes in the same sequence type indicates that ST15 has a great capacity to acquire different resistance plasmids, and can successively adapt to continuous antibiotic pressure. Second, VIM-4 production in ST15 seems to be confined to our region suggesting that it might have emerged locally with the attainment of In238b circulating in our country. The In238b was identified earlier in *Pseudomonas aeruginosa* (2002), *Aeromonas hydrophila* (2005), *K. pneumoniae* ST11 (2009) and *Klebsiella oxytoca* (2009) in Hungary, and its presence in the Clinical Centre University of Pécs was shown for *P. aeruginosa* in 2004. When this integron was introduced to the dominant ESBL producing *K. pneumoniae* clone of our institution, a remarkable expansion of VIM-4 production was observed. During the study period, of the 1654 patients from whom *K. pneumoniae* was isolated, 101 (6.1%) were confirmed by the present study to have VIM-producing isolates. The isolates with proven VIM-production originated from 12 distinct departments indicating a widespread dissemination within the Clinical Centre University of Pécs.

The only VIM negative HEC/ST15 isolate with putative carbapenemase production was negative in the phenotypic inhibition test, was susceptible to all three carbapenem derivatives, and only the ertapenem MIC value was above ECOFF (www.eucast.org). Therefore it can be presumed, that the modified Hodge-test utilised to screen for carbapenemase production gave a false positive result for this isolate. The single isolate belonging to PT-15 harboured *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{TEM}$ and *bla*$_{VIM}$ β-lactamase genes.

When superimposing our results of molecular typing and detection of β-lactamase genes over *ceph*$_{all}$ and *carb*$_{all}$ data series, the following explanations could be found for the observed changes in the rates of acquired β-lactam resistance mechanisms:

- The increment in *carb$_{all}$* in 2009-2011 could be explained by the recent acquisition and clonal expansion of bla$_{VIM-4}$ in the nationally disseminated and regionally dominant CTX-M-15 producing *K. pneumoniae* HEC/ST15.
- The higher *ceph$_{all}$* rate than national data in 2010 was mainly attributable to the surplus generated by the emergence of carbapenemase producing isolates in the Clinical Centre University of Pécs, considering that the majority of the isolates producing carbapenemase expressed *bla*$_{CTX-M-15}$ simultaneously.
- The higher level of *carb$_{all}$* after 2010 than the national rate of non-susceptibility to imipenem can generally be credited to the local expansion of VIM-4 producing HEC/ST15. Furthermore the differences in the calculation of rates might have caused a bias towards higher local percentages, since *carb$_{all}$* shows not only the carbapenem non susceptible isolates (as data by National Bacteriological Surveillance), but also those isolates that are carbapenem susceptible despite the production of a carbapenemase.

Virulence associated trait content and antimicrobial susceptibility of K. pneumoniae isolates with acquired β-lactam resistance mechanisms

In order to look for clonal features, isolates with acquired β-lactamase resistance mechanism were characterised in the aspects of virulence associated trait content and antimicrobial susceptibility. The results and statistical analysis of antimicrobial susceptibility testing and possession of virulence traits are presented in Tables 4-8.

Virulence associated trait content of ESBL producing clones

Considering virulence associated trait content of the major clones (Table 4) , some general attributes could be seen: all three major clones showed high biofilm forming capacity and high rate of type 1 fimbria expression, on the other hand hypermucoviscosity phenotype, K1 (*magA*) or K2 (*k2a*) serotype and aerobactin production was absent or rare. Beside these common features, several clonal characteristics could be recognised. HEC/ST15 showed the highest frequency of enterobactin and type 3 fimbria expression; ECP/ST101 was the only to harbour *irp2-1* coding for yersiniabactin and EC II/ ST147 was the least susceptibly to serum bactericidal killing and lacked Klebsiella Ferric ion Uptake System (*kfuB*).

4. Table Virulence associated factor content of ESBL producing *K. pneumoniae* clones

	HEC ST15 n=69	**ECP ST101 n=10**	**EC II ST147 n=9**	**minor clones n=14**	**p**
string-test	2 (3%)	0 (0%)	0 (0%)	2 (14%)	0.245
rmpA	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-
magA	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-
k2a	0 (0%)	0 (0%)	0 (0%)	0 (0%)	-
enterobactin	67 (97%)	5 (50%)	6 (67%)	14 (100%)	<0.001
aerobactin	1 (1%)	0 (0%)	0 (0%)	1 (7%)	0.566
kfuB	69 (100%)	10 (100%)	0 (0%)	3 (21%)	<0.001
irp2-1	0 (0%)	10 (100%)	0 (0%)	2 (14%)	<0.001
type 1 fimbria	67 (99%)*	10 (100%)	9 (100%)	13 (93%)	0.833
type 3 fimbria	65 (96%)*	4 (40%)	6 (67%)	6 (46%)	<0.001
biofilm (median)	3.526	2.112	2.463	1.262	<0.001
serum resistance at 60 min (median)	15.20%	5.40%	62.11%	1.46%	0.005
serum resistance at 180 min (median)	4.41%	4.09%	8.86%	0.09%	0.087

* n=68. One isolate showed auto-aggregative properties.

While some studies indicated that ESBL producing *K. pneumoniae* isolates (1) had higher rates of co-expression of type 1 and type 3 fimbria, (2) were more resistant to serum bactericidal activity or (3) showed increased adherence to and invasion of human epithelial cells, than non-ESBL producing ones, other studies suggested that different virulence factors might be associated with distinct clones or resistance plasmids. Our results also imply that the distribution of virulence associated traits might be diverse among different ESBL producing *K. pneumoniae* clones.

The virulence associated traits identified in major clones were confirmed to play an important role during the pathogenesis of the following infections: type 1 fimbria in urinary tract infections, type 3 fimbria in catheter associated urinary tract infections, and yersiniabactin in respiratory infections. The possession of such virulence associated traits along with a multiresistant phenotype might render these ESBL producing major clones a successful nosocomial pathogen.

Antimicrobial susceptibility of ESBL producing clones

Although resistance to gentamicin and tobramycin was similarly high in major and minor ESBL producing clones, the rate of susceptibility to amikacin and trimethoprim/sulfamethoxazole varied across different clones (Table 5).

While resistance to ciprofloxacin was universal in major ESBL producing clones, the majority (57.1%) of isolates in minor clones showed wild-type phenotype according to epidemiological cut-off (ECOFF) values determined by EUCAST. Differences in the level of resistance to ciprofloxacin was suggested to be influenced by variations in fitness cost associated with the acquisition of fluoroquinolone resistance, and it was indicated that SHV ESBL plasmids might be lost during the induction of high level resistance. In our study high level ciprofloxacin resistance was not observed in SHV-5 producing isolates. The only resistant isolate showed low level resistance (MIC = 2 mg/L), and belonged to ST113.

The majority of isolates belonging to major clones showed combined resistance to aminoglycosides and fluoroquinolones (ST15: 94%; ST101: 100%; ST147: 100%). Such a combination of resistance mechanism was less frequently seen in minor clone isolates (36%). The high rate of resistance to non-β-lactam agents might have contributed to the over usage of carbapenems.

5. Table Susceptibility to various antimicrobial agents of ESBL producing *K. pneumoniae* clones

	HEC ST15 n=69	**ECP ST101 n=10**	**EC II ST147 n=9**	**minor clones n=14**	**p**
amikacin	51 (74%)	3 (30%)	8 (89%)	8 (57%)	0.018
gentamicin	11 (16%)	0 (0%)	1 (11%)	0 (0%)	0.070
tobramycin	4 (6%)	0 (0%)	0 (%)	0 (0%)	0.361
trimethoprim/ sulfamethoxazole	38 (55%)	1 (10%)	0 (0%)	9 (64%)	<0.001
ciprofloxacin	0 (0%)	0 (0%)	0 (0%)	9 (64%)	<0.001
ciprofloxacin MIC range	≥32 mg/L	≥32 mg/L	4-32 mg/L	0.06-32 mg/L	

Antimicrobial susceptibility of carbapenemase producing isolates

In our study the carbapenem resistance conferred by VIM was low level (Table 6). For the majority of the isolates the MIC values of imipenem and meropenem were near the susceptible clinical breakpoint. The low level of resistance hindered detection by phenotypic inhibition assay as indicated by smaller difference between inhibition zones of meropenem and meropenem + dipicolinic acid (metallo-β-lactamase inhibitor) at lower meropenem MIC (Table 7). This explains why a remarkable portion of isolates (51.0%) was not positive for metallo-β-lactamase production in the phenotypic inhibition assay, despite the production of the VIM enzyme could be demonstrated by the modified Hodge-test. Considering the low level of carbapenem resistance conferred by the VIM enzyme, the usage of meropenem ECOFF value proposed by EUCAST to screen for carbapenemase production, the usage of the modified Hodge-test and the simultaneous testing of susceptibility to the three carbapenem derivatives could be beneficial in the detection of VIM production.

6. Table Susceptibility to carbapenems of VIM producing *K. pneumoniae* isolates (n=101)

	Ertapenem	**Imipenem**	**Meropenem**
range (mg/L)	0.5-32	0.25-32	0.12-32
MIC_{50} (mg/L)	4	2	1
MIC_{90} (mg/L)	32	32	2
susceptible	7 (6.9%)	57 (56.4%)	91 (90.1%)
intermediate	10 (9.9%)	18 (17.8%)	8 (7.9%)
resistant	84 (83.2%)	26 (25.7%)	2 (2.0%)
above ECOFF	101 (100%)	61 (60.4%)	95 (94.1%)

ECOFF = epidemiological cut off value

For serious, life-threatening infections caused by carbapenemase producing *K. pneumoniae* isolates combination therapy should be given. A carbapenem based combination can be considered, if the isolate has a carbapenem MIC ≤4 mg/L. This condition was met for 100/101 (99.0%) of the VIM positive isolates, rendering these compounds to be a considerable choice in combination with other agents like colistin, tigecycline, fosfomycin, chloramphenicol, fluoroquinolones, and aminoglycosides.

7. Table Proportions of metallo-β-lactamase (MBL) positivity in the phenotypic inhibition assay and mean differences between inhibition zones of meropenem and meropenem + inhibitor in relation to meropenem MIC

Meropenem MIC (mg/L)	n	Boronic acid		Dipicolinic acid		Cloxacillin		MBL positive (%)
		mean (mm)	SD	mean (mm)	SD	mean (mm)	SD	
0.12	6	-0.8	1.169	2.2	0.983	0.0	0.693	0.0
0.25	21	0	1.284	2.8	1.209	0.6	1.284	14.3
0.5	21	0.4	1.284	4.6	1.028	1.1	0.944	52.4
1	30	0.6	1.382	4.8	1.315	1.5	1.042	63.3
2	13	0.4	0.870	4.9	1.256	1.2	1.235	69.2
4	8	0.3	1.753	4.9	0.991	1.0	1.512	75.0
32	2	0.5	0.707	6.0	1.414	0.0	0.0	100.0
all	101	0.3	1.300	4.2	1.567	1.0	1.177	49.0

SD = standard deviation

In our study resistance to colistin was rare (1/82) and there were no isolates resistant to tigecycline (Table 8). Despite their good in vitro activity both colistin and tigecycline have drawbacks. Colistin is nephrotoxic and neurotoxic, but recent advances in dosing regimens seem to abate this problem. Tigecycline is approved for just three clinical syndromes (complicated intraabdominal infection, complicated skin and soft tissue infection and community acquired pneumonia) and most importantly lacks indications for treatment of sepsis, ventilator associated pneumonia or urinary tract infections.

Almost two third (65,9%) of the isolates in this study were susceptible to fosfomycin, making it a possible option for treatment of urinary tract infections, but it is only available as a per oral compound for short term treatment in our country, and per oral formulation is only proposed for treatment of uncomplicated urinary tract infections by EUCAST.

High portion of resistance (48.8%) and severe toxicity constrict the possible role of chloramphenicol in the treatment of infections caused by carbapenemase-producing *K. pneumoniae* in our institution.

All isolates were resistant to ciprofloxacin, which indicates resistance to all fluoroquinolone derivatives. Fluoroquinolone resistance in HEC/ST15 was shown to be due to mutations in *gyrA* and *parC* genes by others.

8. Table Susceptibility to non-β-lactam antimicrobial agents of VIM producing *K. pneumoniae* isolates, n=82 (S = susceptible, I = intermediate, R = resistant)

	MIC range	**MIC_{50}**	**MIC_{90}**	**S**	**I**	**R**
ciprofloxacin	-	-	-	0 (0%)	0 (0%)	82 (100%)
gentamicin	-	-	-	6 (7.3%)	0 (0%)	76 (92.7%)
tobramycin	-	-	-	0 (0%)	0 (0%)	82 (100%)
amikacin	4-16 mg/L	8 mg/L	16 mg/L	66 (80.5%)	16* (19.5%)	0 (0%)
chloramphenicol	2-256 mg/L	8 mg/L	64 mg/L	42 (51.2%)	-	40 (48.8%)
colistin	0.5-4 mg/L	1 mg/L	1 mg/L	81 (98.8%)	-	1 (1.2%)
tigecycline	0.03-2 mg/L	0.5 mg/L	2 mg/L	66 (89.5%)	16 (19.5%)	0 (0%)
fosfomycin	4-256 mg/L	16 mg/L	256 mg/L	54 (65.9%)	-	28 (34.1%)

* In the case of six isolates the results for amikacin were modified from susceptible to intermediate as stated in the EUCAST Expert Rule No. 12.7.

The possible usage of aminoglycosides is questioned by the presence of *aac(6')-Ib* in In238b. The AAC(6')-I enzyme is capable of modifying amikacin and tobramycin. All isolates were resistant to tobramycin, but only 10 had amikacin MIC in the non-susceptible range. EUCAST Expert Rule No. 12.7 recommends the modification of amikacin results from susceptible to intermediate when the isolate is tobramycin resistant and gentamicin susceptible in order to indicate the possible modification of amikacin by an AAC(6')-I enzyme. This rule could be applied for six isolates, but for 66 isolates the possible modification of amikacin was not indicated, because the phenotype described in the expert rule was possibly disguised by a gentamicin modifying enzyme.

Based on the results of molecular typing and antimicrobial susceptibility testing it can be affirmed that: if a severe infection by a carbapenemase producing *K. pneumonia*e isolate is suspected in our institution, an imipenem or meropenem plus colistin or tigecycline combination could be applicable as a first-line empiric therapy.

Novel findings

- First report on the presence of CTX-M-15 producing internationally disseminated ST101 high risk *K. pneumoniae* clone in Hungary.
- First detection of VIM-4 metallo-β-lactamase production in ST15 *K. pneumoniae* clone.
- Identification of novel *infB* allele (number: 83) and sequence type (ST1193, allelic profile: 2-83-2-1-9-4-135) in *K. pneumoniae*.
- First comprehensive analysis on trends of β-lactam resistance rates of *K. pneumoniae* at the Clinical Centre University of Pécs.
- First detailed description on dissemination of different multiresistant *K. pneumoniae* clones at the various departments of the Clinical Centre University of Pécs.
- Identification of diversities in virulence associated trait content of multiresistant *K. pneumoniae* clones at the Clinical Centre University of Pécs.

Acknowledgements

First of all I would like to express my sincere gratitude to *Gyula Mestyán* for training me in clinical microbiology and for his continuous professional support.

I would like to thank *Monika Kerényi* and *Levente Emődy* for their guidance throughout my PhD degree acquiring procedures. Moreover I am grateful to *Levente Emődy* for his financial support and thorough reviewing of my manuscripts and abstracts.

I am thankful to my supervisors, *György Schneider* and *Zoltán Tigyi*, for initiating my scientific career; to *István Almási* for introducing me to the every-day practice of infectology; to *Ildikó Denke, Rózsa Lajkó, Marianna Veláncsics* and *Miklós Oldal* for their technical assistance; to my colleagues, *Adrienn Nyul, Krisztina Kovács, Brigitta Patkó* and *Tamás Gám,* for relieving me from routine duties while working on my PhD thesis; and to my fellow PhD students, *Valéria Szijártó, Beáta Kovács, Judit Kovács, Ágnes Dorn, Péter Felső, Mária Kindl* and *Marianna Horváth*, for inspiring me throughout my PhD studies.

Last, but not least, I would like to extended my thanks and show appreciation to my loving partner, *Péter Novák*, and my *caring family* for their selfless help in everyday life.

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