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# IDENTIFICATION AND CHARACTERIZATION OF CTX-M-15 PRODUCING *KLEBSIELLA PNEUMONIAE* CLONE ST101 IN A HUNGARIAN UNIVERSITY TEACHING HOSPITAL

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We investigated the molecular epidemiology of extended spectrum β-lactamase (ESBL) producing *Klebsiella pneumoniae* isolates derived from the teaching hospitals of University of Pécs, Pécs, Hungary in the time period 2004–2008. Molecular typing, antimicrobial susceptibility testing, detection of common β-lactamase genes ($bla_{\text{CTX-M}}$, $bla_{\text{TEM}}$ and $bla_{\text{SHV}}$) and virulence associated traits (hypermucoviscosity, *magA*, *k2a*, *rmpA*, siderophores, type 1 and 3 fimbria, biofilm formation, serum resistance) were performed for 102 isolates. The results showed the presence of three major ciprofloxacin resistant CTX-M-15 producing clones (ST15 n = 69, ST101 n = 10, and ST147 n = 9), of which ST15 was predominant and universally widespread. Considering distribution in time and place, ST101 and ST147 were detected at fewer inpatient units and within a narrower time frame, as compared to ST15. Beside major clones, eleven minor clones were identified, and were shown to harbour the following β-lactamase genes: six clones carried $bla_{\text{CTX-M}}$, four clones harboured $bla_{\text{SHV-5}}$ and one clone possessed both $bla_{\text{CTX-M}}$ and ESBL type $bla_{\text{SHV}}$. Among the SHV-5 producing *K. pneumoniae* clones a novel sequence type was found, namely ST1193, which harboured a unique *infB* allele. Different virulence factor content and peculiar antimicrobial susceptibility profile were characteristic for each clone. In contrast to major clone isolates, which showed high level resistance to ciprofloxacin, minor clone

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isolates displayed significantly lower MIC values for ciprofloxacin suggesting a role for fluoroquinolones in the dissemination of the major *K. pneumoniae* clones. This is the first description of the CTX-M-15 producing *K. pneumoniae* clone ST101 in Hungary.

**Keywords:** ESBL, *K. pneumoniae*, ST101, ST1193, virulence

## Introduction

*Klebsiella pneumoniae* is an opportunistic pathogen, often causing urinary, respiratory and blood stream infections in patients with underlying medical conditions. The number of multiresistant *K. pneumoniae* isolates, including extended spectrum β-lactamase (ESBL) producing ones, is continuously increasing. According to the European Resistance Surveillance Network report altogether 25.7% of invasive *K. pneumoniae* isolates in Europe were resistant to third generation cephalosporins in 2012, of which 62–100% were supposed to produce some sort of ESBL [1]. The most frequently encountered ESBL genes belong to the $bla_{\text{CTX-M}}$, $bla_{\text{TEM}}$ and $bla_{\text{SHV}}$ groups, and their spread is attributed to dissemination of epidemic resistance plasmids and expansion of successful clones [2].

According to nationwide studies, until 2004 the most common ESBL genes in Hungary belonged to the $bla_{\text{SHV}}$ group, and were carried on epidemic resistance plasmids [3–5]. In 2005 the spread of three CTX-M-15 producing epidemic clones (ST11, ST15 and ST147) were detected across the country [6]. One year later the emergence of a fourth epidemic clone (ST274) was observed in various regions of the country [7].

Several studies were engaged with the virulence factors of ESBL producing *K. pneumoniae* isolates, but the results seem to be limited to the specific clones and/or resistance plasmids [8–12].

In order to reveal the local characteristics (clonality, antibiotic susceptibility and virulence factor pattern) we investigated ESBL producing *K. pneumoniae* isolates originating from a single institution (teaching hospitals of University of Pécs, Pécs, Hungary) over a 5-year period (2004–2008).

## Materials and Methods

### *Isolates*

102 ESBL producing *K. pneumoniae* isolates were collected between 2004 and 2008 from human clinical samples (urine n = 57; blood n = 22; lower respira-

tory tract n = 7; wound, pus or wound aspirate n = 10, vascular catheter n = 3, other n = 3) originating from different departments of the Clinical Centre University of Pécs, Pécs, Hungary. The isolates were selected so as to represent the study period regarding time and place of collection. Only one isolate per patient was included. The identification was performed with standard biochemical procedures [13]. Confirmation of ESBL production was accomplished with the combined disc (Bio-Rad, Hercules, CA, USA) method [14].

### *Molecular typing*

Clonal relationships were assessed with pulsed-field gel electrophoresis (PFGE) as described by others [6]. PFGE was evaluated with unweighted pair group method with arithmetic means (UPGMA) using Dice-coefficient. Clones were defined as group of isolates with >85% similar patterns.

Multilocus sequence typing (MLST) was performed for single representatives of seven selected clones (three major clones and four SHV-5 producing minor clones) [15].

### *Detection of β-lactamase genes*

The presence of $bla_{\text{CTX-M}}$, $bla_{\text{TEM}}$ and $bla_{\text{SHV}}$ was investigated by polymerase chain reaction (PCR) [16–18]. Digestion of $bla_{\text{SHV}}$ with *Nhe*I was carried out in order to identify Gly238→Ser mutation associated with the hydrolysis of third generation cephalosporins [19]. Sequencing of $bla_{\text{CTX-M}}$ and $bla_{\text{TEM}}$ genes were performed for single representatives of major clones [17, 20]. The nucleotide sequence of $bla_{\text{SHV}}$ was determined for single representatives of minor clones, where ESBL production was suspected to be solely attributed to SHV-type enzymes [18].

### *Antibiotic susceptibility testing*

Susceptibility to amikacin, ciprofloxacin, gentamicin, tobramycin and trimethoprim/sulfamethoxazole was determined by disc (Bio-Rad, Hercules, CA, USA) diffusion method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards [21].

For minor clone isolates and five representative isolates of major clones minimum inhibitory concentration (MIC) of ciprofloxacin was measured by broth microdilution method, and the results were interpreted according to EUCAST [21].

*Virulence associated traits*

The presence of several factors known or hypothesized to contribute to the virulence of *K. pneumoniae* was investigated. We focused on phenotypic detection in order to verify gene expression. In those cases where phenotypic tests were not available in our department we detected the relevant gene itself by PCR.

Capsular serotypes K1 and K2, which are associated with higher virulence potential than other serotypes, were determined with PCR (*magA* and *k2A,* respectively) [22, 23]. Hypermucoviscosity phenotype was assessed by string-test, and the presence of *rmpA* was detected by PCR [24]. Measurement of susceptibility to human serum bactericidal activity was carried out as described by others [25]. All isolates were tested in triplicates. The ratio of mean number of colony forming units at 60 and 180 minutes after treatment with serum to mean number of colony forming units at 0 minute was evaluated for each isolate. The production of enterobactin and aerobactin was established in cross feeding bioassay [25]. The assay was evaluated after 24 and 48 hours of incubation, and was performed twice for each isolate. PCR was carried out to assess the presence of yersiniabactin *(irp2-1)* and *Klebsiella* ferric iron uptake gene cluster *(kfuB)* [26, 27]. Yeast cell coagglutination in the presence or absence of 1.0% D-mannose was used to detect type 1 fimbria [28, 29]. Expression of type 3 fimbria was evaluated by agglutination of tannic acid treated bovine erythrocytes [25]. All agglutination assays were carried out three times. Microtiter plate biofilm assay was performed in triplicates as described elsewhere [30]. The biofilm formation capacity was approximated by calculating the mean optical density ($OD_{595}$) for each isolate, and isolates with a value of less than 1,000 was considered to be negative in the assay.

*Statistical methods*

In order to test if the three major clones and the minor clones compounded were the same in the aspects of virulence associated factor content and antibiotic susceptibility rates, probability values (p) were calculated with likelihood ratio test for categorical variables and Kruskal–Wallis-test for continuous variables (biofilm, serum resistance). All statistical computations were performed in SPSS 20. Statistical significance was established as $p < 0.05$.

## Results

By PFGE the isolates were grouped into fourteen pulsotypes. The results of MLST and β-lactamase gene detection are presented in Table I. The distribution of the different clones in time and place is shown in Tables II and III. The results and statistical analysis of antimicrobial susceptibility testing and possession of virulence traits are summarised in Tables IV and V. For eight isolates the results of amikacin susceptibility testing was modified from susceptible to intermediate as indicated by EUCAST expert rule No. 12.7 [31].

**Table I.** Results of molecular typing and β-lactamase gene detection

| | Pulsotype (PFGE) | Number of isolates | Result of MLST | β-Lactamase genes detected |
|---|---|---|---|---|
| Major clones | PT-01 | 69 | ST15 | $bla_{SHV}$ (*Nhe*I: negative)<br>$bla_{CTX-M-15}$ |
| | PT-02 | 10 | ST101 | $bla_{SHV}$ (*Nhe*I: negative)<br>$bla_{CTX-M-15}$<br>$bla_{TEM-1}$ (in six isolates) |
| | PT-03 | 9 | ST147 | $bla_{SHV}$ (*Nhe*I: negative)<br>$bla_{CTX-M}$ |
| Minor clones | PT-04 | 2 | – | $bla_{SHV}$ (*Nhe*I: negative)<br>$bla_{CTX-M}$ |
| | PT-05 | 1 | – | $bla_{SHV}$ (*Nhe*I: negative)<br>$bla_{CTX-M}$ |
| | PT-06 | 1 | – | $bla_{SHV}$ (*Nhe*I: negative)<br>$bla_{CTX-M}$ |
| | PT-07 | 1 | – | $bla_{SHV}$ (*Nhe*I: negative)<br>$bla_{CTX-M}$ |
| | PT-08 | 1 | – | $bla_{SHV}$ (*Nhe*I: negative)<br>$bla_{CTX-M}$ |
| | PT-09 | 1 | – | $bla_{SHV}$ (*Nhe*I: negative)<br>$bla_{CTX-M}$ |
| | 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 | – | $bla_{SHV}$ (*Nhe*I: positive)<br>$bla_{CTX-M}$ |

**Table II.** Number of patients infected or colonized by ESBL producing *K. pneumoniae* isolates detected at the Clinical Centre University of Pécs and timely distribution of ESBL producing *K. pneumoniae* clones

| Year of isolation | Number of patients | Number of isolates studied | PT-01 ST15 | PT-02 ST101 | PT-03 ST147 | Minor clones |
|---|---|---|---|---|---|---|
| 2004 | 16 | 2 | 0 | 1 | 0 | 1 |
| 2005 | 34 | 9 | 4 | 5 | 0 | 0 |
| 2006 | 78 | 17 | 12 | 3 | 0 | 2 |
| 2007 | 70 | 47 | 37 | 1 | 2 | 7 |
| 2008 | 74 | 27 | 16 | 0 | 7 | 4 |
| Total | 272 | 102 | 69 | 10 | 9 | 14 |

**Table III.** Number of patients infected or colonized by ESBL producing *K. pneumoniae* isolates detected at the Clinical Centre University of Pécs and spatial distribution of ESBL producing *K. pneumoniae* clones

| Department | Number of patients | Number of isolates studied | PT-01 ST15 | PT-02 ST101 | PT-03 ST147 | Minor clones |
|---|---|---|---|---|---|---|
| Anaesthesia and Intensive Therapy | 22 | 11 | 6 | 0 | 0 | 5 |
| Internal Medicine 1 | 92 | 40 | 27 | 9 | 1 | 3 |
| Internal Medicine 2 | 18 | 5 | 3 | 1 | 1 | 0 |
| Neurology | 23 | 3 | 2 | 0 | 0 | 1 |
| Surgery | 14 | 3 | 3 | 0 | 0 | 0 |
| Urology | 79 | 35 | 27 | 0 | 6 | 2 |
| Other | 24 | 5 | 1 | 0 | 1 | 3 |
| Total | 272 | 102 | 69 | 10 | 9 | 14 |

**Table IV.** Susceptibility to antibiotics of ESBL producing *K. pneumoniae* clones

| | | PT-01 ST15 (n = 69) | PT-02 ST101 (n = 10) | PT-03 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%) | 0.361 |
| trimethoprim / sulfamethoxazole | | 38 (55%) | 1 (10%) | 0 (0%) | 9 (64%) | <0.001 |
| ciprofloxacin | disc diffusion | 0 (0%) | 0 (0%) | 0 (0%) | 9 (64%) | <0.001 |
| | MIC range | ≥32 mg/L | ≥32 mg/L | 4–32 mg/L | 0.06–32 mg/L | |

*P-values were calculated to test whether antimicrobial susceptibility were independent of the clonal grouping.

**Table V.** Virulence trait possession of ESBL producing *K. pneumoniae* clones

| | PT-01 ST15 (n = 69) | PT-02 ST101 (n = 10) | PT-03 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 formation | 67 (97%) | 10 (100%) | 9 (100%) | 8 (57%) | <0.001 |
| biofilm (median of OD) | 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 |

*P-values were calculated to test whether virulence associated trait possession were independent of the clonal grouping.
**n = 68. One isolate showed autoaggregative properties.

## Discussion

Our results show the complexity, and present the local characteristics of molecular epidemiology of ESBL producing *K. pneumoniae* in our institution.

Beside several minor clones, three internationally disseminated CTX-M-15 producing *K. pneumoniae* clones, namely ST15, ST101 and ST147 were present in the Clinical Centre University of Pécs, of which ST15 and ST147 are considered to be epidemic clones in Hungary [6, 32–36].

Until now the presence of ST101 was not reported in Hungary. This sequence type was associated with various β-lactam resistance mechanisms in different parts of the world: CTX-M-15 production in France, Greece and Italy; SHV-12 production in the Czech Republic; OXA-48 production in Spain, Libya, France, Tunisia, Morocco, Switzerland and South Africa; KPC-2 production in Italy and Spain [32, 35–42]. In our institution the presence of CTX-M-15 producing ST101 seemed to be restricted both timely and spatially, as the majority of the isolates originated from a single department with a decreasing incidence (Tables II and III).

Beside $bla_{\text{CTX-M-15}}$ detected in major clones one additional ESBL gene ($bla_{\text{SHV-5}}$) was identified in this study. The $bla_{\text{SHV-5}}$ gene was harboured by four distinct minor clones (ST34, ST113, ST323 and ST1193). Among them ST1193 was identified as a new sequence type (allelic profile: 2-83-2-1-9-4-135), and harboured a novel variant of the *infB* allele (designated as number 83). The new sequence type and *infB* allele were deposited and are publicly available at the MLST database of Institut Pasteur, Paris, France (bigsdb.web.pasteur.fr).

Considering the distribution in time and place (Tables II and III), several clonal characteristics could be assumed. Presumably ST101 was the dominant clone in our institution between 2004 and 2005, and its dissemination was mainly confined to Internal Medicine 1 and 2. ST15 might have emerged during the initial period of our study, and later became the predominant ESBL producing clone of our institution affecting the majority of the departments. The dissemination of ST147 supposedly started around 2007, and since then it has spread to several departments of the Clinical Centre. Minor clones were most commonly detected 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, but 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 (ST11 and ST274) were not observed during our study period, and despite widespread dissemination of 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 [6, 7]. According to our investigations ST15 and CTX-M-15 β-lactamases were the dominant clone and ESBL type in our institution, which matches with national data [6]. Of the SHV-type ESBL genes examined, only $bla_{\text{SHV-5}}$ was found, which is in agreement with the result of a nationwide study [4].

Considering the results of antimicrobial susceptibility testing it can be concluded that non-susceptibility to gentamicin and tobramycin was common for major and minor clone isolates as well. The rate of susceptibility to amikacin and trimethoprim/sulfamethoxazole varied among different clones. While the CTX-M-15 producing major clone isolates were universally resistant to ciprofloxacin, the majority (64%) of minor clones were susceptible to this compound. The rate of susceptibility was even higher (80%), when only SHV-5 producing isolates were taken into account. Corresponding to susceptibility rates, the range of MIC values also showed considerable differences (ST15: ≥32 mg/L; ST101: ≥32mg/L; ST147: 4–32 mg/L; SHV-5 producing minor clones: 0.06–2 mg/L). Dissimilarities in the level of resistance to ciprofloxacin was suggested to be influenced by

variability in fitness cost associated with the acquisition of fluoroquinolone resistance, and it was indicated that SHV-type ESBL plasmids might be lost during the induction of high level fluoroquinolone resistance [43]. Correspondingly high level ciprofloxacin resistance was not observed in SHV-5 producing isolates in our study. The only resistant isolate showed low level resistance (MIC = 2 mg/L), and belonged to ST113.

In our study high rate of type 1 fimbria expression and peculiar siderophore content were characteristic for each clone. All the major clones (ST15, ST101 and ST147) showed high biofilm forming capacity. ST147 was the least susceptible clone to bactericidal activity of human serum. 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 [8–12, 44, 45]. Our results also imply that the distribution of virulence associated traits might be diverse among different ESBL producing *K. pneumoniae* clones.

The ongoing dissemination of internationally successful multiresistant clones and epidemic resistance plasmids in addition to emergence of new antibiotic resistance mechanisms make continuous surveillance and study of molecular epidemiology of multiresistant bacteria mandatory.

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## Conflict of Interest

None.

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