Countrywide dissemination of a DHA-1-type plasmid-mediated AmpC β-lactamase-producing Klebsiella pneumoniae ST11 international high-risk clone in Hungary, 2009–2013

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The first plasmid-mediated AmpC β-lactamase-producing Klebsiella pneumoniae (pAmpC KP) isolate was detected in December 2009 in Hungary. Hungarian microbiological laboratories were asked to send all KP strains showing cefoxitin resistance and decreased susceptibility or resistance to any third-generation cephalosporins to the Reference Laboratories at the National Center for Epidemiology. Investigation was conducted in order to outline spatio-temporal distribution and genetic characterization of pAmpC-KP isolates in Hungary. Between December 2009 and December 2013, 312 consecutive KP clinical isolates were confirmed as producing pAmpCs. All isolates showed resistance to third-generation cephalosporins, aminoglycosides and fluoroquinolones, and 77 % were non-susceptible to at least one carbapenem. Analysis of β-lactamase genes showed blaDHA-1 in all and additionally blaCTX-M-15 in 90 % of isolates. PFGE typing revealed 12 pulsotypes; of these, KP053 (262/312) and KP070 (38/312) belonged to sequence type ST11 and comprised 96 % of the isolates. The blaDHA-1 and blaCTX-M-15 co-producing KP053/ST11 clone affected 234 patients and spread to 55 healthcare centres across Hungary during the study period. Three KP053 isolates were also resistant to colistin. In two of these, the mgrB gene was truncated by IS10R, while in the third isolate, insertional inactivation of mgrB by ISKPn14 was identified. Hungary is the first European country showing endemic spread of blaDHA-1 facilitated by the international high-risk clone ST11. The rapid countrywide spread of this multidrug-resistant clone seriously endangers Hungarian healthcare facilities and warrants strengthening of infection control practices and prudent use of carbapenems and colistin.

INTRODUCTION

Klebsiella pneumoniae (KP) is still one of the leading causes of community-acquired pneumonia and pyogenic liver abscess in some countries and, as an opportunistic pathogen, it is responsible for severe nosocomial infections and large nosocomial outbreaks primarily in neonatal and adult intensive care units (Podschun & Ullmann, 1998). KP strains represent considerable epidemic potential and are a major source of horizontal spread of antimicrobial resistance. The multifarious acquired β-lactamase-producing KP are among the most common multi-resistant Gram-negative bacteria not only in Hungary but also globally (Ramirez et al., 2014).

AmpC type β-lactamases are cephalosporinases belonging to the Ambler-type C molecular class β-lactamases and are generally not inhibited by clavulanic acid. They are mainly chromosomally encoded, but plasmid-encoded versions more often occur (Bush et al., 1995; Jacoby, 2009). pAmpCs were reported for the first time in 1989 (Bauernfeind et al., 1989). Based on differences in the amino acid sequences, seven enzyme families are currently described: CMY, FOX, ACC, LAT, MIR, ACT, MOX and DHA (Jacoby, 2009). The pAmpC enzymes are usually constitutively produced (rarely inducible, e.g. ACT-1, DHA-1, DHA-2) and confer resistance...
to penicillins, broad-spectrum cephalosporins, cefamycins and variably to aztreonam, and usually remain sensitive to ceftazidime and carbapenems (Jacoby, 2009). They often occur together with other resistance genes (e.g. conferring resistance to aminoglycosides, chloramphenicol, quinolones, sulfonamides, tetracyclines and numerous other β-lactamases, such as TEMs, PSEs, CTX-Ms, SHVs, VIMs).

Although less frequent than extended-spectrum β-lactamases (ESBLs), the pAmpCs are globally distributed (Jacoby, 2009; EUCAST, 2013b). CMY-2 showed the widest distribution and susceptibility to antimicrobials (Jacoby, 2009). They often occur together with other resistance genes (e.g. conferring resistance to aminoglycosides, chloramphenicol, quinolones, sulfonamides, tetracyclines and numerous other β-lactamases, such as TEMs, PSEs, CTX-Ms, SHVs, VIMs).

Methods

Patients and bacterial isolates. All Hungarian microbiological laboratories were asked, using the official Microbiological Circular of NCE in 2010, to send all KP strains during 2009 period and (ii) to identify phylogenetic relationships between isolates using molecular typing.

Antibiotic susceptibility testing and resistance determinants. Antimicrobial susceptibility testing to ceftazidime, cefotaxime, FOX, ertapenem (ETP), imipenem (IMP), meropenem, ciprofloxacin (CIP), amikacin (AMK), gentamicin (GEN) and tobramycin was performed using the standard disk diffusion method (www.eucast.org) or to colistin (COL) by using the MIC gradient test (bioMérieux). Results were interpreted according to the EUCAST guidelines (EUCAST, 2013a). The putative pAmpC-KP isolates were preliminarily identified using the following phenotypic criteria: resistance to FOX and non-susceptibility to at least one third-generation cephalosporin.

Phenotypic confirmation of the putative AmpC-producing isolates was performed by ESBL combined disk test and ESBL&AMP ID test (Mast Diagnostics). The presence of pAmpC encoding genes was verified using PCR (Pérez-Pérez & Hanson, 2002) and sequencing.

For detection of alterations in the mcrB region causing COL resistance, PCR and sequencing of amplicons were performed as previously described by Olaitan et al. (2014) and Cheng et al. (2015). Insertion sequences (ISs) in donor isolates were analysed using the IS finder web tool (https://www.is.biotoul.fr/), while searching for such sequences in the GenBank database of the National Center for Biotechnology Information (NCBI). Levels of similarity between ISs were assessed using the Basic Local Alignment Search Tool (BLAST). Levels of similarity were calculated using the Dice coefficient, and unweighted pair group method with arithmetic averages was used for the cluster analysis of the pAmpC-KP isolates within the species K. pneumoniae.

The presence of genes encoding for 16S rRNA methylases (mttB, mttC and arnA) was confirmed using multiplex PCR as described previously by Cuzon et al. (2010). The presence of pAmpC-KP isolates selected according to their plasmid content, and PT-E. coli K12 J5-3− and E. coli DH5α were used as recipients (Sambrook et al., 1989).

Main plasmid incompatibility groups including F, FIA, FIB, FIC, H11, H12, H11-1c, L/M, N, P, T, A/C, K, B/O, X, Y and FII were determined using the PCR-based replicon typing schemes as described by Carattoli et al. (2005), Villa et al. (2010) and Poirel et al. (2012).

Data analysis. For statistical analysis, chi-square or Fisher’s exact test was performed in SPSS 17.0 for Windows. There were two approaches used for statistical tests: (i) test for independence between two
variables (gender or antibiotic susceptibilities compared to two epidemic clones); and (ii) to test for equality of proportions between two groups (clinical samples or age groups compared to two epidemic clones). *P*<0.05 was considered significant.

**RESULTS**

**Molecular typing of isolates**

The 312 consecutive isolates originated from 275 patients who attended 60 HCU[s hospitals and general practitioner (GP) clinics] around the entire country.

All investigated isolates were pAmpC producers; 86 isolates were collected in 2010, 93 in 2011, 72 in 2012 and 60 in 2013. The only pAmpC β-lactamase identified was the DHA-1-type, and in the majority of isolates (*n* = 264, 85%) it was accompanied by CTX-M-15-type ESBL. Fourteen different PTs were identified; of these, 12 were represented by one single isolate (S PT, Z PT, KP055, KP071, KP073, KP079, KP102, KP119, KP122, KP180, KP184 and KP189), while KP053 and KP070 PTs accounted for 84% (*n* = 262) and 12% (*n* = 38) of all isolates, respectively. Both KP053 and KP070 PTs belonged to ST11. For further investigations, we focused on these two PTs. Fig. 1 shows the distribution of KP053 and KP070 PTs by year.

**Demographic characteristics**

The median age of patients was 59.4 years (range 0–100 years), and 55% of these were male. A similar gender distribution (*P* = 0.604) was observed when comparing the two patient groups to KP053 and KP070 isolates (Table 1). Fifty percent of KP070 isolates originated from infants (0–1 years, *n* = 20) from a nosocomial cluster, while KP053 was only found in four patients (1.5%) within this age group. The KP053 and KP070 isolates were recovered from lower respiratory sputum (19 and 2), upper respiratory sputum (17 and 0), blood/cerebrospinal fluid (CSF) (33 and 6), wounds (30 and 6), stools (63 and 15), urine (92 and 8) and other specimen types (8 and 1), respectively.

Twenty-seven patients had two isolates and three patients had three isolates each during the study period, which did not comply with the exclusion criteria (Table S1, available in the online Supplementary Material). The median age of these patients was 64.5 years (range 0–86), and 57% of them were male. The average time between two isolations of pAmpC KP from the same patient was 55 days (range 0–644 days). From twenty-five patients only KP053 isolates were obtained, one patient had two isolations of KP070, while for four patients, besides KP053, their second DHA-1-producing isolates belonged to several PTs (Z PT, KP070, KP119 and KP122) (Table S1).

**Spatio-temporal distribution of pAmpC-producing isolates**

The majority of the KP070 isolates (33/38) were recovered from two large tertiary hospitals. Thirteen isolates – all from adult patients – originated from a hospital in Fejér county (FJ1) between 2010 and 2012 (Fig. 2a–d). Twenty isolates were recovered from a hospital in Szabolcs-Szatmár-Bereg county (SS1) in 2013, where 19 of them were from patients in the neonatal intensive care unit and 1 from an adult patient in surgery ward. The remaining five isolates originated from small hospitals and GP practices located in the surroundings of these two tertiary hospitals.

The KP053 PT accounted for 262 isolates during the 4-year study period and spread to 55 hospitals and healthcare facilities (of these, 25 are located in Budapest city) in 9 of 19 Hungarian counties (Fig. 2a–d). The first and only pAmpC-KP isolate recovered in 2009 belonged to KP053 PT and was isolated from a patient attended a large tertiary specialized teaching hospital in Budapest (BP1).

In 2010, a total of 69 KP053 isolates were recovered. Of these 31 were from BP1 and 38 originated from 15 further hospitals mainly in Budapest city (nine HCU[s] (Fig. 2a). During 2011, this PT disseminated to 14 more hospitals and GP clinics located in the middle and in the north-eastern part of the country with 24 isolates. In total, 84 isolates were recovered in that year (Fig. 2b). During 2012 and 2013, a further 70 and 38 KP053 PT isolates were confirmed, respectively, of which 20 were from 12 other HCU[s] in 2012 and 20 from 13 additional HCU[s] (of these, five were GP clinics) in 2013 (Fig. 2c, d).

**Susceptibility to antimicrobials**

All KP053 and KP070 KP isolates showed resistance to third-generation cephalosporins and CIP and the majority (97.6%) to any of three investigated aminoglycosides. Seventy-seven per cent were non-susceptible to at least one carbapenem. The KP053 PT showed a significantly higher non-susceptibility rate to ETP (*P* = 0.001), IMP (*P* = 0.015), GEN (*P* < 0.001) and AMK (*P* < 0.001) compared to KP070.
PT (Table 1). Additionally, significant differences were also observed with CTX-M-15-type ESBL production (the only ESBL identified among isolates) (KP053 PT 94.3 % vs KP070 PT 15.8 %; \( P < 0.001 \)) and between ESBL production and resistance to GEN \( (P < 0.001) \).

Regarding isolates from the same patient showing identical PTs, different antibiotic susceptibility patterns were obtained for isolates from 11 patients, and susceptibility to carbapenems varied (Table S1).

One hundred and eight DHA-1-type and CTX-M-15-type \( \beta \)-lactamase co-producing isolates showed resistance to all aminoglycosides investigated. Of these, 20 isolates were randomly selected for 16S rRNA methylase PCR, but none of these yielded positive result.

Three KP053 KP isolates showed resistance to COL (MIC range 8–32 mg l\(^{-1}\)), and all harboured alterations in their \( mgrB \) region. Truncation of the \( mgrB \) gene by IS\( 10R \), a 1329 bp IS belonging to the IS\( 4 \) family, was found between nucleotide positions +76 and +77 in two isolates, while insertion inactivation of \( mgrB \) by IS\( Kn14 \), a 768 bp IS belonging to the IS\( 1 \) family, was identified between nucleotide positions −16 and −15 in one isolate when referring to the start codon of \( mgrB \) as +1.

### Plasmid replicon typing and transfer of resistance determinants

The mating assays and electroporation were performed on randomly selected KP053 \((n=7)\) and KP070 \((n=3)\) isolates. Only the electroporation of CTX-M-15 \( \beta \)-lactamase harbouring plasmid was successful for three KP053 isolates. The PCR-based plasmid replicon typing showed that the selected isolates were positive only for Inc\( FII_k \) plasmid irrespective of the presence of ESBL, while those isolates subjected to electroporation carried non-typeable CTX-M-15 plasmids. The results of the plasmid profile analysis combined with those results of plasmid replicon typing suggested that the \( \text{bla}_{DHA-1} \) gene is located on an approximately 170 kbp-size Inc\( FII_k \) plasmid, while the \( \text{bla}_{CTX-M-15} \) gene is on an approximately 90 kbp-size Inc\( NT \) plasmid. PCR searching for IS elements revealed the presence of both IS\( 10R \) and IS\( Kn14 \) in each of the donor KP053 isolates and their electroporants (containing only \( \text{bla}_{CTX-M-15} \)-bearing plasmids).
IncNT plasmid). KP070 isolates showed no amplicons for the sought IS elements.

**DISCUSSION**

This is the first comprehensive study on the spatio-temporal distribution of pAmpC β-lactamases in Hungary. Grundmann et al. (2010) proposed a five-stage epidemiological scale from 0 (no cases reported) to 5 (endemic situation) in order to categorize the nationwide expansion of healthcare-associated carbapenem-non-susceptible Enterobacteriaceae. Using this categorization, Freitas et al. (2014) classified the pAmpC epidemiological situation internationally. Based on this scheme, Hungary is the first European country showing endemic spread of *bla*<sub>bla</sub>DHA-1, which was facilitated by the international high-risk clone ST11. The first ST11 strain was isolated in 1997 in France and was assigned to ST11 in 2005 after development of the MLST scheme. In Hungary, MLST was performed in the same year for the first time for identification of MDR KP epidemic clones so as to provide evidence for the existence of potential genetically stable clones among the KP population. In 2005, three epidemic clones were identified in Hungary, which spread successfully in and among Hungarian healthcare facilities, and ST11 was identified as one of those clones (Damjanova et al., 2008). During subsequent years, MDR KP strains were extensively typed worldwide by MLST and it became evident that ST11 was a widespread MDR clone in several countries and continents. In 2011, ST11 was considered a ‘high-risk’ international clone and today it is assessed as one of the most dangerous microorganisms in hospital settings, owing to its high-level antibiotic resistance and virulence potential (Woodford et al., 2011).

The explosive spread of the DHA-1-producing KP ST11 clone in Hungary occurred between 2010 and 2012. The process of expansion appeared to be both clonal and lateral, similar to our previous investigations on the nationwide spread of CTX-M-15-type β-lactamase via three MDR KP epidemic clones (Damjanova et al., 2008). The *bla*<sub>bla</sub>DHA-1 encoding plasmids that occurred in KP053 and KP070 PTs

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**Fig. 2.** Spatio-temporal distribution of DHA-1-producing KP053 and KP070 PTs.
were also found in 12 other isolates belonging to 12 different PTs, which indicated sufficient evidence for horizontal gene transfer. This is be supported by the simultaneous identification of KP053 and KP122 from a single blood sample from a patient. On the other hand, from the identification and monitoring of KP053/ST11, we witnessed the clonal propagation of an MDR strain that became a nationwide epidemic clone over 4 years (2009–2013). Moreover, the vast majority of KP053 isolates were also CTX-M-15 producers, which could additionally promote their spread. This assumption is be supported by the fact that the ESBL-non-producing KP070, which belongs to the same ST11 sequence type, spread to only two hospitals and in small numbers during the study period. Additionally, Shin & Ko (2014) studied the genotype and virulence differences between non-ESBL- and ESBL-producing KP ST11 and found that the plasmid harbouring the blaCTX-M-15 gene confers virulence and antimicrobial resistance; thus, the CTX-M-15-producing clones may have a selective advantage without any existing antibiotic pressure. KP053 is the first DHA-1-producing epidemic clone in the international literature.

Both vertical and horizontal dissemination of blaDHA_1 gene have also been reported; in the Czech Republic, 15 DHA-1-producing KP isolates belonged to ST11 and co-produced SHV-5 ESBL (Chudácková et al., 2010; Empel et al., 2010). In contrast, in Spain, 26 DHA-1-producing KP isolates belonged to eight different STs (of these, three isolates co-produced CTX-M-15-type ESBL and belonged to ST326) (Diestra et al., 2010).

The national spread of KP053 PT was probably promoted by the transfer of patients, mainly from the BP1 centre – where the first isolate was identified in November 2009 – to other HCUs in the country. This is supported by the identification of two – indistinguishable by PFGE – KP053 isolates from urine samples of the same patient (P4) where the first isolate was recovered in the BP1 centre, while the second was recovered 2 months later in another hospital. Moreover, KP053 was continuously isolated from the BP1 centre during the study period, and in total, 48 isolates were identified. Another alarming observation was a probable 644-day carriage of KP053/ST11 – in two urine samples taken from a patient in August 2010 and in May 2012, where isolates showed 100% similarity in their macrorestriction profiles (Table S1). Additionally, 16 KP053 isolates were submitted from GPs from all nine affected counties and Budapest city. These findings suggested that the MDR KP053/ST11 epidemic clone has recently spread into the community, and this is of considerable public health concern.

Plasmid replicon typing of selected KP070 PT and KP053 PT and electroporants of KP053 PT suggested that the blaDHA_1 gene resided on an IncFIIK plasmid of approximately 170 kbp in size in both PTs. The IncF plasmids play an important role in the dissemination of antibiotic resistance and virulence factors among Enterobacteriaceae strains. Among these, the IncFIIK plasmids are common to KP species (Villa et al., 2010). In Europe, the DHA-type AmpC genes were detected mainly on IncL/M plasmids (Compain et al., 2014; Mata et al., 2011), while in South Korea and China, they were found on IncFIIKs and IncFII-plasmids, respectively (Tamang et al., 2008; Guo et al., 2012). To the best of our knowledge, there is only one publication in Europe where the DHA gene was found on the IncFIIK plasmid in one KP isolate in Spain (Compair et al., 2014).

The high carbapenem non-susceptibility found among our DHA-1-producing KP053/ST11 KP isolates is a cause for concern. The reliability of our observations is supported by data from Japan, where Matsumura et al., (2015) reported 30% carbapenem resistance among 27 DHA-1-producing ST11 KP outbreak isolates. The frequent occurrence of carbapenem non-susceptibility among DHA-producing KP isolates is associated mainly with the loss of OmpK36 and OmpK35 outer membrane proteins (Shin et al., 2012, Tsai et al., 2011). According to Shin et al. (2012), clinical outcomes of infections caused by these micro-organisms were very poor, with an extremely high crude mortality rate. The loss of OmpK36 plays a key role in the development of carbapenem non-susceptibility, while the loss of Omp35 in a supplementary role in this process. It is interesting that the loss of these outer membrane proteins leads to the development of reduced susceptibility to carbapenems only in strains producing cephalosporinase (ESBL and/or pAmpC) (Tsai et al., 2011).

The three KP053 PT isolates that showed resistance to COL were not confined to one healthcare facility; they originated from three different centres and were isolated throughout the study period. Furthermore, the macro-restriction profiles of these isolates demonstrated 87% similarity which suggested that mutations leading to COL resistance in different members of KP053 PT occurred independently, as opposed to being enhanced by the spread of a particular subtype. This is a much more alarming situation, as it appears that the KP053 subtype of ST11 was able to develop COL resistance on several occasions. The worldwide emergence of COL resistance owing to inactivation of the PhoP/PhoQ regulator mgrB was recently described in KP (Olaitan et al., 2014) and even in ST11 clone in Taiwan where, similarly to our findings, the IS10R was found between +68 and +76 nucleotide positions in the coding region of mgrB (Cheng et al., 2015). The ISKpn14 element was rarely found as inactivator of the mgrB gene. There is one CTX-M-15-type ESBL-producing ST101 KP isolate from Colombia (Poirel et al., 2015) where the ISKpn14 truncated the mgrB gene between +127 and +128 nucleotide positions and another KPC-2-producing ST147 KP isolate from Greece (Cannatelli et al., 2014) where this insertion element was found at the +124 nucleotide position. To our knowledge, we found for the first time ISKpn14 in a DHA-1 pAmpC-producing ST11 KP isolate and in the promoter region of the mgrB gene. It is of special interest that both IS elements involved in the emergence of COL resistance among
KP053/ST11 isolates resided on the CTX-M-15 harbouring plasmid. It seems that the maintenance of CTX-M-15-producing plasmids is highly beneficial for their bacterial host when survival of the cell is additionally promoted by resistance genes and IS elements. These findings permit deeper insight into trans-hierarchical interactions of evolutionary individuals involved in antibiotic resistance evolution dynamics (Campos et al., 2015).

However, our study has some limitations. During the study period, ertapenem non-susceptibility testing was recommended for screening of putative carbapenemase-producing Enterobacteriaceae isolates in Hungary, which is a very sensitive method with very low specificity (EUCAST, 2013b). Many isolates were received to confirm the suspected carbapenemase production and, based on this selection bias, it can be assumed that carbapenem non-susceptibility was slightly overestimated in our strain collection.

In Hungary, submission of isolates to the National Reference Laboratory for investigation of resistance mechanisms was not mandatory; thus, submission of isolates is variable between laboratories and could also cause selection bias.

In conclusion, our results will contribute towards the knowledge on population dynamics, molecular epidemiology and antibiotic resistance of one of the most important nosocomial pathogens in Hungary and worldwide. Furthermore, the data could help clinicians, infectologists and infection control specialists in overcoming the extremely worrisome Hungarian situation. The rapid and widespread dissemination of pAmpC KP poses a serious threat to Hungarian healthcare institutions, warranting continuous monitoring to control spread. Moreover, it is very important to inform other European countries about this endemic situation in order to prevent probable cross-border transmission of this carbapenem-non-susceptible DHA-1 and CTX-M-15 co-producing KP ST11 high-risk clone and possible European or intercontinental outbreaks.

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REFERENCES


