AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

MECHANISMS OF RESISTANCE

Characterization of KPC-Encoding Plasmids from *Enterobacteriaceae* Isolated in a Czech Hospital

Rudolf Kukla,[a] Katerina Chudejova,[b,c] Costas C. Papagiannitsis,[a,b,c] Matej Medvecky,[d,e] Katerina Habalova,[a] Lenka Hobzova,[f] Radka Bolehovska,[g] Lenka Pliskova,[g] Jaroslav Hrabak,[b,c] Helena Zemlickova[a,h]

[a]Department of Clinical Microbiology, University Hospital and Faculty of Medicine in Hradec Kralove, Charles University, Hradec Kralove, Czech Republic

[b]Department of Microbiology, Faculty of Medicine and University Hospital in Pilsen, Charles University, Pilsen, Czech Republic

[c]Biomedical Center, Faculty of Medicine, Charles University, Pilsen, Czech Republic

[d]Veterinary Research Institute, Brno, Czech Republic

[e]National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Brno, Czech Republic

[f]Department of Hospital Hygiene, University Hospital, Hradec Kralove, Czech Republic

[g]Department of Clinical Biochemistry and Diagnostics, University Hospital, Hradec Kralove, Czech Republic

[h]National Reference Laboratory for Antibiotics, National Institute of Public Health, Prague, Czech Republic

ABSTRACT Ten *Enterobacteriaceae* isolates collected in a Czech hospital carried bla_{KPC}-positive plasmids of different sizes (~30, ~45, and ~80 kb). Sequencing revealed three types of plasmids (A to C) with the Tn*4401a* transposon. Type A plasmids comprised an IncR backbone and a KPC-2-encoding multidrug resistance (MDR) region. Type B plasmids were derivatives of type A plasmids carrying an IncN3-like segment, while type C plasmids were IncP6 plasmids sharing the same KPC-2-encoding MDR region with type A and B plasmids.

KEYWORDS *Citrobacter freundii*, Tn*4401a*, IncR, ST18, Illumina sequencing

KPC-type β-lactamases comprise a distinct group of plasmid-borne enzymes, with carbapenemase activity mainly occurring in *Klebsiella pneumoniae*. KPC-producing *Enterobacteriaceae* have emerged as challenging pathogens causing health care-associated infections, due to their extremely drug-resistant phenotypes and ability to cause infections associated with high mortality (1). KPC producers have disseminated worldwide and currently constitute an important public health problem (2). In Europe, Greece and Italy are the most affected countries, with high proportions of KPC-producing *K. pneumoniae* (3). In the Czech Republic, however, the occurrence of KPC producers has been rare. A sporadic case of KPC-2-producing *K. pneumoniae* recovered from a patient, who had been previously hospitalized in Greece, was detected in the Czech Republic in 2009 (4). Additionally, in 2011, an outbreak of KPC-3-producing *K. pneumoniae* was observed in another Czech hospital (5), with the index case being a patient repatriated from Italy.

In the present study, we describe the molecular characterization of KPC-2-producing *Enterobacteriaceae* isolates, mainly of the species *Citrobacter freundii*, recovered in the University Hospital of Hradec Kralove (Czech Republic).

From 2014 until 2016, a total of 10 nonrepetitive *Enterobacteriaceae* isolates showing carbapenemase activity on matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) meropenem hydrolysis assay (6) were detected. Among them, 7 of the isolates were identified to be *C. freundii*, 1 was identified to be *K. pneumoniae*, 1 was identified to be *Escherichia coli*, and 1 was identified to be *Morganella morganii* (Table 1). Phenotypic testing, PCR screening, and sequencing (7) showed that all isolates were positive for the presence of the bla_{KPC-2} gene. The 10

Received 18 October 2017 **Returned for modification** 9 November 2017 **Accepted** 2 December 2017

Accepted manuscript posted online 20 December 2017

Citation Kukla R, Chudejova K, Papagiannitsis CC, Medvecky M, Habalova K, Hobzova L, Bolehovska R, Pliskova L, Hrabak J, Zemlickova H. 2018. Characterization of KPC-encoding plasmids from *Enterobacteriaceae* isolated in a Czech hospital. Antimicrob Agents Chemother 62:e02152-17. https://doi.org/10.1128/AAC.02152-17.

Address correspondence to Costas C. Papagiannitsis, c.papagiannitsis@gmail.com.

R.K. and K.C. contributed equally to this work.

TABLE 1 Characteristics of KPC-2-producing *Enterobacteriaceae* isolates

Species and isolate	Hospital department[a]	Isolation date	Clinical material	ST[b]	Size of bla_{KPC} plasmid (kb)	Type of plasmid sequence (replicon)	MIC (μg/ml) of[c]:													
							Ctx	Caz	Fep	Atm	Imp	Mem	Etp	Gen	Amk	Tob	Sxt	Cip	Col	Tgc	Fos
C. freundii																					
Cfr-27569	ICU	11/2014	Urine	18	46.826	A (IncR)	>8	>16	>16	>16	16	>16	>4	>32	16	>8	>64	>8	0.25	0.25	≤2
Cfr-31260	ICU	09/2015	Urine	18	46.826	A (IncR)	>8	>16	>16	>16	>32	>16	>4	>32	32	>8	>64	>8	0.12	0.25	≤2
Cfr-31816	ICU	10/2015	Urine	18	46.826	A (IncR)	>8	>16	>16	>16	>32	16	4	>32	>32	>8	>64	>8	0.12	0.12	≤2
Cfr-33038[d]	ICU	11/2015	Rectal swab	18	46.826	A (IncR)	>8	>16	>16	>16	>32	>16	>4	>32	16	>8	>64	>8	4	1	8
Cfr-33795	ICU	04/2016	Urine	142	30.051	C (IncP6)	>8	>16	>16	>16	4	8	4	4	32	>8	>64	4	0.12	0.5	≤2
Cfr-36049	ICU	09/2016	Wounds	87	81.348	B (IncR and IncN3)	>8	>16	>16	>16	8	8	>4	1	16	>8	1	≤0.06	1	0.06	≤2
Cfr-36808	HD	11/2016	Rectal swab	18	46.826	A (IncR)	>8	16	16	>16	12	16	>4	>32	>32	>8	>64	>8	0.25	0.12	≤2
K. pneumoniae																					
Kpn-35786[d]	ICU	09/2016	Catheter	11	46.826	A (IncR)	>8	>16	>16	>16	>32	>16	2	>32	16	>8	>64	>8	>16	1	>128
E. coli																					
Eco-36682[d]	ICU	11/2016	Catheter	216	81.348	B (IncR and IncN3)	4	4	2	>16	32	4	2	2	>32	>8	0.06	≤0.06	≤0.06	0.25	≤2
M. morganii																					
Mmo-37590[d]	ICU	12/2016	Urine	NA	30.051	C (IncP6)	>8	8	>16	>16	16	8	1	2	32	8	>64	8	>16	0.5	>128

[a]ICU, intensive care unit; HD, hematology department.

[b]ST, sequence type; NA, not applicable.

[c]Ctx, cefotaxime; Caz, ceftazidime; Fep, cefepime; Atm, aztreonam; Imp, imipenem; Mem, meropenem; Etp, ertapenem; Gen, gentamicin; Amk, amikacin; Tob, tobramycin; Sxt, trimethoprim-sulfamethoxazole; Cip, ciprofloxacin; Col, colistin; Tgc, tigecycline; Fos, fosfomycin.

[d]KPC-2-like-producing isolates recovered from the same patient.

KPC-2-producing isolates were recovered from 7 patients, 6 of which were hospitalized in the same unit (Table 1). In addition, 5 of the patients had overlapping stays in several combinations, suggesting transmission of KPC-2 producers.

Susceptibility to various antimicrobial agents was determined by the broth dilution method (8). MICs, interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (http://www.eucast.org/), showed that all KPC-2 producers exhibited resistance to aminopenicillins, aminopenicillin-inhibitor combinations (data not shown), cephalosporins, and aztreonam, and were nonsusceptible to carbapenems. Additionally, KPC-2 producers also exhibited resistance to several non-β-lactam antibiotics, whereas all isolates remained susceptible to tigecycline (Table 1).

The population structure of KPC-2-producing isolates studied by multilocus sequence typing (MLST) (9–11) is shown in Table 1. The *C. freundii* isolates comprised three sequence types (STs). ST18 was the most prevalent, accounting for five isolates. ST18 was previously found among NDM-1-producing isolates from Denmark and VIM-1-producing isolates from Spain (12, 13). The *K. pneumoniae* isolate was assigned to the high-risk clone ST11, previously associated with the production of several carbapenemases (14), while the *E. coli* isolate belonged to ST216.

None of the clinical isolates was capable of transferring the bla_{KPC-2} gene to the *E. coli* A15 laboratory strain by conjugation. Plasmid DNAs from clinical isolates were extracted using a Qiagen maxikit (Qiagen, Hilden, Germany) and used to transform *E. coli* DH5α cells. Transformants were selected on Luria-Bertani agar plates with ampicillin (50 μg/ml), confirmed to be KPC-2 producers by PCR (7), and MALDI-TOF MS meropenem hydrolysis assay (6), and tested for antimicrobial susceptibility (see Table S1 in the supplemental material). The plasmid location of the bla_{KPC-2} genes was demonstrated by S1 nuclease analysis of clinical and recombinant strains (15), followed by hybridization with a digoxigenin-labeled bla_{KPC} probe. Plasmid analysis revealed the transfer of plasmids, most of which ($n = 6$) were ~45 kb in size. The remaining plasmids were ~80 kb ($n = 2$) or ~30 kb ($n = 2$) in size. Replicon typing showed that eight of the plasmids, including those ~45 kb and ~80 kb in size, were positive for the IncR replicon (16) (Table 1), whereas the two remaining plasmids were nontypeable by the PCR-based replicon typing (PBRT) method (17, 18).

Plasmid DNAs from all KPC-2-producing transformants were extracted using a Qiagen large-construct kit (Qiagen, Hilden, Germany). Multiplexed plasmid DNA libraries were prepared using the Nextera XT library preparation kit, and 300-bp paired-end sequencing was performed on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) using the MiSeq v3 600-cycle reagent kit. Initial paired-end reads were quality trimmed using the Trimmomatic tool v0.33 (19) with a sliding window size of 4 bp, required average base quality ≥17, and minimum read length of 48 bases. For assembly of the plasmids, reads were mapped to the reference *E. coli* strain K-12 substrain MG 1655 genome (GenBank accession no. U00096) using the BWA-MEM algorithm (20), in order to filter out the chromosomal DNA. Then, all of the unmapped reads were assembled by use of the de Bruijn graph-based *de novo* assembler SPAdes v3.9.1 (21), using k-mer sizes 21, 33, 55, and 77. *De novo* assembly resulted in sets of contigs with length-weighted average k-mer coverage ranging from 23× to 95×. The sequence gaps were filled by a PCR-based strategy and Sanger sequencing. For sequence analysis and annotation, the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), the ISfinder database (www-is.biotoul.fr/), and the ORF (open reading frame) Finder tool (www.bioinformatics.org/sms/) were utilized. Comparative genome alignments were performed using the Mauve (version 2.3.1) program (22).

Plasmid analysis revealed three types of bla_{KPC-2}-carrying plasmid sequences (types A to C; Table 1), with type A being the most prevalent. All plasmids contained the Tn*4401a* isoform of the Tn*4401* transposon, which is similar to that described in plasmid pNYC, lacking 100 bp upstream of bla_{KPC-2} (23).

All bla_{KPC-2}-carrying plasmids that were ~45 kb in size belonged to type A and showed high degrees of similarity to each other. The plasmids included a contiguous segment of 12,036 bp (nucleotide [nt] 1 to 10294 and 45085 to 46826; GenBank

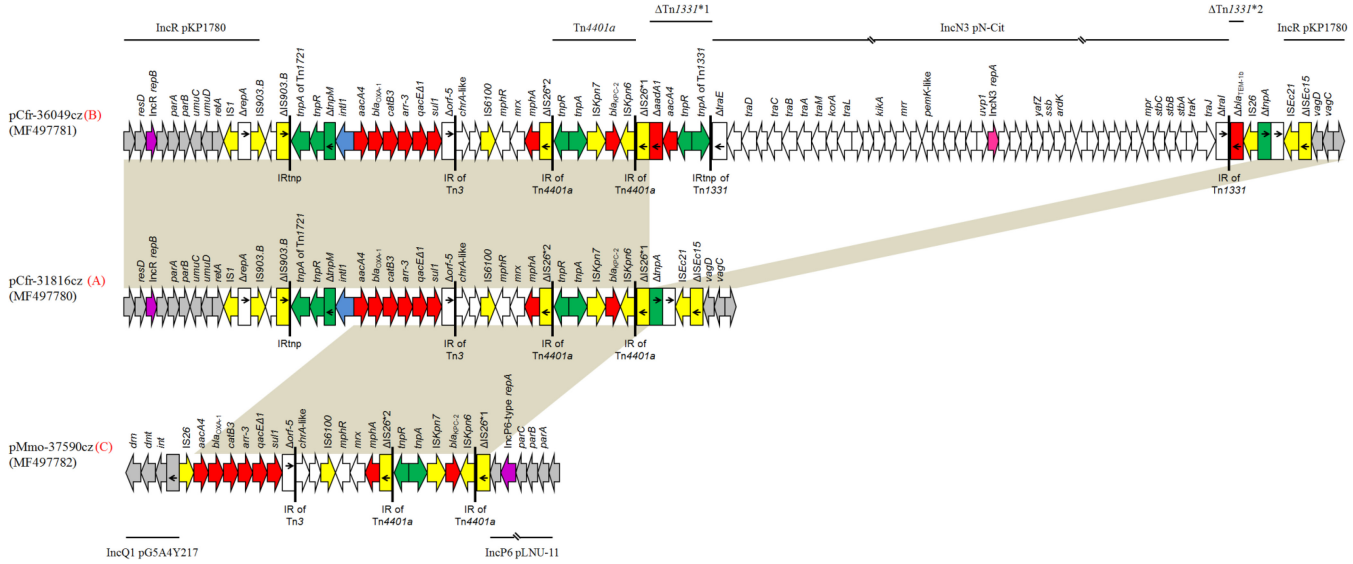

FIG 1 Linear maps of the bla_{KPC-2}-carrying plasmids. For each plasmid, the type of plasmid sequence is indicated in red next to the plasmid name. Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). Resistance genes, insertion sequence (IS) elements, and transposases are shown in red, yellow, and green, respectively. *intI1* genes are shaded blue. Gray arrows or rectangles indicate plasmid scaffold regions; the replication genes are shown in purple. The remaining genes are shown in white. Homologous segments (representing ≥99% sequence identity) are indicated by light gray shading. Thin lines above and below the maps correspond to highly similar sequences from other plasmids.

accession number MF497780) sharing extensive similarity with the backbone of the recently described IncR plasmids (24). This segment was composed of regions responsible for the replication (*repB* gene and iteron region), maintenance (*resD* gene), and stability (*parAB*, *vagCD*, and *umuDC* operons) of the plasmids (Fig. 1).

In the remaining 34,790-bp sequence (nt 10295 to 45084; GenBank accession number MF497780) adjoining the boundaries of the IncR backbone, a multidrug resistance (MDR) region containing the KPC-2-encoding transposon Tn*4401a* was identified. The Tn*4401a* transposon was localized within a copy of insertion sequence IS*26* (ΔIS*26*). Target site duplications of 5 bp (ATGCA) at the boundaries of Tn*4401a* indicated insertion by transposition. Upstream from ΔIS*26**1, an IS*Ec21*-like element and a 916-bp fragment of an IS*Ec15*-like element (ΔIS*Ec15*) were found. The IS*Ec21*-ΔIS*Ec15* structure was at the boundary of the plasmid backbone, downstream of *vagCD*, in the same configuration previously described in the IncR MDR plasmid pKP1780 (24).

In the remaining part of the MDR region, an IS*1* that was followed by a 674-bp segment of the IncN replication region (Δ*repA*) (25) was found at the boundary of the plasmid backbone, downstream of *retA*. The IS*1*-Δ*repA* structure was also identified in pKP1780 at a similar position (24). Next to this sequence, an intact *IS903.B*-like element and a second copy of a *IS903.B*-like element truncated at the 3′ end (ΔIS*903.B*) were found. The deleted part of IS*903.B* was occupied by a Tn*1721*-like fragment (ΔTn*1721*-like) consisting of the 38-bp inverted repeat (IRtnp) of the transposon, *tnpA*, *tnpR*, and *tnpM*. The Tn*1721*-like sequence also included an integron similar to In37 from pHSH2, the variable region of which comprised the *aacA4*, bla_{OXA-1}, *catB3*, and *arr-3* cassettes (26). The IRi of In37 was located within the *tnpM* gene of the Tn*1721*-like transposon, while the 3′CS of In37 was truncated 174 bp after the start codon of *orf5* (Δ*orf5*). Immediately downstream of Δ*orf5*, a Tn*501*-like sequence including the 38-bp inverted repeat (IR) of the transposon, a *chrA*-like gene encoding a chromate ion transporter, an IS*6100*, a macrolide resistance operon [*mph(A)*], and the remaining part of IS*26* (ΔIS*26**2) was found. A similar structure, which confers resistance to ampicillin, streptomycin, sulfonamides and mercury, has also been observed in plasmid pLEW517 from the primate intestinal *E. coli* strain 517-2H1 (27).

The type B plasmids pCfr-36049cz and pEco-36682cz appeared to be derivatives of

type A IncR KPC-2-encoding plasmids characterized during the present study. Type B plasmids differed from type A plasmids by the presence of an additional 34,522-bp sequence (nt 41399 to 75920; GenBank accession number MF497781) upstream of ΔIS*26**1. This sequence comprised two fragments of the Tn*1331* transposon flanking a central sequence (Fig. 1). The central sequence (nt 46232 to 74780; GenBank accession number MF497781) shared extensive similarity with the sequence of pN-Cit (96% coverage and 95% identity), an IncN3-type plasmid originally described from *C. freundii* STE strain collected in France from a patient who had been transferred from India (28). The IncN3-derived sequence possessed genes encoding a transfer locus, and a *repA* gene that was 98% similar to the respective region of pN-Cit. However, a part of the IncN3 transfer system was missing, explaining the inability of pCfr-36049cz and pEco-36682cz to transfer via conjugation.

Plasmids pCfr-33795cz and pMmo-37590cz, which were assigned to type C, included a contiguous segment of 4,062-bp (nt 261 to 4322; GenBank accession number MF497782) containing the partitioning genes, *parA*, *parB*, and *parC*, and the replication gene *repA* (Fig. 1). The *parABC* operon of pCfr-33795cz and pMmo-37590cz was identical to those of IncP6-type plasmids like pCOL-1 described from the KPC-2-producing *Pseudomonas aeruginosa* COL-1 strain isolated in Colombia (29) and to pLNU-11 (GenBank accession number KX863568), which was identified from a *C. freundii* ATetA strain captured from the sediments of an urban coastal wetland. The putative *repA* product of pCfr-33795cz and pMmo-37590cz showed high amino acid sequence similarity (99%) with the replication initiation protein of pLNU-11. Additionally, type C plasmids included a 3,835-bp segment (nt 1 to 260 and 26477 to 30051; GenBank accession number MF497782) consisting of genes encoding a DNA invertase/recombinase (*int*), a deoxymethyltransferase (*dmt*), and a DNase (*drn*) of type II restriction module. The *int-dmt-drn* region has also been observed in IncQ1 bla_{GES-5}-carrying plasmids isolated from *E. coli* and *Serratia marcescens* strains persisting in Canada (30). The remaining 22,154-bp sequence of pCfr-33795cz and pMmo-37590cz (nt 4323 to 26476; GenBank accession number MF497782), which contained the KPC-2-encoding transposon Tn*4401a*, was identical to the MDR region of type A and B plasmids (Fig. 1). In contrast, in plasmid pCOL-1, the bla_{KPC-2} gene was part of the Tn*4401b* isoform of the transposon and was located in a different insertion site.

In conclusion, the present study reports the "hidden outbreak" of ST18 KPC-2-producing *C. freundii* isolates in a Czech hospital. However, the bla_{KPC-2} gene was also identified in other STs of *C. freundii* and other species of *Enterobacteriaceae*. In one of the patients, four different KPC-2 producers were identified during the hospitalization, implying *in vivo* horizontal transfer of the bla_{KPC-2}-carrying plasmid. Sequencing data confirmed the presence of the same bla_{KPC-2}-carrying plasmid in two of these isolates (Table 1), further supporting this hypothesis. Of note was that, in the remaining two isolates recovered from the same patient, two different types of bla_{KPC-2}-carrying plasmids were identified, indicating the ability of enterobacterial plasmids to further evolve through reshuffling.

Illumina analysis results showed that, in 6 out of the 10 isolates, the KPC-2-encoding transposon Tn*4401a* was localized on an IncR-type plasmid (type A). To our knowledge, this is the first report on complete sequences of IncR plasmids carrying Tn*4401a* transposon. However, previous studies have reported the presence of multireplicon $IncFII_{K2}$-IncR KPC-2-encoding plasmids from ST101 *K. pneumoniae* isolated in Italian hospitals (31, 32). In addition, type B plasmids were derivatives of type A IncR bla_{KPC-2}-positive plasmids carrying an IncN3-derived segment. Type C plasmids belonged to the IncP6 group and shared the same KPC-2-encoding MDR region with type A and B plasmids. Therefore, *en bloc* acquisition of the KPC-2-encoding MDR region by an InpP6-type replicon from type A or B plasmids is a plausible hypothesis regarding the formation of type C bla_{KPC-2}-carrying plasmids. All three types of plasmids were noncapable of transferring the bla_{KPC-2} gene via conjugation, due to partial deletion or absence of the transfer system genes. Thus, the hypothesis of mobilization in *trans* of the bla_{KPC-2}-carrying plasmids by a coresident plasmid cannot be excluded.

The data presented here contribute to the current knowledge of KPC-2-producing *Enterobacteriaceae*. In agreement with the results of previous studies (16, 24, 31, 32), our findings underline the increasing clinical importance of the IncR plasmid family as well as the spreading potential of large MDR segments through reshuffling of enterobacterial plasmids.

Accession number(s). The nucleotide sequences of pCfr-31816cz, pCfr-36049cz, pMMO-37590cz, pCfr-27569cz, pCfr-31260cz, pCfr-33038cz, pCfr-36808cz, pKpn-35786cz, pEco-36682cz, and pCfr-33795cz have been deposited in GenBank under the accession numbers MF497780, MF497781, MF497782, MG557994, MG557995, MG557996, MG557997, MG557998, MG557999, and MG558000, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02152-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Ø. Samuelsen and J. Janice for helpful suggestions. We also thank Dana Kralova for technical assistance.

This work was supported by the Medical Research Foundation of the Czech Republic (grants 15-28663A and 17-29239A), the National Sustainability Program I (NPU I) grant LO1503 provided by the Ministry of Education Youth and Sports of the Czech Republic, the Charles University Research Fund—PROGRES (grant Q39), and the Norwegian Financial Mechanism (grant NF-CZ07-MOP-4-254-2015).

We have no conflicts to declare.

REFERENCES

1. Tumbarello M, Trecarichi EM, De Rosa FG, Giannella M, Giacobbe DR, Bassetti M, Losito AR, Bartoletti M, Del Bono V, Corcione S, Maiuro G, Tedeschi S, Celani L, Cardellino CS, Spanu T, Marchese A, Ambretti S, Cauda R, Viscoli C, Viale P; ISGRI-SITA. 2015. Infections caused by KPC-producing *Klebsiella pneumoniae*: differences in therapy and mortality in a multicentre study. J Antimicrob Chemother 70:2133–2143. https://doi.org/10.1093/jac/dkv086.
2. Grundmann H, Livermore DM, Giske CG, Canton R, Rossolini GM, Campos J, Vatopoulos A, Gniadkowski M, Toth A, Pfeifer Y, Jarlier V, Carmeli Y; CNSE Working Group. 2010. Carbapenem-nonsusceptible *Enterobacteriaceae* in Europe: conclusions from a meeting of national experts. Euro Surveill 15(46):pii=19711. https://doi.org/10.2807/ese.15.46.19711-en.
3. Albiger B, Glasner C, Struelens MJ, Grundmann H, Monnet DL; EuSCAPE working group. 2015. Carbapenemase-producing *Enterobacteriaceae* in Europe: assessment by national experts from 38 countries, May 2015. Euro Surveill 20(45):pii=30062. https://doi.org/10.2807/1560-7917.ES.2015.20.45.30062.
4. Hrabak J, Niemczykova J, Chudackova E, Fridrichová M, Studentová V, Cervená D, Urbášková P, Zemličková H. 2011. KPC-2-producing *Klebsiella pneumoniae* isolated from a Czech patient previously hospitalized in Greece and *in vivo* selection of colistin resistance. Folia Microbiol (Praha) 56:361–365. https://doi.org/10.1007/s12223-011-0057-6.
5. Hrabak J, Papagiannitsis CC, Studentova V, Jakubu V, Fridrichová M, Zemlickova H; Czech Participants of European Antimicrobial Resistance Surveillance Network. 2013. Carbapenemase-producing *Klebsiella pneumoniae* in the Czech Republic in 2011. Euro Surveill 18(45):pii=20626. https://doi.org/10.2807/1560-7917.ES2013.18.45.20626.
6. Papagiannitsis CC, Studentova V, Izdebski R, Oikonomou O, Pfeifer Y, Petinaki E, Hrabak J. 2015. MALDI-TOF MS meropenem hydrolysis assay with NH_4HCO_3, a reliable tool for the direct detection of carbapenemase activity. J Clin Microbiol 53:1731–1735. https://doi.org/10.1128/JCM.03094-14.
7. Papagiannitsis CC, Izdebski R, Baraniak A, Fiett J, Herda M, Hrabák J, Derde LP, Bonten MJ, Carmeli Y, Goossens H, Hryniewicz W, Brun-Buisson C, Gniadkowski M, Grabowska A, Nikonorow E, Dautzenberg MJ, Adler A, Kazmas M, Navon-Venezia S, Malhotra-Kumar S, Lammens C, Legrand P, Annane D, Chalfine A, Giamarellou H, Petrikkos GL, Nardi G, Balode A, Dumpis U, Stammet P, Arag I, Esteves F, Muzlovic I, Tomic V, Torres Mart A, Lawrence C, Salomon J, Paul M, Lerman Y, Rossini A, Salvia A, Vidal Samso J, Fierro J. 2015. Survey of metallo-β-lactamase-producing *Enterobacteriaceae* colonizing patients in European ICUs and rehabilitation units, 2008–11. J Antimicrob Chemother 70:1981–1988. https://doi.org/10.1093/jac/dkv055.
8. European Committee on Antimicrobial Susceptibility Testing (EUCAST). 2003. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clin Microbiol Infect 9:ix–xv. https://doi.org/10.1046/j.1469-0691.2003.00790.x.
9. Bai L, Xia S, Lan R, Liu L, Ye C, Wang Y, Jin D, Cui Z, Jing H, Xiong Y, Bai X, Sun H, Zhang J, Wang L, Xu J. 2012. Isolation and characterization of cytotoxic, aggregative *Citrobacter freundii*. PLoS One 7:e33054. https://doi.org/10.1371/journal.pone.0033054.
10. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, Karch H, Reeves PR, Maiden MC, Ochman H, Achtman M. 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol 60:1136–1151. https://doi.org/10.1111/j.1365-2958.2006.05172.x.
11. Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. 2005. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. J Clin Microbiol 43:4178–4182. https://doi.org/10.1128/JCM.43.8.4178-4182.2005.
12. Hammerum AM, Hansen F, Nielsen HL, Jakobsen L, Stegger M, Andersen PS, Jensen P, Nielsen TK, Hansen LH, Hasman H, Fuglsang-Damgaard D. 2016. Use of WGS data for investigation of a long-term NDM-1-producing *Citrobacter freundii* outbreak and secondary *in vivo* spread of bla_{NDM-1} to *Escherichia coli*, *Klebsiella pneumoniae* and *Klebsiella oxytoca*. J Antimicrob Chemother 71:3117–3124. https://doi.org/10.1093/jac/dkw289.
13. Villa J, Arana DM, Viedma E, Perez-Montarelo D, Chaves F. 2017. Characterization of mobile genetic elements carrying VIM-1 and KPC-2 carbapenemases in *Citrobacter freundii* isolates in Madrid. Int J Med Microbiol 307:340–345. https://doi.org/10.1016/j.ijmm.2017.07.001.
14. Woodford N, Turton JF, Livermore DM. 2011. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol Rev 35:736–755. https://doi.org/10.1111/j.1574-6976.2011.00268.x.
15. Barton BM, Harding GP, Zuccarelli AJ. 1995. A general method for

detecting and sizing large plasmids. Anal Biochem 226:235–240. https://doi.org/10.1006/abio.1995.1220.
16. Garcia-Fernandez A, Fortini D, Veldman K, Mevius D, Carattoli A. 2009. Characterization of plasmids harbouring *qnrS1*, *qnrB2* and *qnrB19* genes in *Salmonella*. J Antimicrob Chemother 63:274–281. https://doi.org/10.1093/jac/dkn470.
17. Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. 2005. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods 63:219–228. https://doi.org/10.1016/j.mimet.2005.03.018.
18. Johnson TJ, Bielak EM, Fortini D, Hansen LH, Hasman H, Debroy C, Nolan LK, Carattoli A. 2012. Expansion of the IncX plasmid family for improved identification and typing of novel plasmids in drug-resistant *Enterobacteriaceae*. Plasmid 68:43–50. https://doi.org/10.1016/j.plasmid.2012.03.001.
19. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120. https://doi.org/10.1093/bioinformatics/btu170.
20. Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv arXiv:1303.3997 [q-bio.GN]. https://arxiv.org/abs/1303.3997.
21. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comp Biol 19:455–477. https://doi.org/10.1089/cmb.2012.0021.
22. Darling AE, Mau B, Perna NT. 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One 5:e11147. https://doi.org/10.1371/journal.pone.0011147.
23. Naas T, Cuzon G, Villegas MV, Lartigue MF, Quinn JP, Nordmann P. 2008. Genetic structure at the origin of acquisition of the beta-lactamase bla_{KPC} gene. Antimicrob Agents Chemother 52:1257–1263. https://doi.org/10.1128/AAC.01451-07.
24. Papagiannitsis CC, Miriagou V, Giakkoupi P, Tzouvelekis LS, Vatopoulos AC. 2013. Characterization of pKP1780, a novel IncR plasmid from the emerging *Klebsiella pneumoniae* ST147, encoding the VIM-1 metallo-β-lactamase. J Antimicrob Chemother 68:2259–2262. https://doi.org/10.1093/jac/dkt196.
25. Miriagou V, Papagiannitsis CC, Kotsakis SD, Loli A, Tzelepi E, Legakis NJ, Tzouvelekis LS. 2010. Sequence of pNL194, a 79.3-kilobase IncN plasmid carrying the bla_{VIM-1} metallo-β-lactamase gene in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 54:4497–4502. https://doi.org/10.1128/AAC.00665-10.
26. Wang M, Tran JH, Jacoby GA, Zhang Y, Wang F, Hooper DC. 2003. Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China. Antimicrob Agents Chemother 47:2242–2248. https://doi.org/10.1128/AAC.47.7.2242-2248.2003.
27. Williams LE, Detter C, Barry K, Lapidus A, Summers AO. 2006. Facile recovery of individual high-molecular-weight, low-copy-number natural plasmids for genomic sequencing. Appl Environ Microbiol 72:4899–4906. https://doi.org/10.1128/AEM.00354-06.
28. Villa L, Carattoli A, Nordmann P, Carta C, Poirel L. 2013. Complete sequence of the IncT-type plasmid pT-OXA-181 carrying the $bla_{OXA-181}$ carbapenemase gene from *Citrobacter freundii*. Antimicrob Agents Chemother 57:1965–1957. https://doi.org/10.1128/AAC.01297-12.
29. Naas T, Bonnin RA, Cuzon G, Villegas MV, Nordmann P. 2013. Complete sequence of two KPC-harbouring plasmids from *Pseudomonas aeruginosa*. J Antimicrob Chemother 68:1757–1762. https://doi.org/10.1093/jac/dkt094.
30. Boyd D, Taylor G, Fuller J, Bryce E, Embree J, Gravel D, Katz K, Kibsey P, Kuhn M, Langley J, Mataseje L, Mitchell R, Roscoe D, Simor A, Thomas E, Turgeon N, Mulvey M; Canadian Nosocomial Infection Surveillance Program. 2015. Complete sequence of four multidrug-resistant MOBQ1 plasmids harboring bla_{GES-5} isolated from *Escherichia coli* and *Serratia marcescens* persisting in a hospital in Canada. Microb Drug Resist 21:253–260. https://doi.org/10.1089/mdr.2014.0205.
31. Frasson I, Lavezzo E, Franchin E, Toppo S, Barzon L, Cavallaro A, Richter SN, Palù G. 2012. Antimicrobial treatment and containment measures for an extremely drug-resistant *Klebsiella pneumoniae* ST101 isolate carrying pKPN101-IT, a novel fully sequenced bla_{KPC-2} plasmid. J Clin Microbiol 50:3768–3772. https://doi.org/10.1128/JCM.01892-12.
32. Papagiannitsis CC, Di Pilato V, Giani T, Giakkoupi P, Riccobono E, Landini G, Miriagou V, Vatopoulos AC, Rossolini GM. 2016. Characterization of KPC-encoding plasmids from two endemic settings, Greece and Italy. J Antimicrob Chemother 71:2824–2830. https://doi.org/10.1093/jac/dkw227.