Journal of Antimicrobial Chemotherapy Advance Access published September 2, 2016

J Antimicrob Chemother
doi:10.1093/jac/dkw350

Journal of Antimicrobial Chemotherapy

Low biological cost of carbapenemase-encoding plasmids following transfer from *Klebsiella pneumoniae* to *Escherichia coli*

Maria Chiara Di Luca[1]¶, Vidar Sørum[2]¶, Irina Starikova[2]†, Julia Kloos[2], Nils Hülter[2]‡, Umaer Naseer[3]§, Pål J. Johnsen[2] and Ørjan Samuelsen[1,2]*

[1]*Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø, Norway;* [2]*Department of Pharmacy, Faculty of Health Sciences, UiT The Arctic University of Norway, Tromsø, Norway;* [3]*Department of Medical Biology, Faculty of Health Sciences, UiT The Arctic University of Norway, Tromsø, Norway*

*Corresponding author. Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø, Norway. Tel: +47-77627043; E-mail: orjan.samuelsen@unn.no

†Present address: K. G. Jebsen–Thrombosis Research and Expertise Center (TREC), Department of Clinical Medicine, UiT The Arctic University of Norway, Tromsø, Norway and Division of Internal Medicine, University Hospital of North Norway, Tromsø, Norway.

‡Present address: Genomic Microbiology, Institute of Microbiology, Christian-Albrechts-University Kiel, Kiel, Germany.

§Present address: Department of Food-borne Infections, Norwegian Institute of Public Health, Oslo, Norway.

¶These authors contributed equally to this work.

Received 5 February 2016; returned 4 April 2016; revised 20 June 2016; accepted 27 July 2016

Objectives: The objective of this study was to determine the biological cost, stability and sequence of two carbapenemase-encoding plasmids containing bla_{KPC-2} (pG12-KPC-2) and bla_{VIM-1} (pG06-VIM-1) isolated from *Klebsiella pneumoniae* when newly acquired by uropathogenic *Escherichia coli* clinical isolates of different genetic backgrounds.

Methods: The two plasmids were transferred into plasmid-free *E. coli* clinical isolates by transformation. The fitness effect of newly acquired plasmids on the host cell was assessed in head-to-head competitions with the corresponding isogenic strain. Plasmid stability was estimated by propagating monocultures for ~312 generations. Plasmid nucleotide sequences were determined using next-generation sequencing technology. Assembly, gap closure, annotation and comparative analyses were performed.

Results: Both plasmids were stably maintained in three of four *E. coli* backgrounds and resulted in low to moderate reductions in host fitness ranging from 1.1% to 3.6%. A difference in fitness cost was observed for pG12-KPC-2 between two different genetic backgrounds, while no difference was detected for pG06-VIM-1 between three different genetic backgrounds. In addition, a difference was observed between pG12-KPC-2 and pG06-VIM-1 in the same genetic background. In general, the magnitude of biological cost of plasmid carriage was both host and plasmid dependent. The sequences of the two plasmids showed high backbone similarity to previously circulating plasmids in *K. pneumoniae*.

Conclusions: The low to modest fitness cost of newly acquired and stably maintained carbapenemase-encoding plasmids in *E. coli* indicates a potential for establishment and further dissemination into other Enterobacteriaceae species. We also show that the fitness cost is both plasmid and host specific.

Introduction

Plasmids can carry multiple resistance genes that can disseminate multidrug resistance (MDR) through horizontal gene transfer in and between bacterial populations.[1] The emergence and acquisition of plasmid-mediated carbapenemases in Gram-negatives represent a serious clinical challenge.[2] Carbapenemases have broad-spectrum activity making virtually all clinically relevant β-lactams ineffective, resulting in frequent treatment failures.[2] Dissemination of acquired carbapenemases is enhanced by their presence on plasmids with different genetic backbones and association with other mobile genetic elements (MGEs), creating a potential for further spread of these enzymes to other Enterobacteriaceae. Following successful transfer events, the stability and persistence of newly acquired plasmids largely depend on the fitness cost imposed on new hosts in the absence of selective pressure on traits harboured by the plasmid.[3,4] Thus, understanding transmission dynamics of plasmids between species and interactions between plasmids and host genetic backgrounds might contribute to minimize the dissemination of these MDR plasmids.

Here, we investigated the fitness effect, stability and sequence of two carbapenemase-encoding plasmids, pG12-KPC-2 and pG06-VIM-1, isolated from *Klebsiella pneumoniae* when newly acquired by uropathogenic *Escherichia coli* of different genetic backgrounds.

Materials and methods

Bacterial isolates, transformants and susceptibility testing

Bacterial strains used in this study and their relevant characteristics are given in Table S1 (available as Supplementary data at *JAC* Online). Strains were grown under aeration at 37°C in LB and/or Super Optimal Broth (SOB) agar or broth. Plasmids were transferred into *E. coli* of various genetic backgrounds by electroporation and transformants selected on lactose agar with 100 mg/L ampicillin. Transformants were screened by PCR for bla_{VIM}[5] or bla_{KPC}[6] genes and further analysed using S1 nuclease PFGE followed by hybridization with bla_{VIM} and bla_{KPC} probes[7] (data not shown). Susceptibility profiles were determined by Etest (bioMérieux, France).

Plasmid stability, fitness measurements and statistical analysis

Transformants were propagated by serial transfer[4] for ~312 generations and plated on non-selective LB agar. One hundred colonies were patched on LB agar with 100 mg/L ampicillin. Plasmid presence was checked by colony PCR targeting bla_{VIM}[5] or bla_{KPC}[6] and *repB* or *repA*.[8,9] Relative fitness of the newly acquired plasmids was determined in 24 h head-to-head competitions with their plasmid-free isogenic ancestors[4] in both LB and minimal (M9 with 0.2% glucose) media. Initial and final titres of each competitor were determined by selective (100 mg/L ampicillin) and non-selective plating and the relative fitness calculated.[10] Fitness data were assessed using a two-way ANOVA with Tukey's honest significant difference (HSD) correction between the different transformants. Fitness cost of plasmid carriage compared with the plasmid-free ancestor was assessed with one-sample *t*-tests. Statistical analysis was performed in R (https://www.r-project.org/).

Generation of rifampicin-resistant mutants and filter mating

Spontaneous rifampicin-resistant mutants were obtained by spreading 100 μL of overnight cultures onto LB agar with 100 mg/L rifampicin. Transferability of the plasmids from the transformants to the corresponding rifampicin-resistant plasmid-free recipients was tested by filter mating. Transconjugants were selected on LB agar containing 100 mg/L rifampicin and 100 mg/L ampicillin.

DNA extraction and plasmid sequencing

Genomic DNA was isolated using anion exchange columns (Qiagen, Germany). Plasmid DNA from donor strains was isolated using the NucleoBond Xtra Midi/Maxi kit (Macherey-Nagel, Germany) following the manufacturer's instructions with proteinase K added during the lysis step and an extended incubation of 40 min.

Generation of long paired-end libraries and multiplexed sequencing using Roche/454 GS FLX Titanium technology were performed by Eurofins MWG Operon (Germany). Reads were *de novo* assembled using Newbler (454 Life Sciences, USA) and contigs were ordered relative to reference sequences using progressiveMauve (http://darlinglab.org/mauve/mauve.html). Assembly gaps were closed by Sanger sequencing (Applied

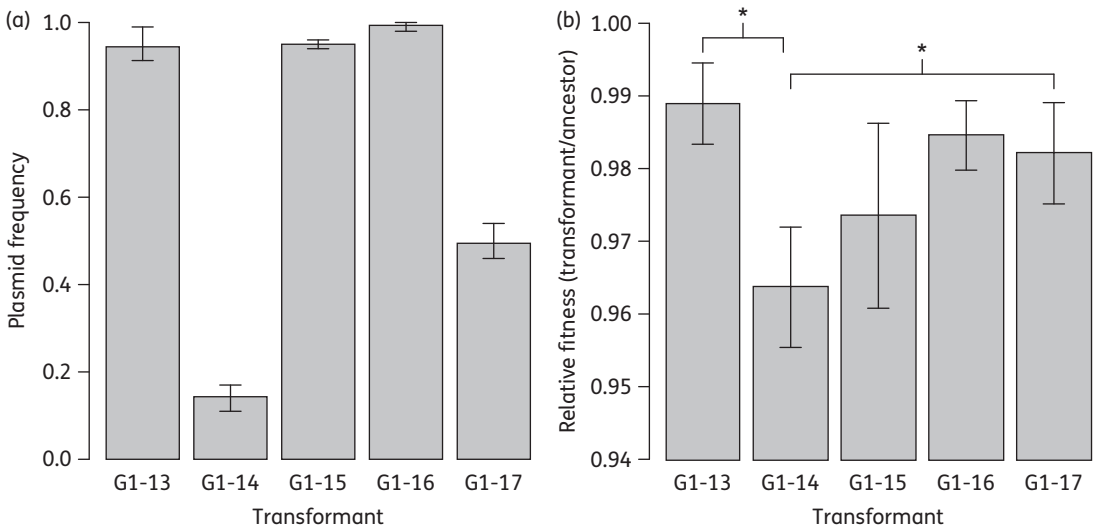

Figure 1. (a) Frequency of *E. coli* cells within a population still harbouring plasmids bla_{KPC-2} (G1-13 and G1-14) and bla_{VIM-1} (G1-15, G1-16 and G1-17) after ~312 generations in the absence of antibiotic pressure. Both plasmids were stably maintained except in the ST69 background (G1-14 with pG12-KPC-2 and G1-17 with pG06-VIM-1). Error bars denote maximum and minimum values. Transformant populations were propagated in triplicate. (b) Relative fitness (w) of *E. coli* harbouring newly acquired plasmids carrying bla_{KPC-2} (G1-13 and G1-14) and bla_{VIM-1} (G1-15, G1-16 and G1-17). The results were obtained by 18 independent competitions in each group. Plasmid acquisition reduced host fitness in all transformants (relative fitness below 1, $P=0.001$). The error bars denote the 95% CIs: G1-13 ($w=0.989\pm0.006$); G1-14 ($w=0.964\pm0.008$); G1-15 ($w=0.974\pm0.013$); G1-16 ($w=0.985\pm0.005$); and G1-17 ($w=0.982\pm0.007$). Asterisks denote a significant relative fitness difference of pG12-KPC-2 carriage between G1-13 and G1-14 ($P<0.001$) as well as between G1-14 and G1-17 (same strain, different plasmids, $P=0.01$). All the other transformants did not display different costs of carriage as identified by multiple comparisons with Tukey's HSD correction with relative fitness as the response variable and strain (four levels), plasmid (two levels) and growth medium (two levels) as explanatory variables.

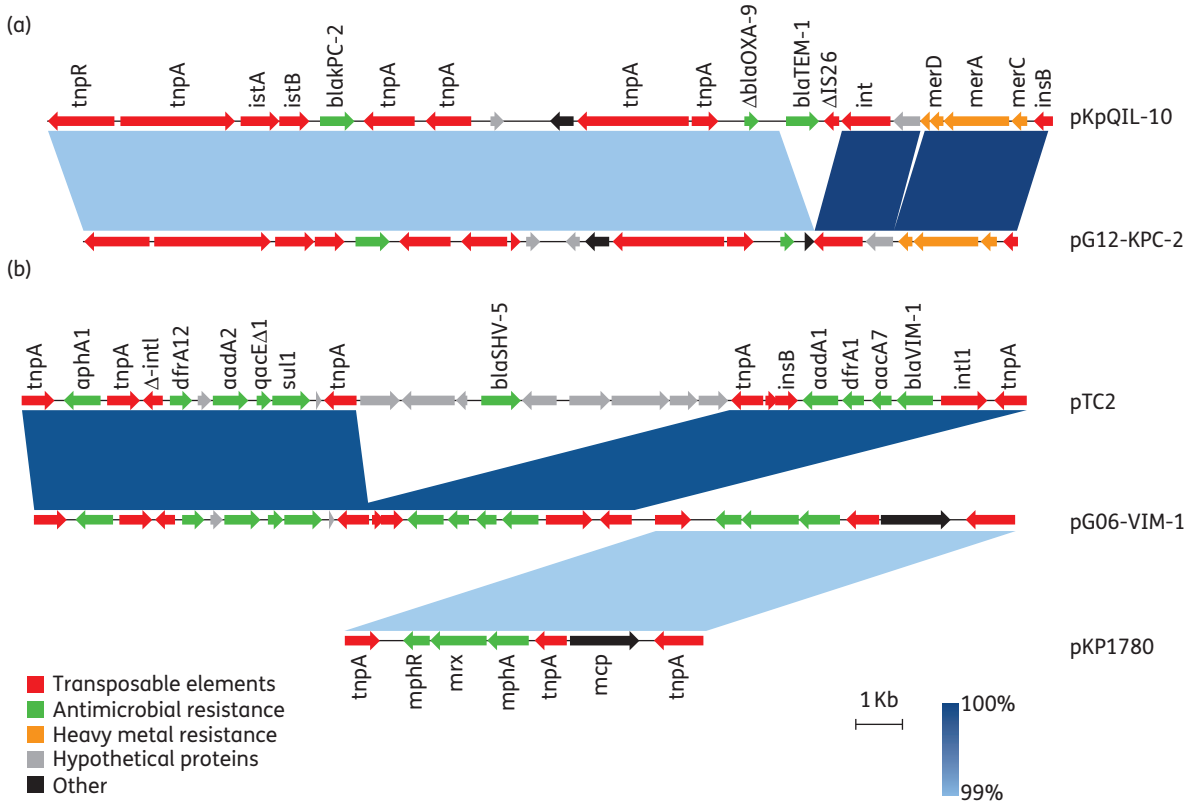

Figure 2. Comparative genomics of pG12-KPC-2 (GenBank accession number KU665642) and pG06-VIM-1 (GenBank accession number KU665641) MDR regions. (a) Physical map comparing the genes encoding the MDR region in plasmid pG12-KPC-2 with the corresponding regions in plasmid pKpQIL-10 (GenBank accession number KJ146687). (b) Physical map comparing the genes encoding the MDR region in plasmid pG06-VIM-1 with the corresponding regions in plasmids pTC2 (GenBank accession number JQ824049) and pKP1780 (GenBank accession number JX424614). Blue areas indicate shared regions of homology (>99% nucleotide similarity).

Biosystems, USA). Coding sequences were identified and annotated using RAST (http://rast.nmpdr.org), BLAST and Artemis. Sequence comparisons were carried out using Easyfig 2.1 (https://mjsull.github.io/Easyfig/). Plasmid sequences are deposited in GenBank under accession numbers KU665641 and KU665642.

Results and discussion

Characterization of transformants

We transferred plasmids pG12-KPC-2 and pG06-VIM-1 from two carbapenemase-producing *K. pneumoniae* clinical donor strains into plasmid-naive uropathogenic *E. coli* recipient strains of various genetic backgrounds by electroporation. pG12-KPC-2 was successfully transformed into two different genetic backgrounds (ST10 and ST69). pG06-VIM-1 was transformed into three different genetic backgrounds (ST537, ST95 and ST69). The recipient strain *E. coli* K56-75 (ST69) was successfully transformed with pG12-KPC-2 or pG06-VIM-1 (Table S1). The activity profile against β-lactams for the transformants was consistent with the expected activity profiles of KPC-2 and VIM-1 (Table S2). No plasmid transfer was detected in filter mating assays between transformants and corresponding rifampicin-resistant plasmid-free mutants.

Plasmid stability

We also propagated the plasmid-containing *E. coli* in monocultures for ∼312 generations in the absence of antibiotics. Both plasmids were stably maintained in their respective backgrounds except in the ST69 background (Figure 1a). No plasmid loss was observed during the time course of the 24 h competition experiments.

Biological costs of plasmid carriage

The relative fitness of plasmid carriage was estimated in mixed culture competitions with the corresponding plasmid-free isogenic ancestors in LB and M9 media. Costs of plasmid carriage were independent of LB and M9 media ($P=0.42$) and data were pooled in the analysis. Both plasmids reduced fitness in their new hosts ranging from 1.1% to 3.6% (all P values <0.001) (Figure 1b).

A host-dependent difference was observed between strains G1-13 (phylogroup A, ST10) and G1-14 (phylogroup D, ST69) ($P<0.001$) carrying pG12-KPC-2 (Figure 1b). This is consistent with recent reports demonstrating that fitness costs of newly acquired MGEs depend on the genetic background and/or environmental factors.[4,11] When in the same genetic background (phylogroup D, ST69), pG12-KPC-2 and pG06-VIM-1 reduced fitness

by 3.6% (G1-14-pG12-KPC-2) and 1.8% (G1-17-pG06-VIM-1), respectively ($P=0.01$), revealing a plasmid-dependent difference. No difference in relative fitness was observed between strains G1-15 (phylogroup B2, ST537), G1-16 (phylogroup B2, ST95) and G1-17 (phylogroup D, ST69) harbouring pG06-VIM-1 (all P values >0.27).

pG12-KPC-2 and pG06-VIM-1 imposed low to modest reductions in fitness in *E. coli* of different genetic backgrounds including strains from phylogroups A and B2, frequently associated with human and animal commensal strains.[12] These results are worrying since commensal *E. coli* and *K. pneumoniae* coinhabit the gastrointestinal tract, a hotspot for horizontal gene transfer.[13] Even though our *in vitro* plasmid transfers to clinical *E. coli* were unsuccessful, conjugative transfer of pG12-KPC-2 to a laboratory *E. coli* has previously been shown.[14] Further, pG12-KPC-2 was responsible for a long-term plasmid outbreak where both inter- and intraspecies transfer were observed.[14] Transfer of other carbapenemase-encoding plasmids between *K. pneumoniae* and *E. coli* as well as other Enterobacteriaceae has been shown to occur *in vivo*.[15] Thus, the transformants characterized here provide important insight into carbapenemase-encoding plasmids and their interactions with *E. coli* hosts when newly acquired.

Sequence analysis of pG12-KPC-2 and pG06-VIM-1

pG12-KPC-2 was recovered from a KPC-producing *K. pneumoniae* strain, isolated from a hospital outbreak in southern Norway in 2007–10.[14] It is 111 926 bp in length and belongs to both IncFI and IncFII groups (Figure S1a). It encodes antirestriction proteins ArdA and ArdB, stability proteins StbA and StbB, a copy of the single strand binding protein Ssb and the *parAB* partitioning operon. The transfer region comprises 37 genes and makes up ∼30% of the plasmid. The backbone sequence is 99% identical to the KPC-2-encoding pKpQIL-10 (accession number KJ146687)[16] and highly similar to KPC-3-encoding pKpQIL-like plasmids identified in the *K. pneumoniae* epidemic ST258 clone[17,18] and to pKPN4 carrying bla_{SHV-12} (accession number CP000649). The bla_{KPC-2} region revealed the structure of the Tn*4401a* transposon (Figure 2a), including a resolvase and a transposase, and IS*Kpn6* and IS*Kpn7*. The association between bla_{KPC-2} and Tn*4401* is worrisome due to the potential evolution of structural variations caused by recombination/transposition events and further mobilization of carbapenem resistance in Enterobacteriaceae. pG12-KPC-2 also harbours a disrupted bla_{OXA-9} as part of Tn*501*.

pG06-VIM-1 was isolated from a *K. pneumoniae* collected in 2006 in Sweden from a patient who had undergone medical treatment in Greece.[19] The plasmid is a 53 618 bp circular molecule and belongs to the IncR group (Figure S1b). pG06-VIM-1 exhibits 99% nucleotide identity to bla_{VIM-1}-encoding pKP1780 (accession number JX424614). The IncR fragment encodes a set of genes controlling RepB expression and plasmid copy number, the *parAB* operon, a VagCD toxin–antitoxin system, a plasmid stabilization system specified by ResD and a *umuDC* operon. No conjugative transfer region was identified, explaining its inability to be transferred through conjugation. In pG06-VIM-1, bla_{VIM-1} is part of a mosaic structure similar to that of the VIM-1-encoding pTC2 (accession number JQ824049)[20] (Figure 2b). We hypothesize that this MDR region resulted from multiple genetic rearrangements that have led to the mosaic structure of pG06-VIM-1 and to its plasticity. pG06-VIM-1 also harbours a macrolide inactivation cluster [*mphR, mrx* and *mph*(A)] and a composite transposon (Tn*4352*) containing the aminoglycoside resistance gene *aphA1*.

Conclusions

In conclusion, we show that the fitness cost of plasmid carriage depends on the individual plasmid–host combinations. In addition, the combination of stable VIM- and KPC-plasmid maintenance in the absence of antibiotic selective pressures is worrying since it may indicate the potential for further dissemination and establishment in *E. coli* and other Enterobacteriaceae.

Acknowledgements

Bettina Aasnæs is acknowledged for the construction of transformants.

Funding

M. C. D. L. is supported by a grant from the Northern Norway Regional Health Authority (SFP1168-14). P. J. J. acknowledges financial support from Tromsø Research Foundation (project TFS A23245), the Research Council of Norway (project 204263/F20) and a joint grant from North Norway Regional Health Authority/University Hospital North Norway and UiT The Arctic University of Norway (project A23270).

Transparency declarations

None to declare.

Supplementary data

Table S1, Table S2 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Schultsz C, Geerlings S. Plasmid-mediated resistance in Enterobacteriaceae: changing landscape and implications for therapy. *Drugs* 2012; **72**: 1–16.

2 Tzouvelekis LS, Markogiannakis A, Psichogiou M *et al*. Carbapenemases in *Klebsiella pneumoniae* and other Enterobacteriaceae: an evolving crisis of global dimensions. *Clin Microbiol Rev* 2012; **25**: 682–707.

3 San Millan A, Peña-Miller R, Toll-Riera M *et al*. Positive selection and compensatory adaptation interact to stabilize non-transmissible plasmids. *Nat Commun* 2014; **5**: 5208.

4 Starikova I, Al-Haroni M, Werner G *et al*. Fitness costs of various mobile genetic elements in *Enterococcus faecium* and *Enterococcus faecalis*. *J Antimicrob Chemother* 2013; **68**: 2755–65.

5 Mendes RE, Kiyota KA, Monteiro J *et al*. Rapid detection and identification of metallo-β-lactamase-encoding genes by multiplex real-time PCR assay and melt curve analysis. *J Clin Microbiol* 2007; **45**: 544–7.

6 Naas T, Cuzon G, Villegas M-V *et al*. Genetic structures at the origin of acquisition of the β-lactamase bla_{KPC} gene. *Antimicrob Agents Chemother* 2008; **52**: 1257–63.

7 Samuelsen Ø, Naseer U, Tofteland S *et al*. Emergence of clonally related *Klebsiella pneumoniae* isolates of sequence type 258 producing plasmid-mediated KPC carbapenemase in Norway and Sweden. *J Antimicrob Chemother* 2009; **63**: 654–8.

8 Garcia-Fernandez A, Fortini D, Veldman K *et al*. Characterization of plasmids harbouring *qnrS1*, *qnrB2* and *qnrB19* genes in *Salmonella*. *J Antimicrob Chemother* 2009; **63**: 274–81.

9 Chen L, Chavda KD, Melano RG *et al*. Complete sequence of a bla_{KPC-2}-harboring IncFII(K1) plasmid from a *Klebsiella pneumoniae* sequence type 258 strain. *Antimicrob Agents Chemother* 2013; **57**: 1542–5.

10 Lenski RE. Quantifying fitness and gene stability in microorganisms. *Biotechnology* 1991; **15**: 173–92.

11 Humphrey B, Thomson NR, Thomas CM *et al*. Fitness of *Escherichia coli* strains carrying expressed and partially silent IncN and IncP1 plasmids. *BMC Microbiol* 2012; **12**: 53.

12 Escobar-Páramo P, Le Menac'h A, Le Gall T *et al*. Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. *Envir Microbiol* 2006; **8**: 1975–84.

13 Stecher B, Maier L, Hardt W-D. 'Blooming' in the gut: how dysbiosis might contribute to pathogen evolution. *Nat Rev Microbiol* 2013; **11**: 277–84.

14 Tofteland S, Naseer U, Lislevand JH *et al*. A long-term low-frequency hospital outbreak of KPC-producing *Klebsiella pneumoniae* involving intergenus plasmid diffusion and a persisting environmental reservoir. *PLoS One* 2013; **8**: e59015.

15 Gottig S, Gruber TM, Stecher B *et al*. *In vivo* horizontal gene transfer of the carbapenemase OXA-48 during a nosocomial outbreak. *Clin Infect Dis* 2015; **60**: 1808–15.

16 Chen L, Chavda KD, Melano RG *et al*. Comparative genomic analysis of KPC-encoding pKpQIL-like plasmids and their distribution in New Jersey and New York hospitals. *Antimicrob Agents Chemother* 2014; **58**: 2871–7.

17 Leavitt A, Chmelnitsky I, Carmeli Y *et al*. Complete nucleotide sequence of KPC-3-encoding plasmid pKpQIL in the epidemic *Klebsiella pneumoniae* sequence type 258. *Antimicrob Agents Chemother* 2010; **54**: 4493–6.

18 García-Fernández A, Villa L, Carta C *et al*. *Klebsiella pneumoniae* ST258 producing KPC-3 identified in Italy carries novel plasmids and OmpK36/OmpK35 porin variants. *Antimicrob Agents Chemother* 2012; **56**: 2143–5.

19 Samuelsen Ø, Toleman MA, Hasseltvedt V *et al*. Molecular characterization of VIM-producing *Klebsiella pneumoniae* from Scandinavia reveals genetic relatedness with international clonal complexes encoding transferable multidrug resistance. *Clin Microbiol Infect* 2011; **17**: 1811–6.

20 Drieux L, Decré D, Frangeul L *et al*. Complete nucleotide sequence of the large conjugative pTC2 multireplicon plasmid encoding the VIM-1 metallo-β-lactamase. *J Antimicrob Chemother* 2013; **68**: 97–100.