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Gaëlle Cuzon, Thierry Naas, Michele Guibert, Patrice Nordmann. In vivo selection of imipenem-resistant producing extended-spectrum β -lactamase CTX-M-15 and plasmid-encoded DHA-1 cephalosporinase. *International Journal of Antimicrobial Agents*, 2010, 35 (3), pp.265. 10.1016/j.ijantimicag.2009.10.021 . hal-00556383

HAL Id: hal-00556383

<https://hal.science/hal-00556383v1>

Submitted on 16 Jan 2011

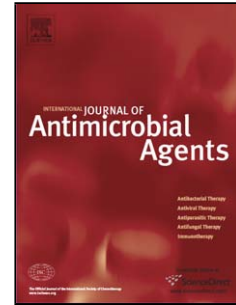
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PII: S0924-8579(09)00508-1
DOI: doi:10.1016/j.ijantimicag.2009.10.021
Reference: ANTAGE 3176

To appear in: *International Journal of Antimicrobial Agents*

Received date: 30-6-2009
Revised date: 29-10-2009
Accepted date: 30-10-2009

Please cite this article as: Cuzon G, Naas T, Guibert M, Nordmann P, In vivo selection of imipenem-resistant *Klebsiella pneumoniae* producing extended-spectrum β -lactamase CTX-M-15 and plasmid-encoded DHA-1 cephalosporinase¹, *International Journal of Antimicrobial Agents* (2008), doi:10.1016/j.ijantimicag.2009.10.021

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In vivo selection of imipenem-resistant *Klebsiella pneumoniae* producing extended-spectrum β -lactamase CTX-M-15 and plasmid-encoded DHA-1 cephalosporinase [☆]

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ARTICLE INFO

Article history:

Received 30 June 2009

Accepted 30 October 2009

Keywords:

ESBL

AmpC

Multidrug resistance

Carbapenem resistance

Outer membrane proteins

OMPs

Antimicrobial resistance mechanisms

Carbapenems

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☆ This work was presented in part at the 19th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), 16–19 May 2009, Helsinki, Finland.

Accepted Manuscript

ABSTRACT

Four *Klebsiella pneumoniae* isolates (KP1–4) were recovered sequentially from an infected patient. Whilst KP1–3 were resistant to all β -lactams except carbapenems, KP4 recovered after 24 days of imipenem-containing treatment showed additional resistance to carbapenems. No carbapenem hydrolysis could be identified in KP4. Molecular characterisation revealed that KP1–4 were indistinguishable by pulsed-field gel electrophoresis, contained a 95-kb self-transferable plasmid harbouring *bla*_{CTXM-15} and *bla*_{TEM-1} genes and a 65-kb plasmid that was not transferred by conjugation into *Escherichia coli*, and harboured the plasmid-mediated *bla*_{DHA-1} AmpC β -lactamase gene. In addition, KP4 failed to express OmpK36 owing to a point mutation leading to a premature stop of the protein. This study demonstrates development of carbapenem resistance related to loss of OmpK36 expression in a *K. pneumoniae* isolate harbouring extended-spectrum β -lactamase and plasmid-mediated cephalosporinase genes following prolonged imipenem exposure.

1. Introduction

Carbapenems have the most consistent in vitro activity against extended-spectrum β -lactamase (ESBL)-producing *Klebsiella pneumoniae* [1]. Resistance to carbapenems, while uncommon in Enterobacteriaceae, can be mediated by two main mechanisms. The first involves production of a β -lactamase without carbapenem-hydrolysing activity but combined with decreased permeability due to loss or alteration of porins. *Klebsiella pneumoniae* expresses two main porins in its outer membrane, OmpK35 and OmpK36, which play an important role in penetration of antibiotics into the cell. Loss of one or more of these porins contributes to antimicrobial resistance, particularly when additional mechanisms of resistance are present such as ESBLs or plasmid-encoded AmpC enzymes [1–4]. The second mechanism involves the production of an acquired carbapenem-hydrolysing β -lactamase (carbapenemase) [1]. The carbapenemases identified in *K. pneumoniae* are either metallo- β -lactamases (IMP, VIM), rarely reported in *K. pneumoniae*, expanded-spectrum oxacillinases, in particular OXA-48, and plasmid-mediated Ambler class A enzymes (NMCA, IMI, SME, GES and KPC) [1,5].

Here we report the in vivo modification of porin expression in *K. pneumoniae* isolates with ESBL and AmpC-type β -lactamase activities, isolated sequentially following exposure to imipenem. A 79-year-old man with a 12-year history of cancer treatment with numerous hospitalisations was hospitalised in the Intensive Care Unit (ICU) of Bécélère Hospital (suburbs of Paris, France) for brain infarction and sepsis. After 1 week of hospitalisation, rectal screening revealed an ESBL-producing *Escherichia coli* and a meticillin-resistant *Staphylococcus aureus* (MRSA). Two weeks later, the

patient was admitted to the visceral surgery department and two multidrug-resistant ESBL-producing *K. pneumoniae* (KP1 and KP2) were isolated from urine and blood cultures, respectively. The patient received imipenem 500 mg every 8 h for 24 days. He was subsequently re-admitted to the Surgical ICU for sepsis due to *Candida tropicalis*. Upon admission at the ICU, rectal swabs were positive for MRSA and ESBL-producing *K. pneumoniae* KP3 susceptible only to imipenem. The patient again received imipenem for 3 days along with amphotericin B. One month later, a skin swab around the colostomy was positive for an ESBL-producing *E. coli* and an ESBL-producing *K. pneumoniae* KP4 that was resistant to imipenem. The patient died a few days later of uncontrolled septic shock.

2. Materials and methods

2.1. Bacterial strains, antimicrobial agents and minimum inhibitory concentration (MIC) determination

Klebsiella pneumoniae isolates KP1–4 were recovered from colonisation and clinical samples and were identified using the API 20E system (bioMérieux, Marcy-l'Etoile, France). Electrocompetent *E. coli* DH10B (Invitrogen, Éragny, France) was used as a recipient in electroporation experiments, and *E. coli* J53AzR strain, which is resistant to sodium azide, was used for conjugation experiments [6]. *Escherichia coli* 50192 was used as a reference strain for plasmid extraction [6].

Routine antibiograms were determined by the disk diffusion method on Mueller–Hinton (MH) agar (Bio-Rad, Marnes-la-Coquette, France) using susceptibility breakpoints as recommended by the Clinical and Laboratory Standards Institute

(CLSI) [7]. The presence of an ESBL was suggested by a synergy image with the double-disk synergy test performed with cefepime, ceftazidime and ticarcillin/clavulanic acid disks on cloxacillin (200 mg/L)-containing MH agar plates, which partially inhibits cephalosporinase activity [8]. Metallo- β -lactamase Etests were also performed according to the manufacturer's instructions.

2.2. Isoelectric focusing (IEF) and biochemical analysis

Crude β -lactamase extracts were obtained from 10 mL cultures of *K. pneumoniae* KP1 and KP4 and analytical IEF was performed with an ampholine-polyacrylamide gel as described previously [9]. Specific activities for third- and fourth-generation cephalosporins and imipenem were measured spectrophotometrically as described previously [9].

2.3. Analysis of outer membrane protein (OMP) profiles and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

OMP profiles of the four isolates were examined as described previously [10].

2.4. Polymerase chain reaction (PCR) analysis and sequencing

Whole-cell DNA extraction and PCR experiments were performed as described previously [6]. β -Lactamase genes of Ambler classes A, B and D carbapenemases were sought by PCR with primers specific for the *bla*_{GES}, *bla*_{KPC}, *bla*_{IMP}, *bla*_{VIM} and *bla*_{OXA-48} genes. Detection of Ambler class A *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} and of *bla*_{AmpC}-type genes was performed with internal primers as described previously [9].

OMP genes were amplified and sequenced. Primers OmpK36-F (ACA GAG GGT TAA TAA CAT GAA) and OmpK36-R (TAG AAC TGG AAA CCA GGC) were used to amplify a 1107-bp fragment from the OmpK36 gene (GenBank accession no. Z33506), whereas OmpK35-F (CCG GAG TCA TGT TGT AAG TCT) and OmpK35-R (TGA TCC CTG CCC TGC TGG T) were used to amplify a 738-bp fragment from the OmpK35 gene.

The following amplification program was used for each PCR: 10 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 3–5 min at 72 °C; followed by a final extension of 10 min at 72 °C. For SHV PCR, 10% dimethyl sulphoxide (DMSO) was added to the PCR reaction.

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and were subsequently sequenced on both strands on an ABI Prism[®] 3100 sequencer (Applied Biosystems, Les Ulis, France). Nucleotide sequence analysis was performed at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

2.5. Plasmid content and mating-out experiments

Extraction of plasmid DNA from *K. pneumoniae* KP1–4 and direct transfer of resistance into azide-resistant *E. coli* J53 were attempted as previously reported [6,11].

2.6. Southern hybridisation

DNA–DNA hybridisations were performed as described with a Southern transfer of an agarose gel containing extracted DNA by the Kieser extraction method [6]. Two probes consisted of 383-bp and 550-bp PCR-generated fragments and were internal to *bla*_{DHA-1} and *bla*_{CTX-M} genes, respectively. Labelling of the probes and signal detection were carried out using the ECL non-radioactive labelling and detection kit (Amersham Biosciences) according to the manufacturer's instructions.

2.7. Pulsed-field gel electrophoresis (PFGE) analysis

Whole-cell DNA of culture extracts from each isolate embedded in 1% agarose plugs (Bio-Rad) were digested with *Xba*I restriction enzyme (Amersham Pharmacia Biotech) and separated using a CHEF-DR II System (Bio-Rad) as described previously [11]. Following migration, the gel was stained with a 0.5 mg/L ethidium bromide solution and PFGE results were analysed by eye as described previously [11].

3. Results and discussion

3.1. Antimicrobial susceptibility

Routine antibiograms performed by the disk diffusion method on MH agar revealed that strains KP1–3 were cephamycin-resistant and susceptible only to imipenem and colistin. No synergy image could be observed between clavulanic acid and cefepime or ceftazidime. In contrast, use of cloxacillin-containing MH plates (200 mg/L) allowed detection of a synergy image, indicating the presence of an ESBL, but also

the likely presence of an AmpC enzyme. Compared with the other three isolates, KP4 was resistant to carbapenems (imipenem MIC = 12 mg/L) and did not appear to produce metallo- β -lactamases since the MIC of imipenem was not reduced by more than eight-fold in the presence of ethylene diamine tetra-acetic acid (EDTA) (Table 1). Moreover, the MIC of imipenem was reduced on cloxacillin-containing MH plates to 1 mg/L. MICs of tigecycline for the four isolates were identical (2 mg/L).

3.2. Molecular analysis

PCR and sequencing analysis indicated that KP1–4 produced the β -lactamases TEM-1 and CTX-M-15 and a plasmid-mediated DHA-1 β -lactamase. Neither metallo- β -lactamases nor KPC- or OXA-associated carbapenem-hydrolysing β -lactamase genes were found.

3.3. Biochemical analysis

IEF results identified four isoelectric points (pI) (8.1 for SHV-1, >8.5 for DHA-1, 7.6 for CTX-M-15 and 5.3 for TEM-1) in all isolates. A crude β -lactamase extract of KP4 culture had no significant imipenem-hydrolysing activity, suggesting that the carbapenem resistance was not mediated by a carbapenemase. In addition, measurement of specific activities for third- and fourth-generation cephalosporins did not reveal significant differences between KP1 and KP4, suggesting similar levels of expression. Furthermore, induction studies suggested that AmpC is constitutively expressed in KP1 and KP4 at similar levels, ruling thus out that KP4 hyperproduces its AmpC and that this hyperproduction contributes to imipenem resistance.

3.4. Genetic support

KP1–4 all carried two large plasmids (65 kb and 95 kb). The 95-kb plasmid was successfully transferred to *E. coli* J53 by mating-out assay (Fig. 1A). DNA–DNA hybridisation revealed that this plasmid was common to the four isolates and accommodated both the *bla*_{TEM-1} and *bla*_{CTX-M-15} genes. The plasmid-mediated DHA-1 β -lactamase gene was harboured on the 65-kb plasmid but was not self-conjugative to *E. coli*. Antimicrobial susceptibility testing and PCR sequencing analysis of the transformants confirmed DNA–DNA hybridisation results.

3.5. Porin and PFGE analysis

Indistinguishable PFGE patterns were found for KP1–4, suggesting that they belong to the same clone. OMP analysis revealed that KP1–3 produced OmpK35 and OmpK36. For KP4, OmpK36 was not expressed (Fig. 1B). Sequencing analysis found a point mutation in the OmpK36 coding region leading to a premature stop of the protein.

4. Conclusion

Genotypic analysis indicated that the carbapenem-resistant isolate KP4 was a derivative of carbapenem-susceptible *K. pneumoniae* KP1–3, differing by the loss of OmpK36 in KP4.

The OmpK36 deficiency in KP4 was due to disruption of the coding sequence owing to the presence of a point mutation. Several mechanisms, including point mutations, deletions and insertions, have been associated with OmpK36 deficiency, but

insertional interruptions appear to be the most frequent type of change [2]. Different insertion sequences (IS1, IS5, IS26, IS102 and IS903) have been reported and the insertion positions are usually within the first 100 bp from the OmpK36 start codon [2,4].

This mutation in OmpK36 must have occurred/been selected following prolonged imipenem exposure and resulted in carbapenem resistance. In vivo selection of carbapenem-resistant *K. pneumoniae* isolates has already been described during ertapenem treatment as well as with cephamycins [12–14]. In each case, carbapenem resistance was related to selection of OmpK36-deficient strains [12–14].

Selection of porin mutants might be enhanced by antibiotic pressure. Prolonged use of cephamycins, which are poor substrates for ESBLs and thus might in theory be a therapeutic option in this case, is associated with in vivo development of porin-deficient mutants and acquisition of plasmids encoding class C β -lactamases in ESBL-producing organisms [12,15]. Here, both ESBL and plasmid-encoded AmpC were already present in the same isolate, resulting in easy selection of carbapenem resistance. As collateral damage, these plasmid-mediated class C β -lactamases provide not only a broader spectrum of resistance than ESBLs owing to their hydrolysing activity against cephamycins and resistance to β -lactamase inhibitors, but in conjunction with porin losses may also confer higher resistance levels to carbapenems [1]. Expression of ESBLs associated with porin deficiency usually yields ertapenem resistance and reduced susceptibility to imipenem. The diminished production of outer membrane porins, together with the expression of a class C

cephalosporinase and CTX-M-15, appeared to be important factors leading to carbapenem resistance. Induction experiments and specific activity measurements revealed similar expression levels both of AmpC and CTX-M-15 in KP1 and in its derivative KP4.

In the present case, the KP1–3 isolates were multidrug-resistant, being susceptible only to carbapenems. Treatment of serious infections with such bacterial isolates requires carbapenems as monotherapy. The increasing isolation of ESBLs and the increasing prevalence and diversity of acquired AmpC genes is a source of concern since carbapenem resistance may be more often selected [12,15].

This study demonstrated the development of imipenem resistance in an OmpK36-deficient ESBL- and AmpC-producing *K. pneumoniae* strain. Carbapenem resistance in Enterobacteriaceae is due not only to carbapenemases but also to a combination of non-carbapenem-hydrolysing β -lactamases and a permeability defect.

Funding

This work was funded by a grant from the Ministère de l'Éducation Nationale et de la Recherche (UPRES-EA3539), Université Paris XI, Paris, by the Assistance Publique/Hôpitaux de Paris, France, and by the European Community (6th PCRD, LSHMCT- 2003-503-335, TROCAR contract HEALTH-F3-2008-223031).

Competing interests

None declared.

Ethical approval

Not required.

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References

- [1] Queenan AM, Bush K. Carbapenemases: the versatile β -lactamases. *Clin Microbiol Rev* 2007;20:440–58.
- [2] Kaczmarek FM, Dib-Hajj F, Shang W, Gootz TD. High-level carbapenem resistance in a *Klebsiella pneumoniae* clinical isolate is due to the combination of *bla*_{ACT-1} β -lactamase production, porin OmpK35/36 insertional inactivation, and down-regulation of the phosphate transport porin *phoE*. *Antimicrob Agents Chemother* 2006;50:3396–406.
- [3] Mena A, Plasencia V, García L, Hidalgo O, Ayestarán JI, Alberti S, et al. Characterization of a large outbreak by CTX-M-1-producing *Klebsiella pneumoniae* and mechanisms leading to in vivo carbapenem resistance development. *J Clin Microbiol* 2006;44:2831–7.
- [4] Martínez-Martínez L. Extended-spectrum β -lactamases and the permeability barrier. *Clin Microbiol Infect* 2008;14(Suppl 1):82–9.
- [5] Nordmann P, Cuzon G, Naas T. The real threat of KPC carbapenemase-producing bacteria. *Lancet Infect Dis* 2009;9:228–36.
- [6] Naas T, Cuzon G, Villegas MV, Lartigue MF, Quinn JP, Nordmann P. Genetic structures at the origin of acquisition of the β -lactamase *bla*_{KPC} gene. *Antimicrob Agents Chemother* 2008;52:1257–63.
- [7] Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing*. Fifteenth informational supplement. Document M100-S15. Wayne, PA: CLSI; 2005.

- [8] Drieux L, Brossier F, Sougakoff W, Jarlier V. Phenotypic detection of extended-spectrum β -lactamase production in Enterobacteriaceae: review and bench guide. *Clin Microbiol Infect* 2008;14(Suppl 1):90–103.
- [9] Cuzon G, Naas T, Demachy MC, Nordmann P. Plasmid-mediated carbapenem-hydrolyzing β -lactamase KPC-2 in *Klebsiella pneumoniae* from Greece. *Antimicrob Agents Chemother* 2008;52:796–7.
- [10] Filip C, Fletcher G, Wulff JL, Earhart CF. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. *J Bacteriol* 1973;115:717–22.
- [11] Naas T, Zerbib M, Girlich D, Nordmann P. Integration of a transposon Tn 1-encoded inhibitor-resistant β -lactamase gene, *bla*_{TEM-67} from *Proteus mirabilis*, into the *Escherichia coli* chromosome. *Antimicrob Agents Chemother* 2003;47:19–26.
- [12] Elliott E, Brink AJ, van Greune J, Els Z, Woodford N, Turton J, et al. In vivo development of ertapenem resistance in a patient with pneumonia caused by *Klebsiella pneumoniae* with an extended-spectrum β -lactamase. *Clin Infect Dis* 2006;42:e95–8.
- [13] Lee CH, Chia JH, Chu C, Wu TL, Liu JW, Su LH. In vivo selection of OmpK35-deficient mutant after cefuroxime therapy for primary liver abscess caused by *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2006;58:857–60.
- [14] Lee CH, Chu C, Liu JW, Chen YS, Chiu CJ, Su LH. Collateral damage of flomoxef therapy: in vivo development of porin deficiency and acquisition of *bla*_{DHA-1} leading to ertapenem resistance in a clinical isolate of *Klebsiella pneumoniae* producing CTX-M-3 and SHV-5 β -lactamases. *J Antimicrob Chemother* 2007;60:410–3.

- [15] Mata C, Miró E, Rivera A, Mirelis B, Coll P, Navarro F. Prevalence of acquired AmpC β -lactamases in Enterobacteriaceae lacking inducible chromosomal *ampC* genes at a Spanish hospital from 1999 to 2007. Clin Microbiol Infect 2009 June 11 [Epub ahead of print]. doi: 10.1111/j.1469-0691.2009.02864.x

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Fig. 1. Plasmid and outer membrane analysis. (A, top) Plasmid analysis of *Klebsiella pneumoniae* isolates and transconjugants. (A) Kieser extraction method. Lane 1, *K. pneumoniae* KP1; lane 2, transconjugant TcKP1; and lane 3, *Escherichia coli* 50192 harbouring four plasmids. (b,c) DNA–DNA hybridisation with probes internal to *bla*_{CTX-M} (b) and *bla*_{DHA} (c). (B, lower) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel of outer membrane proteins. Lane 1, *K. pneumoniae* wild-type; lane 2, *K. pneumoniae* KP1; lane 3, *Klebsiella pneumoniae* KP4; lane 4, pre-stained protein weight marker.

Table 1

Minimum inhibitory concentrations (MICs) for imipenem (IMP)

Isolate	Date of isolation	Site of isolation	MIC (mg/L)			
			MH		MH + cloxacillin ^a	
			IMP	IMP + EDTA	IMP	IMP + EDTA
KP1	5/05/2007	Urine	0.125	0.125	0.125	0.125
KP2	5/05/2007	Blood culture	0.125	0.125	0.125	0.125
KP3	13/06/2007	Faeces	0.125	0.125	0.125	0.125
KP4	15/06/2007	Colostomy	12	12	1.0	1.0

MH, Mueller–Hinton; EDTA, ethylene diamine tetra-acetic acid.

^a Cloxacillin was added to the plates at a concentration of 200 mg/L.

Figure 1A

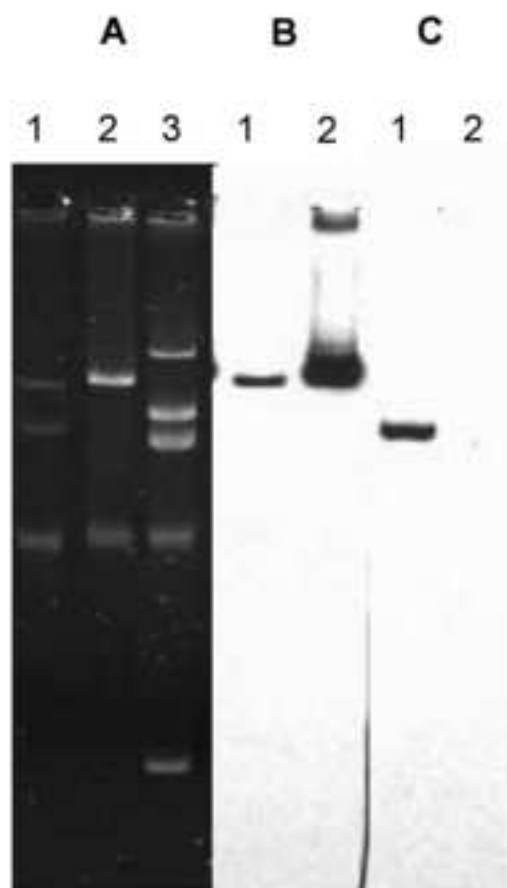


Figure 1B

