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Carbapenem-resistant *Klebsiella pneumoniae* Clinical Isolate Harbouring a blaIMP-4- and *qnrS*-carrying Plasmid, as well as a Conjugative blaKPC-2 Plasmid

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ABSTRACT

Objective: To characterize a carbapenem-resistant *K. pneumoniae* carrying multiple carbapenemase-encoding genes isolated from a hospitalized patient in Shenzhen, China. Multidrug resistance (MDR) phenotypes among Enterobacteriaceae isolates have increasingly become a healthcare problem. KPC and IMP variants have been frequently reported worldwide, while combination of carbapenemases has begun to be described in China and elsewhere.

Methods: During the SENTRY Antimicrobial Surveillance Program for 2011, Enterobacteriaceae displaying meropenem and imipenem MIC results ≥2 mg/L were screened for extended-spectrum β-lactamase (ESBL) and carbapenemases. Susceptibility testing (broth microdilution) and interpretations followed the CLSI guidelines (M07-A9) and EUCAST (2014). Selected isolate (*K. pneumoniae* 27243D) was screened for plasmid AmpC and ESBL genes by Check-Points, according to the manufacturer's instructions, and additional custom PCR assays for detection of β-lactam and fluoroquinolone resistance genes. Carbapenemase gene locations and plasmid sizes were determined by S1 digestion, followed by PFGE, Southern blot and hybridization. Plasmid extraction was carried out by Plasmid MIDI kit. Plasmid transfers were performed by conjugation and electroporation. Genes and surrounding regions were sequenced using generic primers and a primer walking approach, respectively. Plasmid incompatibility groups were assessed by a PCR method.

Results: *K. pneumoniae* 27243D was recovered from an abscess in a 58-year-old female patient. This strain was resistant to β-lactams and fluoroquinolones, while remaining susceptible to aminoglycosides and colistin. PCR and sequencing reactions detected KPC-2- and IMP-4-encoding genes, while negative results were obtained for plasmid AmpC or ESBL genes. Strain 27243D showed three plasmid bands of 50-, 80- and 200-kb, whereas the 50- and 80-kb plasmids carried bla_{IMP-4} and bla_{KPC-2}, respectively. The former plasmid was transferred by electroporation, while transfer of the latter occurred by conjugation. Transconjugant and transformant strains showed elevated MIC values for β-lactams, except that the latter strain carrying bla_{IMP-4}, displayed a low aztreonam MIC result. This same transformant strain also exhibited slightly elevated MIC results to ciprofloxacin and levofloxacin, and further investigations confirmed the presence of *qnrS*. The bla_{IMP-4}- (and *qnrS*-) carrying plasmid belonged to an IncN incompatibility group and the carbapenemase gene was surrounded by IS*26*. Sequencing analysis of bla_{KPC-2} plasmid demonstrated the presence of a Tn*3*-based transposon and partial Tn*4401* segment, consisting of Tn*801*-transposase, Tn*3*-resolvase, IS*Kpn8*, the bla_{KPC-2} gene, and IS*Kpn6*-like. This genetic context was most similar to that of pKP048 (FJ628167) observed in Enterobacteriaceae from China.

Conclusions: This study reports the detection of a MDR *K. pneumoniae* co-harbouring bla_{KPC-2} and bla_{IMP}-genes. bla_{KPC-2} was detected in a conjugative plasmid, which can be easily transferred between species and the bla_{KPC-2} environment was similar to that observed among isolates from China. These findings have been previously reported; however, the bla_{IMP-4}-carrying plasmid also harboured a fluoroquinolone resistance determinant, emphasizing the ability of this species for acquiring and expressing resistance determinants.

INTRODUCTION

Enterobacteriaceae species producing extended-spectrum β-lactamase enzymes are often associated with community- and hospital-acquired infections. However, multiple studies have now reported the detection of carbapenem-resistant Enterobacteriaceae due to the production of numerous carbapenemase enzymes in several countries worldwide. Carbapenemases belong to the Ambler class A (enzymes such as KPC), Ambler class B (metallo-β-lactamases [MBL]; such as NDM, VIM and IMP) or Ambler class D β-lactamases (OXA-48). Overall, Ambler class A (KPC) and class B (MBL) carbapenemase enzymes hydrolyze all clinically available β-lactam agents, except for class B enzymes that do not recognize monobactams. In contrast, OXA-48 shows a different hydrolysis profile, with high activity against penicillins, but low hydrolytic activity against carbapenems.

Carbapenemase-encoding genes are usually located within mobile genetic elements and bla_{KPC} genes have been consistently associated with Tn*4401*. Tn*4401* is a Tn*3*-like transposon that has been identified in distinct plasmids carried by KPC-producing Enterobacteriaceae from various geographic areas. MBL-encoding genes, such as bla_{VIM} and bla_{IMP} are usually detected as part of gene cassettes within Class 1 integrons, while bla_{NDM} can be carried by different plasmid types (IncA/C, IncF, IncL/M, or untypable) and, more rarely, becomes chromosomally integrated. In contrast, the current spread of bla_{OXA-48} gene seems to be associated with the wide dissemination of an identical IncL/M plasmid scaffold. In this study, we report the detection of a carbapenem-resistant *K. pneumoniae* carrying multiple carbapenemase-encoding genes isolated from a hospitalized patient in Shenzhen, China.

MATERIALS AND METHODS

Bacterial strains. During 2011, a total of 867 Enterobacteriaceae isolates collected from hospitalized patients in 12 medical centres in China were submitted to a central monitoring laboratory (JMI Laboratories, North Liberty, USA) as part of SENTRY Antimicrobial Surveillance Program.

Antimicrobial susceptibility test methods. Isolates were tested for susceptibility by broth microdilution following the Clinical and Laboratory Standards Institute (CLSI) M07-A9 document. Bacterial inoculum density was monitored by colony counts to assure an adequate number of cells for each testing event. Validation of the MIC values was performed by concurrent testing of CLSI-recommended quality control (QC) reference strains. All QC results were within published acceptable ranges (M100-S24). MIC interpretations were based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST; 2014) breakpoint criteria, as available.

METHODS-CONTINUED

Screening for antimicrobial resistance genes. Enterobacteriaceae displaying elevated MIC values (≥2 mg/L) for imipenem and meropenem were selected for further investigations. Isolates were screened for the presence of plasmid AmpC genes (ACC-, ACT/MIR-, CMY-, DHA-, and FOX-encoding genes), bla_{CTX-M} (Groups 1, 2, 8+25 and 9), bla_{TEM}, bla_{SHV}, bla_{KPC} and bla_{NDM} using Check-MDR CT101 kit (Check-Points, Wageningen, Netherlands). Additional ESBL (bla_{GES}, bla_{VEB}, bla_{PER}, bla_{PSE}, bla_{BEL}) and carbapenemase (bla_{OXA-48}, bla_{IMP} and bla_{VIM})-encoding genes were screened by custom PCR assays, as well as fluoroquinolones genes (*qnrA*, *qnrB* and *qnrS*). All PCR experiments included reactions containing target DNA templates for each screening primer set utilized. Amplicons generated were sequenced on both strands; nucleotide and deduced amino acid sequences were analysed using the Lasergene software package (DNASTAR, Madison, Wisconsin, USA). Amino acid sequences were compared with those available via internet sources (http://www.ncbi.nlm.nih.gov/blast/).

Genetic location of antimicrobial resistance genes. Agarose embedded total DNA was subjected to partial digestion with S1 nuclease. DNA digests were resolved by electrophoresis on CHEF DRII (BioRad, Richmond, California), followed by Southern blotting and hybridization with digoxigenin labeled (Roche Diagnostics GmbH, Mannheim, Germany) bla_{KPC}- and bla_{IMP}-specific probes. Plasmid sizes were estimated using concatenated Lambda DNA ladder.

Transfer of resistance-carrying plasmid. Plasmid extractions were carried out by using the Plasmid MIDI kit (QIAGEN; Hilden, Germany). Plasmid conjugation was attempted by filter matting using *Escherichia coli* J53 and selected *Klebsiella pneumoniae* 27243D as recipient and donor strains, respectively. Electroporation was performed by standard methods using *E. coli* DH5α as a recipient strain. Selection of transconjugants and transformants were performed using meropenem (1 mg/L) with or without aztreonam (4 mg/L).

Surrounding carbapenemase DNA sequences. The carbapenemase-carrying plasmids were sequenced using a primer walking strategy.

Molecular typing. Multilocus sequence typing (MLST) was performed for the *K. pneumoniae* isolate according to instructions on the website http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html.

RESULTS

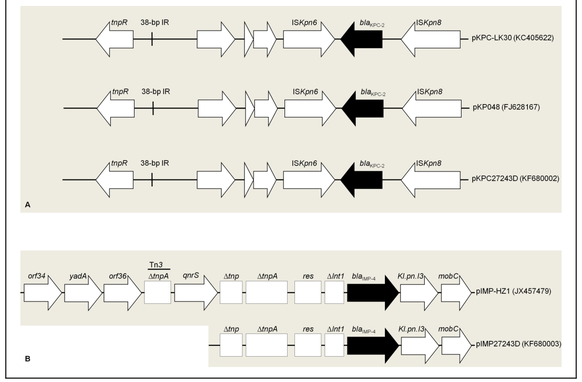
- A total of 18 (2.1%) Enterobacteriaceae demonstrated elevated MIC values (≥2 mg/L) for imipenem and meropenem. During further β-lactamase screening, *K. pneumoniae* 27243D was found to harbour KPC-2- and IMP-4-encoding genes, while negative results were obtained for plasmid AmpC or ESBL genes.
- *K. pneumoniae* 27243D (MLST, 1136) was recovered from an abscess in a 58-year-old female patient. This strain demonstrated a resistance phenotype to β-lactams, while remaining susceptible to amikacin, colistin and tigecycline (**Table 1**).
- Strain 27243D showed three plasmid bands of 50-, 80- and 200-kb in sizes. Hybridization signals were observed from the 50- and 80-kb plasmids when using the bla_{IMP-4}- and bla_{KPC-2}-specific probes, respectively. The former plasmid was transferred by electroporation, while transfer of the bla_{KPC-2}-carrying plasmid occurred by conjugation.
- Transconjugant and transformant strains showed elevated MIC values for β-lactams, except that the *E. coli* DH5α strain carrying bla_{IMP-4}, displayed a low aztreonam MIC result (i.e. 0.06 mg/L). This same transformant strain also exhibited slightly elevated MIC results for ciprofloxacin (0.5 mg/L) and levofloxacin (1 mg/L; **Table 1**). Investigation of fluoroquinolone resistance genes identified the presence of *qnrS*.
- bla_{IMP-4} was the first gene cassette with a truncated integrase gene upstream and a type II intron (*KI.pn.I3*) downstream that disrupted the integron *attC* recombination site. The 8-kb bla_{IMP-4} surrounding region was identical to that previously described in a 50-kb IncN incompatibility group plasmid (also harbouring *qnrS*) found in a *K. pneumoniae* strain associated with medical travel in China (**Figure 1**).
- Sequencing analysis of bla_{KPC-2} plasmid demonstrated the presence of a Tn*3*-based transposon and partial Tn*4401* segment, consisting of Tn*801*-transposase, Tn*3*-resolvase, IS*Kpn8*, the bla_{KPC-2} gene, and IS*Kpn6*-like. This genetic context was most similar to those of pKP048 (FJ628167) and pKP0C-LK30 (KC405622) observed in *K. pneumoniae* from China (**Figure 1**).

Table 1. Antimicrobial susceptibility profiles of *K. pneumoniae* 27243D, and transformant and transconjugant strains.

Antimicrobial agent	MIC (mg/L) [susceptibility category; EUCAST][a]				
	K. pneumoniae 27243D	*E. coli* J53 (bla_{KPC-2})	*E. coli* DH5α (bla_{IMP-4})	*E. coli* J53	*E. coli* DH5α
β-lactam					
Piperacillin/tazobactam	>256 [R]	8 [S]	4 [S]	2 [S]	0.5 [S]
Cefoxitin	>16	16	>16	8	4
Aztreonam	>64 [R]	64 [R]	0.06 [S]	0.06 [S]	0.12 [S]
Ceftriaxone	>8 [R]	>8 [R]	>8 [R]	0.12 [S]	≤0.06 [S]
Ceftazidime	>32 [R]	16 [R]	>32 [R]	0.25 [S]	0.12 [S]
Cefepime	64 [R]	16 [R]	4 [I]	≤0.03 [S]	≤0.03 [S]
Imipenem	>8 [R]	8 [R]	2 [S]	0.25 [S]	0.25 [S]
Doripenem	>8 [R]	8 [R]	4 [R]	≤0.06 [S]	≤0.06 [S]
Meropenem	32 [R]	8 [I]	4 [I]	0.03 [S]	0.03 [S]
Fluoroquinolone					
Ciprofloxacin	2 [R]	0.015 [S]	0.5 [S]	0.015 [S]	0.03 [S]
Levofloxacin	2 [I]	0.06 [S]	1 [S]	0.06 [S]	0.06 [S]
Moxifloxacin	1 [I]	≤0.12 [S]	2 [R]	≤0.12 [S]	≤0.12 [S]
Other					
Amikacin	1 [S]	2 [S]	0.5 [S]	1 [S]	≤0.25 [S]
Colistin	0.5 [S]	0.5 [S]	1 [S]	1 [S]	0.5 [S]
Tigecycline	0.5 [S]	0.12 [S]	0.12 [S]	0.25 [S]	0.12 [S]

S, susceptible; I, intermediate; R, resistant.
a. MIC interpretive criteria as published by EUCAST (2014), as available.

Figure 1. Comparative analysis of linear plasmid representations for (A) bla_{KPC-2}- and (B) bla_{IMP-4}-carrying plasmids.



CONCLUSIONS

- This study reports the detection of a MDR *K. pneumoniae* co-harbouring bla_{KPC-2}- and bla_{IMP-4}-carrying plasmids. The 50-kb bla_{IMP-4}-carrying plasmid also had a fluoroquinolone resistance determinant (*qnrS*) and demonstrated a genetic context identical to that reported in a *K. pneumoniae* strain associated with medical travel in China.
- bla_{KPC-2} was detected in a conjugative plasmid, which can be easily transferred between Enterobacteriaceae species and the bla_{KPC-2} environment was similar to that observed among isolates from China.
- These findings have been previously reported; however, the bla_{IMP-4}-carrying plasmid also harboured a fluoroquinolone resistance determinant, emphasizing the ability of this species for acquiring and expressing resistance determinants.

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