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SHORT REPORT

Simultaneous three Enterobacteriaceae with different *bla* $_{NDM-1}$-encoding plasmids in a patient transferred from mainland China to Taiwan

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Abstract: New-Delhi metallo-β-lactamase1 (NDM-1) *Enterobacteriaceae* are increasing worldwide. Herein, we describe a single patient who carried three unusual NDM-1 carbapenem-resistant *Enterobacteriaceae – Enterobacter cloacae (E. cloacae)* yielded from a urine specimen and *Klebsiella pneumoniae (K. pneumoniae)* and *Escherichia coli (E. coli)* from stool specimens. For *E. cloacae*, its *bla* $_{NDM-1}$-encoding plasmid was pKP04NDM with a size of ~54 kb replicons with an IncN backbone. For *K. pneumoniae*, its *bla* $_{NDM-1}$-encoding plasmid was pNDM-BTR with a size of ~59.6 kb and belonged to IncN. For *E. coli*, its main *bla* $_{NDM-1}$-encoding plasmid was pIMP-HK1500, and the NDM-1 gene was obtained from a part of pNDM-BTR (8439 bp). These three clinical strains are reported for the first time and are assumed to be imported from mainland China to Taiwan. The three different plasmids were never reported in *K. pneumoniae, E. coli,* and *Citrobacter spp* before. Owing to their associated multidrug resistance, appropriate measures of periodic, targeted surveillance, and development of new antimicrobial agents are urgently needed.
Keywords: carbapenem-resistant *Enterobacteriaceae*, NDM-1, *K. pneumoniae*, *E. coli*, *Citrobacter*, plasmid

Introduction

Carbapenem-resistant *Enterobacteriaceae* are increasing worldwide and have become a severe public health threat.[1] Several mechanisms of carbapenem resistance, including the production of extended spectrum β-lactamase (ESBL) and AmpC enzymes, and secretions of carbapenemase, have been reported. In terms of carbapenemases, *Klebsiella pneumoniae* carbapenemase (KPC) and New-Delhi metallo-β-lactamase1 (NDM-1) are the most notorious because they are associated with high-level carbapenem resistance and can spread between different species of *Enterobacteriaceae*.[2] This critical condition was also noted in Taiwan,[3,4] and the National Task Force of the Carbapenem Resistance Monitoring Program was implemented. Through this program, we first detected a single patient who carried three unusual NDM-1 producing carbapenem-resistant *Enterobacteriaceae*.

Methods

Bacterial strains

Three strains, carbapenem-resistant *Enterobacter cloacae (E. cloacae)* CRE961, *K. pneumoniae* CRE967, and *Escherichia. coli (E. coli)* CRE968, were isolated from a 30-year-old, previously healthy woman who was transferred from mainland China and hospitalized at Chi-Mei Medical Center in Taiwan for spontaneous brainstem hemorrhage. Because of her vegetative status, she received a tracheostomy and prolonged

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mechanical ventilation in mainland China. The patient was initially hospitalized in mainland China for one month and then transferred to Taiwan for long-term care. *E. cloacae* CRE961 was isolated from the first urine specimen in Taiwan and persisted during the whole course. One month after admission in Taiwan, the patient had diarrhea, and we sent the stool specimen for an initial examination. *E. cloacae* CRE961 was yielded from the urine specimen on April 24 and May 3 in 2017 and *K. pneumoniae* CRE967 and *E. coli* CRE968 were yielded from stool specimens on May 25. These bacterial species were confirmed by a VITEK 2 automated system (bioMe ´rieux, Marcy l'Etoile, France) with VITEK®2 GN ID card. These isolates were stored at –80°C in Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, UK) before investigation. The patient's written informed consent was obtained; however, no ethical approval was required for this case from the Chi Mei Medical Center as this study was based on retrospective design with routine laboratory work.

Antimicrobial susceptibility testing and minimum inhibitory concentration (MIC) measurement

Standard powders of amikacin, ciprofloxacin, doxycycline, ertapenem, gentamicin, imipenem (U.S. Pharmacopeia, Rockville, MD, USA), ampicillin, cephalothin, cefuroxime, ceftriaxone, ceftazidime, colistin sulfate, doripenem, meropenem, trimethoprim/sulfamethoxazole (Sigma-Aldrich, St. Louis, MO, USA), fosfomycin (Ercros, Barcelona, Spain), and tigecycline (Pfizer, New York, NY, USA) were used for antimicrobial susceptibility tests. MIC determinations and susceptibility interpretation criteria followed the Clinical Laboratory and Standard Institute (CLSI) and Federal Drug Administration standards.[5,6] MICs of the drugs, except tigecycline and colistin, were measured by agar dilution in Mueller–Hinton agar (Oxoid, Basingstoke, UK) according to CLSI recommendations.[5] For fosfomycin susceptibility, glucose-6-phosphate (25 mg/mL) was added to the agar plate. Tigecycline and colistin MICs were determined by broth microdilutions in freshly prepared cation-adjusted Mueller–Hinton broth.[8] *E. coli* ATCC 25922 was used as the control strain.

Multilocus sequence typing (MLST)

MLST was performed with seven housekeeping genes (*E. cloacae* CRE961, including dnaA, fusA, gyrB, leuS, pyrG, rplB, and rpoB; *K. pneumoniae* CRE967, including gapA, infB, mdh, pgi, phoE, rpoB, and tonB, and *E. coli* CRE968, including adk, fumC, gyrB, icd, mdh, purA, and recA) as previously described.[7–9] The allele sequences and STs were verified at http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html.

DNA manipulation and PCR amplification

Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Plasmid DNA was also extracted by a QIAprep Spin Miniprep kit (Qiagen). PCR amplifications were performed using specific primers as previously described.[7]

PCR detection and sequencing of antibiotic resistance genes

PCR was used to amplify the ESBL genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M}), ampC genes (bla_{DHA-1} and bla_{CMY-2}) and to screen the representative carbapenemase gene (bla_{KPC-2}, bla_{NDM}) using specific primers as previously published.[10–13] Amplicons of β-lactamase genes were purified with PCR clean-up kits (Roche Diagnostics GmbH, Penzberg, Germany) and were sequenced on an ABI PRISM 3730 sequencer analyzer (Applied Biosystems, Foster City, CA, USA).[7]

S1-nuclease pulsed-field gel electrophoresis (PFGE)

Plasmid DNA was extracted from bacteria with the Qiagen Midi Kit (Qiagen). Plasmid sizing was performed using S1-nuclease (Promega, Madison, WI, USA) digested plasmid DNA, and then separated by PFGE using a CHEF mapper system (Bio-Rad, Berkeley, CA, USA) as previously described.[14]

Southern Blot of NDM-1

Southern Blotting was performed using semi-dry transfer system (Bio-Rad) and NDM-containing plasmids were identified by hybridization with Dig-labeled bla_{NDM}-specific probe generated by the PCR DIG Probe Synthesis Kit, and Detection Starter Kit II (Roche Applied Sciences, Mannheim, Germany) as previously reported.[14]

Plasmid sequencing

Bacterial pellets in centrifuge tubes were resuspended in buffer. Cell wall was removed by enzymatic digestion in the presence of RNase. Cell lysis and chromosome removal was achieved by alkaline lysis, followed by acid aggregation and centrifugation. DNA in the supernatant was extracted using organic solvent and recovered by ethanol precipitation. Concentration of samples was determined by fluorescence

quantification. Purified DNA was analyzed by electrophoresis in 0.4% agarose gel matrix. The Illumina MiSeq System (Illumina, San Diego, CA, USA) was used for plasmid sequencing. The derived reads were assembled using the CLC Genomics Workbench 5.51 (CLC bio, Aarhus, Denmark).[15]

Results

Antibiotic susceptibility

The MIC values of the antibiotics against three CRE strains are shown in Table 1. All of them were susceptible to tigecycline and colistin but resistant to all carbapenems, including imipenem, meropenem, ertapenem, and doripenem. In addition, these three strains were resistant to most of other antibiotics.

Molecular characteristics

Based on the MLST, these three strains belonged to different ST types, *E. cloacae* (ST932), *K. pneumoniae* (ST656), and *E. coli* (ST131), which were detected by different housekeeping genes. Plasmid DNA was converted to a linear form by S1-nuclease on PFGE and agarose gel showing the S1-nuclease PFGE-based sizing of plasmids for 3 isolates (Figure 1A, arrows). Figure 1B shows the corresponding gel after Southern Blotting, and the plasmids with NDM-1 were detected by Southern Blot with a specific probe.

Antibiotic-resistant gene

All three isolates were positive for bla_{ESBL}, and the gene encoding CTX-M-55 was detected for *K. pneumoniae* CRE967, and *E. coli* CRE968. The genes encoding SHV-12 and SHV-31 were detected for *E. cloacae* CRE961, and *K. pneumoniae* CRE967, respectively. Only one strain, *K. pneumoniae* CRE967, was positive for bla_{ampc}, which is where the gene encoding DHA-1 was found. In addition, the gene encoding TEM was found for *K. pneumoniae* CRE967, and *E. coli* CRE968.

Sequencing analyses of the carbapenem-resistant plasmids

For *E. cloacae* CRE961, its bla_{NDM-1}-encoding plasmid was pKP04NDM (GenBank accession no. KU314941.1), with a size of ~54 kb, and an IncN backbone (Figure 2A). For

Table 1 Minimal inhibitory concentrations of various antibiotics against three *Enterobacteriaceae*

MIC and β-lactamase gene	*Enterobacter cloacae* CRE961	*Klebsiella pneumoniae* CRE967	*Escherichia coli* CRE968
MIC			
Ampicillin	>128	>128	>128
Cephalothin	>128	>128	>128
Cefuroxime	>128	>128	>128
Ceftriaxone	>128	>128	>128
Ceftazidime	>128	>128	>128
Gentamicin	4	>128	128
Amikacin	64	>128	2
Ciprofloxacin	32	>64	>64
Sulfamethoxazole/trimethoprim	0.25	>128	128
Imipenem	4	8	16
Meropenem	4	8	8
Ertapenem	16	16	16
Doripenem	8	16	16
Doxycycline	32	8	16
Tigecycline	0.5	2	0.5
Fosfomycin	>1,024	8	2
Colistin	0.5	1	1
β-lactamase gene			
DHA	–	1	–
TEM	–	1	1
CMY	–	–	–
SHV	12	31	–
CTX-M3 family	–	55	55
CTX-M14 family	–	–	–

K. pneumoniae CRE967, its bla_{NDM-1}-encoding plasmid was pNDM-BTR (GenBank accession no. KF534788.2) with a size of ~59.6 kb and belonging to IncN (Figure 2B). For *E. coli* CRE968, its main bla_{NDM-1}-encoding plasmid was pIMP-HK1500 (GenBank accession no. KT989599.1), and the NDM-1 gene was obtained from a part of pNDM-BTR (8439 bp). Its size was 60.7 kb and belonged to IncX3 (Figure 2C).

Discussion

Since NDM-1 was first identified in a *K. pneumoniae* isolate in India in 2009,[16] more and more carbapenem-resistant Enterobacteriaceae have been found to carry NDM-1.[17,18] In this report, we present a rare case of three different NDM-1 carbapenem-resistant *Enterobacteriaceae* that were isolated from the clinical specimens of one patient during a 1-month hospitalization. It is difficult to identify the sources of the three different unusual NDM-1 *Enterobacteriaceae* of this patient. Clinically, these three NDM-1 *Enterobacteriaceae* were considered as colonization strains. During the hospitalization in Taiwan, the patient ever received the following antibiotics – levofloxacin, ceftriaxone, minocycline, and amoxicillin/clavulanate. According to the clinical history, it was thought that all of three NDM-1 *Enterobacteriaceae* were carried by this patient from mainland China to Taiwan.

IncX3 and IncN were two major plasmids found in three NDM-1 *Enterobacteriaceae*. IncX plasmids are thought to be narrow-host-range plasmids of *Enterobacteriaceae* and are low in prevalence. Some studies from China have shown that the most common plasmid Inc type to harbor bla_{NDM} was IncX3, indicating that the main manner mediating the transfer

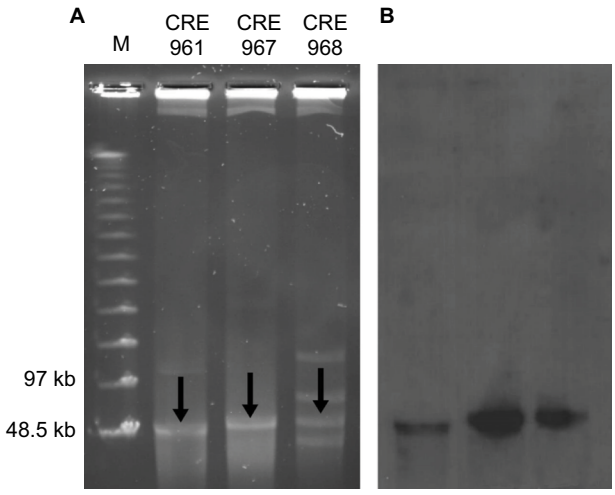

Figure 1 (**A**) S1-nuclease pulsed-field gel electrophoresis profiles and (**B**) Southern Blot of three *Enterobacteriaceae* carrying the NDM-1 plasmid.

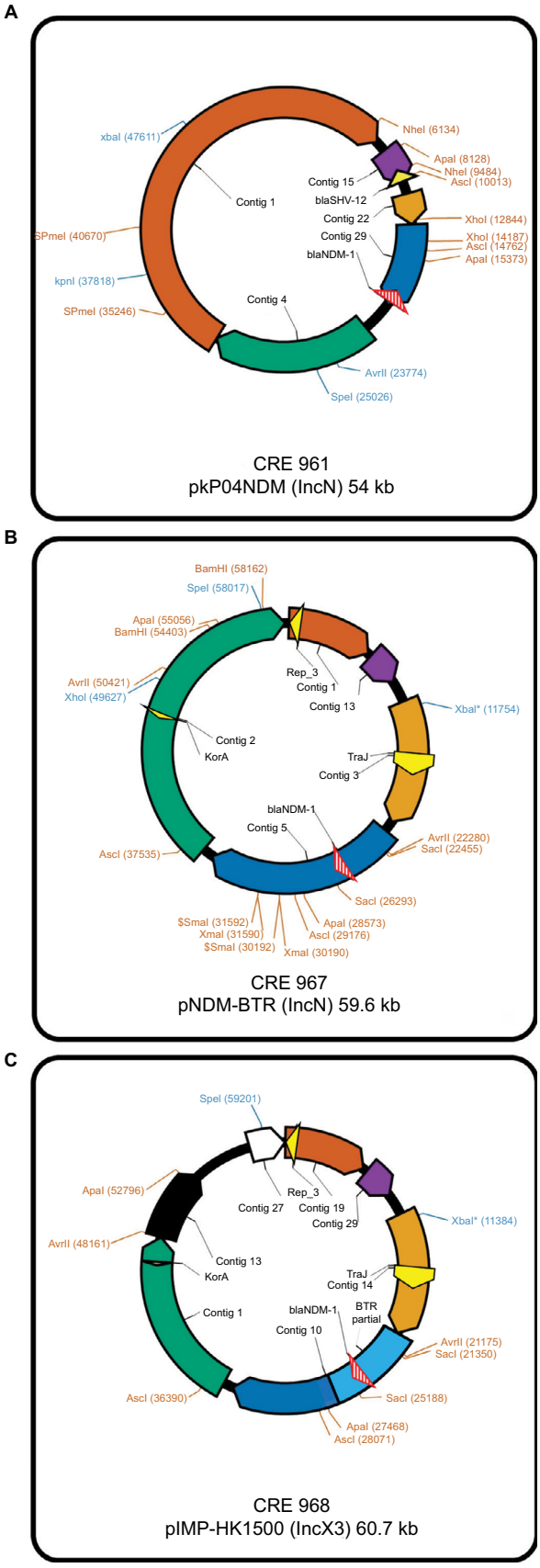

Figure 2 Schematic diagrams of different plasmids (**A**) *Enterobacter cloacae* CRE961 (pKP04NDM), (**B**) *Klebsiella pneumoniae* CRE967 (pNDM-BTR), and (**C**) *Escherichia coli* CRE968 (pIMP-HK1500).

of bla_{NDM} may be the spread of IncX3 plasmids in China.[19] Previous reports also indicated that the IncN plasmid has been shown to encode clinically important resistance determinants among *E. coli* and also in *K. pneumoniae* isolates, such as bla_{CTX-M} and bla_{NDM-1}.[20] Highly efficient transmission of the IncN and IncX plasmids appeared to account for the diversity and worldwide spread of bla_{NDM-1}-carrying *Enterobacteriaceae* just as the results found in our three NDM-1 *Enterobacteriaceae*.

E. cloacae CRE961 belongs to ST932, which has never been identified in Taiwan or the whole world, and its bla_{NDM-1}-encoding plasmid is pKP04NDM. The genetic environment surrounding bla_{NDM-1} was identical to that found in various bla_{NDM-1}-carrying plasmids in *Enterobacteriaceae* in mainland China, including pKP04NDM (KU314941), pNDM-HN380 (JX104760), pKPN5047 (KC311431), and pNDM-SX04 (KC876051) in *K. pneumoniae*.[21] In other words, plasmid pKP04NDM was generally found in *K. pneumoniae,* which is the first reported *E. cloacae* ST 932 that carried such a plasmid with the NDM-1 gene.

K. pneumoniae CRE967, which belongs to ST656, has been found in mainland China[22,23]and the Philippines,[23] but not Taiwan. Its bla_{NDM-1}-encoding plasmid pNDM-BTR has been found in *E. coli* strains in mainland China.[24] Generally, plasmid pNDM-BTR is found in *E. coli* ST131. However, this is the first report of a *K. pneumoniae* ST656 that carried such an NDM-1 plasmid.

E. coli CRE968 belongs to ST131 and has been identified in Thailand[25] and mainland China.[26] Its main bla_{NDM-1}-encoding plasmid is pIMP-HK1500, and the NDM-1 gene is obtained from a part of pNDM-BTR (8439 bp). Insertion sequences were found on both sides of NDM-1 gene in pNDM-BTR. Therefore, we speculate the insertion sequence was inserted into pIMP-HK1500 plasmid direct or indirectly.[27] Plasmid pIMP-HK1500 is generally found in *Citrobacter* spp,[28] but a plasmid that carried the NDM-1 gene has never been reported before among *E. coli* ST131 due to most of them carrying the NDM-1-carrying plasmid pNDM-BTR. In addition to NDM-1, co-carriage of different ESBL and AmpC genes in combination were noted among one of the NDM-1 isolates, *K. pneumoniae* CRE967, in the present report.

Initially, we thought that the three different bla_{NDM-1} isolates in one patient may have been caused by plasmid transformation or conjugation among different bacteria. However, our analysis of different plasmids revealed that they not only harbor the NDM-1 gene but also different characteristics among the plasmids. In addition, according to the plasmid analysis results, we can also find the possible plasmid transformation among different *Enterobacteriaceae*, which seems to be more severe in mainland China; further infection control intervention should be implemented in the future.

In this report, all three NDM-1 carbapenem-resistant Enterobacteriaceae were resistant to all the tested carbapenems, including imipenem, meropenem, and doripenem, but remained susceptible to tigecycline and colistin. This is consistent with previous reports[29] regarding NDM-1 in Taiwan. Although the number is limited, the result suggests that colistin or tigecycline may be the drug of choice for these pathogens in Taiwan.

In conclusion, we identified the first patient with three different NDM-1 carbapenem-resistant *Enterobacteriaceae* isolated during a single 1-month hospitalization. Most importantly, *E. cloacae* ST-932 with a bla_{NDM-1}-encoding plasmid pKP04NDM, *K. pneumoniae* ST-656 with a bla_{NDM-1}-encoding plasmid pNDM-BTR, and *E. coli* ST-131 with a main plasmid pIMP-HK1500 inserted with the NDM-1 gene are reported for the first time. Owing to their associated multidrug resistance, appropriate measures of periodic, targeted surveillance, and development of new antimicrobial agents are urgently needed not only in Taiwan but around the world.

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Disclosure

The authors report no conflicts of interest in this work.

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