

Characterization of an *Enterobacter cloacae* Strain Producing both KPC and NDM Carbapenemases by Whole-Genome Sequencing

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A carbapenem-resistant *Enterobacter cloacae* strain, WCHECI-14653, causing a fatal bloodstream infection, was characterized by genome sequencing and conjugation experiments. The strain carried two carbapenemase genes, *bla*_{NDM-1} and *bla*_{KPC-2}, on separate IncF plasmids. The coexistence of *bla*_{NDM-1} and *bla*_{KPC-2} conferred slightly higher-level carbapenem resistance compared with that of *bla*_{NDM-1} or *bla*_{KPC-2} alone, and the coexistence of two IncF plasmids may generate new platforms for spread- ing carbapenemase genes.

Carbapenem-resistant *Enterobacteriaceae* (CRE) are a serious challenge for clinical management and public health. *Klebsiella pneumoniae* carbapenemase (KPC) and NDM represent two major families of carbapenemases, which are able to confer resistance to almost all β -lactams, including carbapenems, in the *Enterobacteriaceae*. Although isolates producing either KPC or NDM enzymes have been reported worldwide, isolates producing both KPC and NDM enzymes rarely have been detected in the same strain before (1–3). The coproduction of two major carbapenemases in a single strain may be significant, as it may confer higher-level resistance to carbapenems. During the ongoing surveillance of CRE in our hospital, an *Enterobacter cloacae* isolate was found to simultaneously produce a KPC and an NDM carbapenemase and is reported here.

Strain WCHECI-14653 was recovered from a blood sample from a 44-year-old male patient with severe pancreatitis in the intensive care unit (ICU) of West China Hospital, Chengdu, China, in April 2014. The patient had no travel history in the year prior to his admission to the ICU. The strain was identified as *E. cloacae* using the Vitek II system (bioMérieux, Durham, NC, USA), and species identification was confirmed by partially sequencing the *gyrB* gene, as described previously (4). *In vitro* antimicrobial susceptibility tests were performed using the Vitek II system, and the MICs of amikacin, ceftazidime, ciprofloxacin, colistin, imipenem, meropenem, and tigecycline were also determined using the microdilution broth method, according to recommendations of the Clinical and Laboratory Standards Institute (CLSI) (5). Breakpoints defined by CLSI were applied, except for tigecycline and colistin, for which those defined by EUCAST were used. WCHECI-14653 was resistant to ceftazidime (MIC, >512 μ g/ml), ciprofloxacin (64 μ g/ml), imipenem (128 μ g/ml), meropenem (128 μ g/ml), and tigecycline (8 μ g/ml) but susceptible to amikacin (16 μ g/ml) and colistin (2 μ g/ml). WCHECI-14653 was also resistant to ampicillin-sulbactam, aztreonam, cefepime, ertapenem, gentamicin, levofloxacin, nitrofurantoin, piperacillin-tazobactam, tobramycin, and trimethoprim-sulfamethoxazole, as determined by the Vitek II system.

Acquired carbapenemase-encoding genes *bla*_{GES}, *bla*_{IMP}, *bla*_{KPC}, *bla*_{NDM}, *bla*_{OXA-48}, and *bla*_{VIM} were screened using PCR, as described previously (6–9). Both *bla*_{NDM} and *bla*_{KPC} were detected in WCHECI-14653. The complete coding sequences of *bla*_{NDM} and

TABLE 1 MICs of carbapenems and ceftazidime against DH5 α containing one or two carbapenemase gene-carrying plasmids

Strain	MIC (μ g/ml) of:		
	Ceftazidime	Imipenem	Meropenem
<i>E. coli</i> DH5 α	0.5	0.06	0.12
<i>E. coli</i> DH5 α :pNDM1_EC14653	>512	16	32
<i>E. coli</i> DH5 α :pKPC2_EC14653	64	16	16
<i>E. coli</i> DH5 α :pNDM1_EC14653: pKPC2_EC14653	>512	32	64
<i>E. cloacae</i> WCHECI-14653	>512	128	128

*bla*_{KPC} were obtained with additional primers (6, 10), and sequencing revealed the presence of *bla*_{NDM-1} and *bla*_{KPC-2}.

Conjugation experiments were performed using azide-resistant *Escherichia coli* strain J53 as the recipient. Potential transconjugants were selected on medium containing 2 μ g/ml meropenem and 150 μ g/ml sodium azide. Additionally, plasmid DNA from WCHECI-14653 was prepared by alkaline lysis and then electroporated into *E. coli* strain DH5 α . Potential transformants were selected on medium containing 2 μ g/ml meropenem for selection. Transconjugants and transformants were screened for *bla*_{NDM-1} and *bla*_{KPC-2} by PCR. *bla*_{NDM-1} was transferred by conjugation, but *bla*_{KPC-2} was not. In contrast, transformants containing *bla*_{NDM-1} or *bla*_{KPC-2} or both genes were obtained via electroporation. The self-transmissible plasmid carrying *bla*_{NDM-1} was designated pNDM1_EC14653, while the non-self-transmissible plasmid carrying *bla*_{KPC-2} was designated pKPC2_EC14653. The MICs of ceftazidime, imipenem, and meropenem for DH5 α ,

Received 1 June 2015 Returned for modification 28 June 2015

Accepted 19 July 2015

Accepted manuscript posted online 27 July 2015

Citation Wu W, Feng Y, Carattoli A, Zong Z. 2015. Characterization of an *Enterobacter cloacae* strain producing both KPC and NDM carbapenemases by whole-genome sequencing. *Antimicrob Agents Chemother* 59:6625–6628. doi:10.1128/AAC.01275-15.

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doi:10.1128/AAC.01275-15

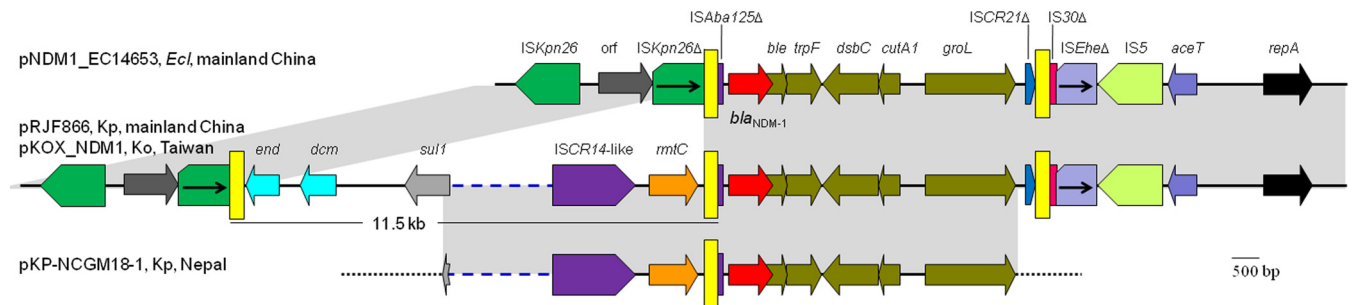


FIG 1 Genetic contexts of *bla*_{NDM-1} on pNDM1_EC14653 and related plasmids. The plasmid names, host species (Ecl, Kp, and Ko stand for *E. cloacae*, *K. pneumoniae*, and *K. oxytoca*, respectively), and locations for each structure are listed. The GenBank accession numbers are [KP868647](#) for pNDM1_EC14653, [KF732966](#) for pRJF866, [JQ314407](#) for pKOX_NDM1, and [AB824738](#) for pKP-NCGM18-1. The identical regions are highlighted in gray. The 256-bp MITEs are indicated as yellow bars. Broken lines in blue, which are not scaled, represent structures that are not shown. Dotted lines in black represent sequences that are not available. *end* and *dcm* form a type II restriction-modification system and encode an endonuclease III and a DNA methyltransferase, respectively.

DH5 α containing pNDM1_EC14653, DH5 α containing pKPC2_EC14653, and DH5 α containing both plasmids were determined using the CLSI microdilution method (5). The presence of two carbapenemase genes in a single strain conferred slightly increased resistance to carbapenems compared to that in the presence of either carbapenemase gene individually; a 2- to 4-fold increase in MIC was observed for imipenem and meropenem (Table 1). This might provide an advantage for the host strain to survive carbapenem exposure during treatment. The coexistence of two carbapenemase genes on separate plasmids may allow new platforms to be generated for mediating the spread of antimicrobial resistance genes.

Whole-genome sequencing of WCHECI-14653 was performed using the HiSeq 2500 sequencer (Illumina, San Diego, CA), with 100 \times coverage. Using the SPAdes program, reads were assembled into 151 contigs, of which 93 were \geq 500 bp in length (N_{50} , 236,790 bp) (11). Strain WCHECI-14653 was of the sequence type 177 (ST177), as determined using the genomic sequence to query the multilocus sequence typing database of *E. cloacae* (<http://pubmlst.org/ecloacae/>). ST177 is a single-allele variant of ST93, which comprises the *E. cloacae* strain ECNIH2 carrying *bla*_{KPC-2}, recovered from a sink at the National Institutes of Health Clinical Center in 2012 (12). The sequences of pNDM1_EC14653 and

pKPC2_EC14653 were completely circularized, with intervals between contigs being filled by PCR and Sanger sequencing.

The 109-kb pNDM1_EC14653 plasmid contained two IncF replicons, FIB (type 36) and FIIY (type 4). The backbone of pNDM1_EC14653 is identical to two FIB36:FIIY4 plasmids, pRJF866 (GenBank accession no. [KF732966](#)) from a *K. pneumoniae* from Shanghai, and pKOX_NDM1 (GenBank accession no. [JQ314407](#)) from a *Klebsiella oxytoca* isolate from Taiwan (13). The genetic context of *bla*_{NDM-1} on pNDM1_EC14653 is also almost identical to that on pRJF866 and pKOX_NDM1 (Fig. 1). In addition, the partially sequenced plasmid pKP-NCGM18-1 (GenBank accession no. [AB824738](#)) from a *K. pneumoniae* isolate from Nepal (14) is likely to be similar to pRJF866 and pKOX_NDM1, based on the genetic context of *bla*_{NDM-1} and its available sequence (13.4 kb), with only three nucleotide differences (Fig. 1). On these plasmids, *bla*_{NDM-1} and with commonly found downstream genes, i.e., *ble-trpF-dsbC-cutA1-groL*, are bracketed by two copies of a 256-bp miniature inverted-repeat transposable element (MITE) (small nonautonomous mobile element containing repeated sequences) (15). The MITE found here is AT rich (AT content, 57.8%), has a 39-bp imperfect inverted repeat (IR) (33 of the 39 bp matched), and contains no open reading frames. It has been suggested that the mobilization of *bla*_{NDM-1} onto the FIB36:FIIY4

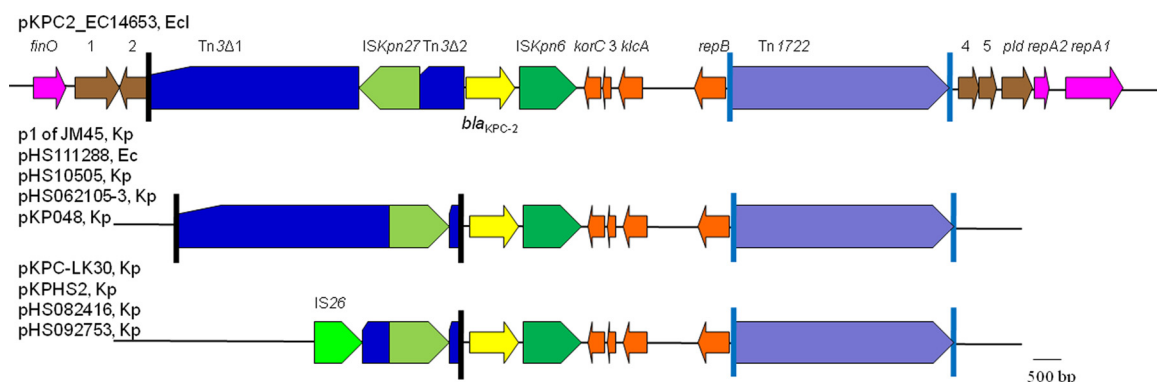


FIG 2 Genetic contexts of *bla*_{KPC-2} on pKPC1_EC14653 and related plasmids. The plasmid names, host species (Ec, Ecl, and Kp stand for *E. coli*, *E. cloacae*, and *K. pneumoniae*, respectively) and locations of each structure are listed. The GenBank accession numbers are [KP868647](#) for pKPC1_EC14653, [CP006657](#) for plasmid p1 of *K. pneumoniae* strain JM45, [KF826291](#) for pHS111288, [KF826292](#) for pHS10505, [KF623109](#) for pHS062105-3, [FJ628167](#) for pKP048, [KC405622](#) for pKPC-LK30, [CP003224](#) for pKPHS2, [KF724507](#) for pHS082416, and [KF826293](#) for pHS092753. All of the plasmids were found in isolates from mainland China, except pKPC-LK30, which was from an isolate from Taiwan. The vertical lines are the IR of Tn3 (black) and Tn1722 (cyan). Open reading frames (ORFs) of unknown function are indicated by numbers 1 to 5.

TABLE 2 Strains carrying *bla*_{NDM-1} and *bla*_{KPC-2}

Strain	Species	Source	Yr	Country	Location of <i>bla</i> _{NDM-1}	Location of <i>bla</i> _{KPC-2}	Reference
IR98	<i>K. pneumoniae</i>	Urine	2010	India	160-kb self-transmissible IncA/C plasmid	70-kb non-transmissible nontypeable plasmid	3
CCBH14397	<i>E. hormaechei</i>	Rectal swab	2013	Brazil	160-kb IncA/C plasmid ^a	50-kb IncN plasmid ^a	1
E134	<i>E. cloacae</i>	Blood	2013	Brazil	30-kb IncP self-transmissible plasmid	Chromosome	2
WCHECI-14653	<i>E. cloacae</i>	Blood	2014	China	109-kb IncF self-transmissible plasmid	88-kb IncF non-transmissible plasmid	This study

^a Whether the plasmid was self-transmissible was not determined.

plasmid was mediated by the transposition of this MITE (13). Compared to pRJF866 and pKOX_NDM1, which have three copies of the MITE, pNDM1_EC14653 has two copies. pRJF866 and pKOX_NDM1 contain a large 11.5-kb region upstream of *bla*_{NDM-1}, containing a 16S rRNA methylase gene, *rmtC* (conferring high-level resistance to aminoglycosides), an ISCR14-like element, *tni*-like transposase/integrase genes, *sul1* (conferring resistance to sulfonamides), and a type II restriction-modification system that is not present on pNDM1_EC14653. The presence or absence of this 11.5-kb region on different plasmids is likely due to homologous recombination between two copies of the MITE. The same plasmid backbone and highly similar regions containing *bla*_{NDM-1} of pNDM1_EC14653, pRJF866, and pKOX_NDM1 suggests that the three plasmids evolved from a common ancestor and diverged recently.

The 88-kb plasmid pKPC2_EC14653 had only a single FIIY replicon, which was classified as a new type, type 5, by a plasmid multilocus sequence typing (MLST) scheme (16). Although both pNDM1_EC14653 and pKPC2_EC14653 contain an FIIY replicon, it has been documented that two plasmids containing FII-like replicons could be compatible in the one isolate (17). FIIY replicons have been thought to originate from *Yersinia* spp., but it is evident now that they are also present in the *Enterobacteriaceae*. The FIIY5 replicon is also present on the *bla*_{KPC-2}-carrying plasmid pKPC-727 of *K. oxytoca* strain KONIH1 (GenBank accession no. CP008791) (12) and the plasmid pNJST258C1, which does not carry *bla*_{KPC-2}, of *K. pneumoniae* strain 30684/NJST258_2 (GenBank accession no. CP006922) (18). Based on available sequences, it appears that *bla*_{KPC-2} is commonly flanked by Tn3 upstream and by Tn1722 downstream, as the genetic context of *bla*_{KPC-2} on pKPC2_EC14653 is similar to several known plasmids found in isolates from China (Fig. 2). However, the Tn3-like transposon on pKPC2_EC14653 was interrupted by the insertion of ISKpn27 and was also truncated at its *bla*_{TEM} end. The Tn3-*bla*_{KPC-2}-Tn1722 region is flanked by various genes on different plasmids (Fig. 2), suggesting that the region has been mobilized onto different plasmids, although the mechanism mediating this event has not been determined. As Tn3 transposons are widespread among the *Enterobacteriaceae*, we speculate that the mobilization of the Tn3-*bla*_{KPC-2}-Tn1722 region might be due to homologous recombination between different Tn3 transposons.

The coexistence of *bla*_{NDM-1} and *bla*_{KPC-2} has been identified in three isolates of the *Enterobacteriaceae* (Table 2). In these isolates, *bla*_{NDM-1} was carried by IncA/C or IncP plasmids, which were generally self-transmissible, while *bla*_{KPC-2} was located either on non-self-transmissible plasmids or on a chromosome (Table 2). On an IncN plasmid of *Enterobacter hormaechei* strain CCBH14397, *bla*_{KPC-2} was carried by a Tn4401 transposon (1), which is different from the Tn3-*bla*_{KPC-2}-Tn1722 structure on pKPC2_EC14653.

Otherwise, the genetic contexts of *bla*_{NDM-1} and *bla*_{KPC-2} are unknown in the three isolates.

In conclusion, the study characterizes a clinical *E. cloacae* strain carrying both *bla*_{KPC-2} and *bla*_{NDM-1}. The coexistence of two carbapenemase genes confers slightly increased resistance to carbapenems, and such a coexistence may generate new platforms to carry both genes and mediate their further spread.

Nucleotide sequence accession numbers. The complete sequences of pKPC2_EC14653 and pNDM1_EC14653 have been deposited into GenBank under the accession numbers KP868646 and KP868647, respectively.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Natural Science Foundation of China (project no. 81222025), the New Century Excellent Talents Program, Ministry of Education, China (project no. NCET-13-0399), and a grant from the Sichuan Bureau of Science, China (project no. 2013JQ0042).

We thank Björn Espedido, University of Western Sydney, Australia, for his critical reading and thoughtful comments.

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