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SHORT REPORT

Characterization of a carbapenem- and colistin-resistant *Enterobacter cloacae* carrying Tn*6901* in bla_{NDM-1} genomic context

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Abstract: We report a clinical strain of *Enterobacter cloacae*, PIMB10EC27, isolated in Vietnam in 2010 that was resistant to 21 of 26 tested antibiotics, including carbapenems (MICs >64 μg/mL) and colistin (MIC >128 μg/mL). The complete genome of strain PIMB10EC27 was sequenced by PacBio RSII and the Illumina Miseq system. Whole-genome analysis revealed that PIMB10EC27 contains a chromosome of the ST513 group (PIMBEC27, length 5,272,177 bp) and two plasmids, pEC27-1 of the IncX3 group (length 62,470 bp) and pEC27-2 of the IncHI1 group (length 84,602 bp). It also revealed that strain PIMB10EC27 carries 15 genes that confer resistance to at least 10 antibiotic groups. Particularly, the insertion of IS*Kpn19* and Tn*6901* into the genomic context of bla_{NDM-1} was first identified and described. In another context, amino acid mutations G273D in PmrB and F515S in PmrC were first identified on the chromosome of PIMB10EC27, which may confer resistance to colistin in this strain.

Keywords: bla_{NDM-1}, colistin, *Enterobacter cloacae*, multidrug-resistance, Tn*6901*

Carbapenems are one of the broad-spectrum groups of β-lactam antibiotics and have been considered the best choice for treatment of infections caused by multidrug-resistant bacteria,[1] however, the recent increase in the rate of carbapenem resistance has been a cause for concern.[2] Encoded by the bla_{NDM-1} gene, New Delhi metallo-β-lactamase 1 (NDM-1), one of the most active and transmissible carbapenemases among the carbapenem-hydrolyzing β-lactamases, was first characterized by Yong et al in 2009[3] and has rapidly spread globally. To date, at least 17 NDM alleles have been characterized[4] and the Tn*125* composite transposon bracketed by two copies of IS*Aba125* appears to be the main vehicle for dissemination of the bla_{NDM-1} gene.[5] The lack of new generations of antibiotics has positioned colistin, a decades-old antibiotic, as one of the treatments of last resort against multidrug-resistant bacteria, particularly carbapenem-resistant Gram-negative bacteria.[6,7] Unfortunately, colistin resistance has been reported and is increasing.[8–11]

Enterobacter cloacae belongs to the *Enterobacteriaceae* family. These Gram-negative bacteria have been a frequent cause of nosocomial multidrug-resistant bacterial infections in the last decade.[12,13] Carbapenem-resistant *E. cloacae* has been commonly reported in Vietnam and many other countries in the world.[14–16] However, colistin resistance in *E. cloacae* has not been reported widely, particularly carbapenemase-producing colistin-resistant *E. cloacae* has very recently been reported from some countries including India,[10] the United States,[17] and China.[18–20]

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In this study, the whole genome of a clinical carbapenem- and colistin-resistant *E. cloacae* strain, PIMB10EC27, was sequenced and analyzed for its genetic characteristics that may associate with its resistance phenotypes.

PIMB10EC27 was recovered from the urine of a 72-year-old male patient admitted to Binh Dan Hospital in Vietnam in December 2010 with diagnoses of invasive prostate cancer and urosepsis, he had undergone prostatectomy at the same hospital 15 days earlier. The patient was discharged at the request of the family in a state of acute urinary retention and respiratory failure 4 days after hospitalization. During hospitalization, the patient was treated with meropenem, cefoperazone, sulbactam, clavulanic acid, and amoxicillin, but not with colistin. The strain was isolated as a part of the routine hospital laboratory procedures and identified as *E. cloacae* using a Vitek II kit (BioMérieux, USA).

The in vitro antibiotic susceptibility of PIMB10EC27 was tested by the Kirby-Bauer (KB) method, and the minimum inhibitory concentration (MIC) using the agar dilution method was also determined according to recommendations of the Clinical and Laboratory Standards Institute (CLSI 2018)[19] except for colistin, which was performed with broth microdilution method and interpreted by the guidelines of European Union Committee for Antimicrobial Susceptibility Testing (EUCAST 2019).[20] The results showed that PIMB10EC27 was resistant to 21 of 26 tested antibiotics, particularly imipenem (MIC >64 μg/mL), ertapenem (MIC =128 μg/mL), meropenem (MIC =128 μg/mL), and colistin (MIC >128 μg/mL); susceptible to amikacin, levofloxacin nitrofurantoin; and showed intermediate resistance to ciprofloxacin, oxfloxacin (Table 1).

Table 1 Antibiotic susceptibility of the *E. cloacae* PIMB10EC27

Antibiotic	Kirby-Bauer method	MIC (μg/mL)
Gentamycin	R	
Tobramycin	R	
Amikacin	S	2
Kanamycin	R	
Piperacillin-tazobactam	R	
Ticarcillin-clavulanic acid	R	
Imipenem	R	>64
Ertapenem	R	128
Meropenem	R	128
Cefuroxime	R	
Cefotaxime	R	>256
Ceftriaxone	R	>256
Ceftazidime	R	>256
Ciprofloxacin	I	
Levofloxacin	S	
Ofloxacin	I	
Trimethoprim-sulfamethoxazole	R	
Sulfonamide	R	
Aztreonam	R	
Nitrofurantoin	S	
Piperacillin	R	
Chloramphenicol	R	>256
Tetracycline	R	>256
Doxycycline	R	
Cefoxitin		>256
Colistin		>128

Abbreviations: R, Resistant; S, Sensitive; I, Intermediate.

A Nextera XT DNA Library Prep Kit (Illumina Inc., USA) and SMRTbell Template Prep Kit (Pacific Biosciences, USA) were used to prepare libraries for the whole genome sequencing of PIMB10EC27 using simultaneously the MiSeq System (Illumina Inc.) with MiSeq Reagent Kit v.2 (2×150 cycles), and the PacBio RSII (Pacific Biosciences) together with Sequel Sequencing Kit 2.0 (8 rxn).

In total, 3.8Gb of filtered subreads were obtained from PacBio RSII sequencing platform. Using PacBio's pre-patching program, we obtained 353Mb of 35,932 reads with the sizes of 500–27,847 bp. The pre-assemble reads were further packaged with HGAP program, which yielded a 5,272,177 bp chromosome and two plasmids of 62,318 bp and 84,480 bp. MiSeq data was used for the error correction of PacBio consensus sequences. By integrating these two platforms, we were able to obtain the complete genome which consisted of a chromosome (5,272,177 bp) belonging to the ST513 group and two plasmids, pEC27-1 of the IncX3 group (62,470 bp) and pEC27-2 of the IncHI1 group (84,602 bp) (Figure 1). The complete genome sequence was annotated with NCBI Prokaryotic Genome Annotation Pipeline servers and analyzed by bioinformatics programs or software, including ResFinder 2.1,[21] MLST,[22] Isaga,[23] Galaxy,[24] and Plasmid Finder[25] for antibiotic resistance genes identification, chromosome classification, sequences of insertion sequence (IS) determination, integrons finding, and plasmids classification, respectively. In total, 15 coding genes conferring resistance to 10 antibiotic groups were found on PIMB10EC27 (Table 2). Among these genes, $bla_{\text{NDM-1}}$, $bla_{\text{SHV-12}}$, bla_{CMH}, $aac_{(3)\text{-ID}}$, *strA, strB, dfrA-14, sul2, catA2*, and *tet(D)* were associated with resistance

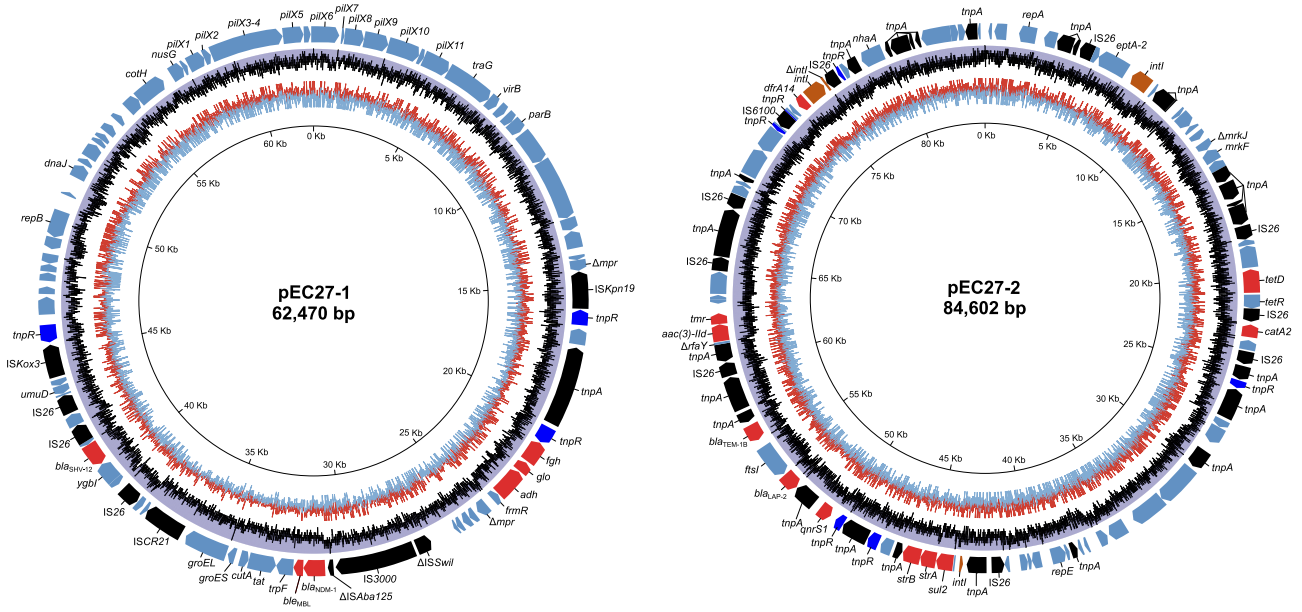

Figure 1 Structure of plasmid pEC27-1 and pEC27-2. pEC27-1 is an IncX3 plasmid that possesses highly syntenic plasmid backbone compare to IncHI1 plasmid pEC27-2. The outer circle shows ORFs on forward and reverse strands. Resistance genes, transposase genes and resolvase genes are depicted by red, black, blue arrows, respectively. The two inner circles show the GC content (purple circle) and GC skew (blue indicates positive values, red indicates negative values) information.

Table 2 Distribution of coding genes conferring to antibiotic resistance in PIMB10EC27

Gene	Description	Resistance mechanism	Antibiotic group/ agent
Chromosome			
bla$_{CMH}$	CMH family class C β-lactamase	Antibiotic inactivation	β-lactams
fosA	FosA/FosA2 family fosfomycin resistance glutathione transferase	Antibiotic inactivation	Fosfomycin
pEC27-1			
bla$_{NDM-1}$	New Delhi metallo-β-lactamase NDM-1	Antibiotic inactivation	β-lactams
bla$_{SHV-12}$	Extended-spectrum beta-lactamase SHV-12	Antibiotic inactivation	β-lactams
ble$_{MBL}$	Bleomycin-resistance protein	Sequestering effect of the bleomycin-binding protein	Bleomycin
pEC27-2			
aac(3)-IId	Aminoglycoside acetyltransferase	Antibiotic inactivation	Aminoglycosides
bla$_{LAP-2}$	Class A β-lactamase LAP-2	Antibiotic inactivation	β-lactams
bla$_{TEM-1B}$	Class A broad-spectrum β-lactamase TEM-1	Antibiotic inactivation	β-lactams
catA2	Chloramphenicol acetyltransferase	Antibiotic inactivation	Phenicols
dfrA14	Dihydrofolate reductase	Altered affinity of reductase	Trimethoprim
qnrS1	Quinolone resistance pentapeptide repeat protein	Antibiotic target protection	Quinolones, fluoroquinolones
strA	Aminoglycoside phosphotransferase	Antibiotic inactivation	Aminoglycosides
strB	Aminoglycoside phosphotransferase	Antibiotic inactivation	Aminoglycosides
sul2	Sulfonamide resistant dihydropteroate synthase	Antibiotic target replacement	Sulphonamides
tet(D)	Tetracycline efflux pump	Antibiotic efflux	Tetracyclines

phenotypes of PIMB10EC27.On the other hand *qnrS1* equivalent to the intermediate fluoroquinolone-resistance phenotype of PIMB10EC27 has been proven to reduce the antibiotic susceptibility of bacteria to quinolones or fluoroquinolone.[26–28] In addition, 160 putative transposase open reading frames (tORFs) were identified in which the number of tORFs located on the chromosome, pEC27-1 and pEC27-2 were 113, 10 and 37, respectively. Antibiotic resistance genes closely located to these transposase sequences may have contributed to the accumulation and increase in the antibiotic resistance of PIMB10EC27. A 1722-bp class 1 integron with gene cassette *dfrA14* flanked by IS6 sequences was also found on plasmid pEC27-2.

We further analyzed the genes in PIMB10EC27 that confer resistance to carbapenem and colistin. As a result, a $bla_{\text{NDM-1}}$ gene was found on pEC27-1 of PIMB10EC27 along with a $bla_{\text{SHV-12}}$ and ble_{MBL}. A comparison of the nucleotide sequence of pEC27-1 and those of other IncX3 plasmids carrying $bla_{\text{NDM-1}}$ and $bla_{\text{SHV-12}}$, including pKPN5047 (KC311431), pNDM-HF727 (KF976405), pNDM-HN380 (JX104760), and RJA274 plasmid NDM-1 (KF877335) was carried out in order to identify if any unique structure characteristics contributed to the multidrug-resistance of PIMB10EC27. Comparative results of the genomic context surrounding the resistance genes revealed that all of the plasmids had a conserved sequence carrying Δ*ISAba125*, $bla_{\text{NDM-1}}$, ble_{MBL}, *trpF, dsbC, cutA1, groES, groEL*, and *ISCR21* (Figure 2). This conserved sequence is also a Tn*125* composite transposon, in which the $bla_{\text{NDM-1}}$ gene lies downstream of a truncated IS*Aba125* element that provides the −35 region for the promoter of $bla_{\text{NDM-1}}$.[29] A 93-bp sequence separates the right-hand inverted repeat of IS*Aba125* from the start codon of $bla_{\text{NDM-1}}$, and the deletion of an IS*5* was identified in the plasmid pEC27-1. An IS*CR21*-like element was also found downstream of $bla_{\text{NDM-1}}$ and it is suggested that this element may be responsible for initial gene capture.[5] The IS26-bounded region of pEC27-1 containing *ygbI, ygbJ*, and $bla_{\text{SHV-12}}$ was found to be very distinct from the other plasmids. This region carried three copies of IS*26*, and the local *ygbJ* gene was split into two parts, whereas other plasmids carried only two copies of IS26 and the full *ygbJ* gene. The isolation and reverse with a copy of IS*26* in the plasmid pEC27-1 of the 424-bp region of the *ygbJ* gene might propose there is an IS*26*-mediated re-organization. Interestingly, an insertion of IS*Kpn19* and Tn*6901* into the *mpr* gene (encoding zinc metalloproteinase) lying further upstream of $bla_{\text{NDM-1}}$ was also observed (Figure 2). Belonging to IS*Kra4* family, IS*Kpn19* is 2851 bp in length and has been found to lie downstream of $bla_{\text{OXA-181}}$ in an IncX3-type plasmid.[30] Tn*6901*, first described in plasmid Rts1 of *Proteus vulgaris*,[31] is 6.9 kb in length and harbors 6 genes, including a transposase gene (*tnpA*), a resolvase gene (*tnpR*), an alcohol dehydrogenase gene (*adh*), a glyoxalase/bleomycin resistance gene (*glo*), an S-formylglutathione hydrolase gene (*fgh*), and a regulatory protein gene (*frmR*). We report here the first case, to our knowledge, of a 9.8-kb insertion sequence harboring IS*Kpn19* and Tn*6901* in the genomic

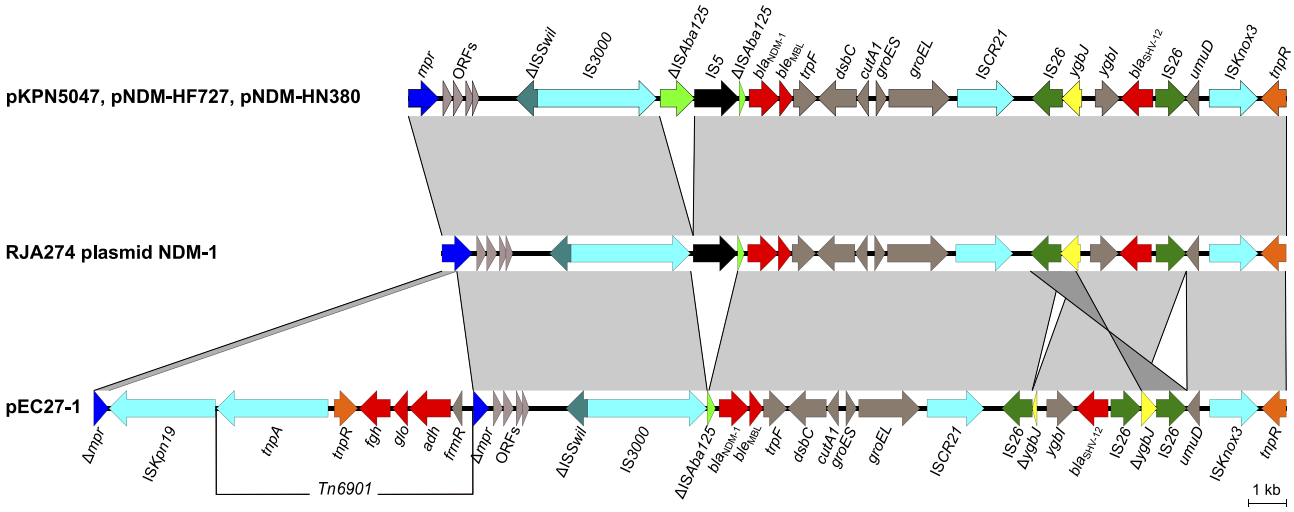

Figure 2 Genetic context of $bla_{\text{NDM-1}}$ on IncX3 plasmids pKPN5047, pNDM-HF727, pNDM-HN380, RJA274 plasmid NDM-1, and pEC27-1. Gray shading denotes shared regions of homology: light gray indicates a forward match and dark gray indicates a reverse match. Notably, *ygbJ* is split into two parts, and the IS*26*-Δ*ygbJ* region is inverted in pEC27-1 relative to the other plasmids. The *mpr* gene in pEC27-1 is interrupted by IS*Kpn19* and Tn*6901* insertions. Open reading frames are portrayed by arrows and are colored. Resistance genes are indicated in red arrows, including $bla_{\text{NDM-1}}$, $bla_{\text{SHV-12}}$, ble_{MBL}, *fgh, glo*, and *adh*.

context of bla_{NDM-1}. This insertion made the genomic context of bla_{NDM-1} in PIMB10EC27 more complex with 6 resistance genes, including *adh, glo, fgh,* bla_{NDM-1}, ble_{MBL}, and bla_{SHV-12}, which may also affect the dissemination and expression of bla_{NDM-1}. Further research is required to clarify these assumptions.

Genome analysis was also performed to identify the genes that confer resistance to colistin of PIMB10EC27. In this research, we did not find any *mcr* gene that recently characterized as a novel mobile gene responsible for colistin resistance among Gram negative bacteria.[32] In addition, our transformation experiments revealed that none of the plasmids of PIMB10EC27 was associated with colistin resistance (data not shown). In another context, encoding genes involved in the attachment of L-Ara-4N and P-EtN to LPSs of PIMB10EC27, including *phoP, phoQ, pmrB, pmrA, pmrC, mgrB, lpx*, and *arn* were examined, and amino acid replacements G273D in PmrB and F515S in PmrC of PIMB10EC27 were recorded. The role of these mutations in colistin resistance remains to be investigated.

In summary, we successfully constructed the complete genome of multidrug-resistant *E. cloacae* strain PIMB10EC27 carrying the novel genomic context of bla_{NDM-1}. To our knowledge, this is the first report in Vietnam of a carbapenemase-producing *E. cloacae* strain that was also resistant to colistin. PIMB10EC27 was isolated from a patient not being treated with colistin and not identified as a case of a plasmid-mediated colistin-resistance mechanism.

In the battle against multidrug-resistant bacteria, particularly carbapenem-resistant bacteria, colistin has been considered to remain effective. In such context, this report of a clinical isolate that is resistant to both colistin and carbapenem raises a high concern over future clinical management and infection control.

Nucleotide sequence accession numbers. The complete nucleotide sequences of PIMB10EC27 have been deposited in GenBank under accession numbers CP020089-CP020091.

(https://www.ncbi.nlm.nih.gov/nuccore/CP020089.1/, https://www.ncbi.nlm.nih.gov/nuccore/CP020090, https://www.ncbi.nlm.nih.gov/nuccore/CP020091).

Abbreviation list

MIC, minimum inhibitory concentration; *E. cloacae, Enterobacter cloacae*; CLSI, Clinical and Laboratory Standards Institute; NDM-1, New Delhi metallo-β-lactamase 1; ORF, Open Reading Frame.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

VC and TI conceived and designed the study. TDLH, PTBT, LL, and LKT collected samples and performed experiments, TDLH, HNLV, PTBT, LL, DM, SN, and MA performed data analysis, TDLH and HNLV wrote the paper. All authors contributed to drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

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