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MECHANISMS OF RESISTANCE

Sequence Type 273 Carbapenem-Resistant *Klebsiella pneumoniae* Carrying bla_{NDM-1} and bla_{IMP-4}

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ABSTRACT A carbapenem-resistant *Klebsiella pneumoniae* isolate was recovered from human blood. Its whole-genome sequence was obtained using Illumina and long-read MinION sequencing. The strain belongs to sequence type 273 (ST273), which was found recently and caused an outbreak in Southeast Asia. It has two carbapenemase genes, bla_{NDM-1} (carried by an ST7 IncN self-transmissible plasmid) and bla_{IMP-4} (located on a self-transmissible IncHI5 plasmid). Non-KPC-producing ST237 may represent a lineage of carbapenem-resistant *K. pneumoniae*, which warrants further monitoring.

KEYWORDS β-lactamases, carbapenemases, resistance, plasmids, *Klebsiella pneumoniae*, *Klebsiella*, carbapenems

Klebsiella pneumoniae is one of the most common pathogens of human infections, and carbapenem-resistant *K. pneumoniae* (CRKP) has emerged as a major challenge to clinical management and global public health (1). Production of carbapenem-hydrolyzing enzymes (carbapenemases) is the major mechanism mediating resistance to carbapenems in *K. pneumoniae*. There are a few types of carbapenemases, and the most common carbapenemase in *K. pneumoniae* is KPC (a group of serine β-lactamases), followed by NDM and IMP (both of which are metallo-β-lactamases). The global dissemination of CRKP is largely mediated by the high-risk clonal complex 258 (CC258), which comprises sequence type (ST) 11, ST258, and a number of closely related sequence types. However, other clones may also contribute to the international spread of CRKP. Recently, ST273 CRKP was found in several countries (2–4), which warrants further investigation. We identified an ST273 CRKP clinical strain carrying both bla_{NDM} and bla_{IMP} genes in our hospital and report its characterization here.

Strain WCHKP020034 was recovered from the blood of a 72-year-old male patient with pancreatitis at West China Hospital. The strain was identified as *K. pneumoniae* by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) (Bruker, Billerica, MA) and Vitek II (bioMérieux, Marcy-l'Étoile, France). MICs of amikacin, aztreonam, aztreonam-avibactam, ceftazidime, ciprofloxacin, colistin, imipenem, meropenem, piperacillin-tazobactam, tigecycline, and trimethoprim-sulfamethoxazole against the isolate were determined using the broth microdilution method of the Clinical and Laboratory Standards Institute (CLSI) (5). Because CLSI does not give breakpoints for colistin and tigecycline, we applied those defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/). The strain was resistant to aztreonam (MIC, 64 μg/ml), ceftazidime (MIC, >256 μg/ml), ciprofloxacin (MIC, 256 μg/ml), imipenem (MIC, 32 μg/ml), meropenem (MIC, 64 μg/ml), piperacillin-

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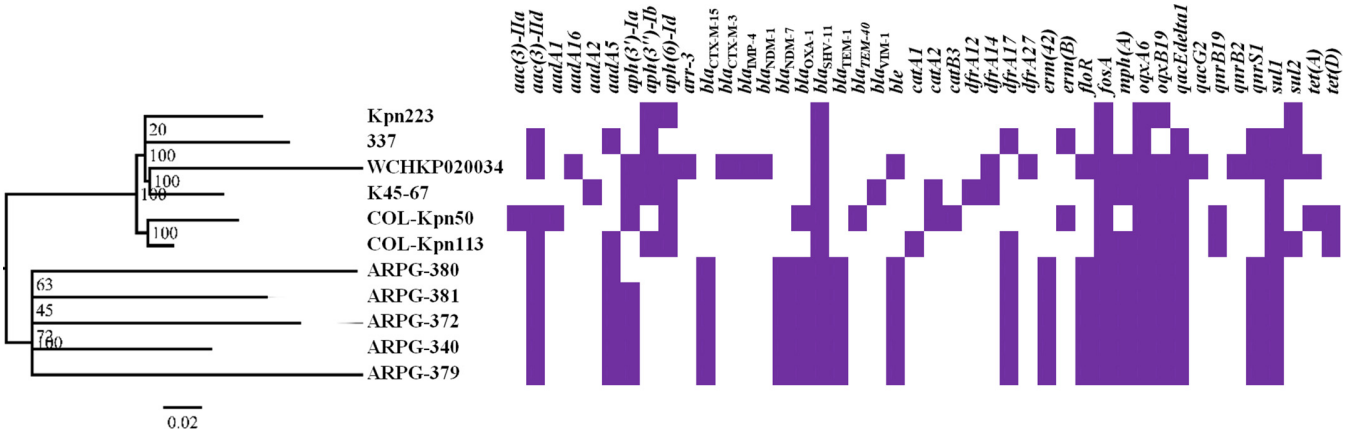

FIG 1 Maximum-likelihood phylogenetic tree of *K. pneumoniae* ST273 strains with genome sequences available in GenBank. The phylogeny is inferred from the recombination-filtered SNP alignment obtained by aligning either a complete or draft genome of *K. pneumoniae* ST273 against the complete genome of WCHKP020034. The annotation denotes the presence of antimicrobial resistance genes as determined by ABRicate.

tazobactam (MIC, >512/4 μg/ml), and trimethoprim-sulfamethoxazole (MIC, 128/2,432 μg/ml) but susceptible to amikacin (MIC, 2 μg/ml), aztreonam-avibactam (MIC, 0.25/4 μg/ml), colistin (MIC, 1 μg/ml), and tigecycline (MIC, 1 μg/ml). Acquired carbapenemase genes bla_{GES}, bla_{KPC}, bla_{IMP}, bla_{NDM}, bla_{OXA-48}, and bla_{VIM} were screened as described previously (6–9), and the strain had bla_{NDM} and bla_{IMP}. bla_{NDM-1} and bla_{IMP-4} were identified by amplifying and sequencing the complete coding sequence of bla_{NDM} and bla_{IMP}.

The strain was subjected to whole-genome sequencing with 150× coverage using the HiSeq X Ten sequencer (Illumina, San Diego, CA), which generated 4,395,250 reads. Reads were trimmed using Trimmomatic (10) and were then assembled to 125 contigs (70 were ≥1,000 bp in length) with a 56.79% GC content using the SPAdes program (11). The *wzi* gene allele, which represents the capsular variation, of strain WCHKP020034 was 50, corresponding to several K types, i.e., K15, K17, K50, K51, and K52, with K15 being the best match predicted using Kaptive (12). None of the K types were K1, K2, or K5, which are proposed as the hypervirulent members of *K. pneumoniae*. With respect to virulence, strain WCHKP020034 had the *mrk* gene cluster (*mrkA-B-C-D-F-H-I-J*), which encodes type 3 fimbrial expression (13) and is seen in almost all *K. pneumoniae* isolates (1). Other known virulence genes, such as those encoding yersiniabactin, colibactin, allantoinase, and aerobactin, were absent from strain WCHKP020034.

Strain WCHKP020034 belonged to ST273, as determined by use of the *de novo* assembled genome sequence to query the MLST database of *K. pneumoniae* (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). There were 10 additional ST273 strains with the whole-genome sequence available in GenBank (see Table S1 in the supplemental material). Genome sequences of ST273 strains were retrieved from GenBank and aligned with that of strain WCHKP020034 using the Harvest Suite with default settings (14). Single nucleotide polymorphisms (SNPs) on recombination sites were removed by Gubbins (15). The filtered SNPs were then used as input for inferring a phylogenetic tree using RAxML (16) with the GTRGAMMA model and 1,000 bootstraps. Antimicrobial resistance genes in these genomes were identified using ABRicate (https://github.com/tseemann/abricate) to query the ResFinder database at the Center for Genomic Epidemiology (http://genomicepidemiology.org/), and the *wzi* gene allele was predicted using Kaptive (12). Five strains carrying bla_{NDM-7}, a point mutant of bla_{NDM-1}, were recovered in 2013 in the Philippines and belonged to a single cluster. No *wzi* allele was identified in these five strains. In contrast, strain WCHKP020034 was clustered with other ST273 strains (Fig. 1) and was closest to strain COL-Kpn113 (carrying no bla_{NDM}, recovered in 2004 in Colombia) and strain K45-67 (carrying no bla_{NDM} but bla_{VIM-1},

TABLE 1 Antimicrobial resistance genes and their locations in strain WCHKP020034

WCHKP020034 chromosome/plasmid	Size (bp)	Replicon type, pMLST	Antimicrobial resistance genes
Chromosome	5,295,791		$bla_{SHV-115}$, *fosA*, *oqxA*, *oqxB*
pNDM1_LL34	58,953	N (ST7)	bla_{NDM-1}, *dfrA14*, *qnrS*
pIMP4_LL34	260,974	IncHI5	*aacA4*, $bla_{CTX-M-3}$, bla_{IMP-4}, *sul1*
pQnrB_LL34	130,688	FII (K2:A−:B−), Q1	*aac(3)-IId*, *ant(3″)-Ih-aac(6′)-IId*, *aadA1*, *aadA16*, *aph(3′)-Ia*, *arr3*, *dfrA27*, *floR*, *mph*(A), *qnrB*, *sul1*, *sul2*, *tet*(A)

recovered in 2007 in Norway), with 116 to 123 SNPs difference, respectively (see Table S2 in the supplemental material). Strains COL-Kpn113 and K45-67 had a *wzi* allele174, which was different from the allele 50 of strain WCHKP020034. The assembled genomes of ST273 strains were also typed using the cgMLST database (http://bigsdb.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public&page=sequenceQuery), and a total of 951 genes were identified in all ST273 genomes. The 951 genes were identical in sequence among all ST273 strains other than WCHKP020034, whereas only 5 out of the 951 genes were different between strain WCHKP020034 and the other 10 ST273 strains (see Table S3 in the supplemental material). The relatively small number of SNPs and almost identical cgMLST results seen in strains from different geographic locations over such a long time frame suggest that ST273 might be highly clonal and merits further focused phylogenetic studies of this lineage. The *wzi* allele was different and even absent in ST273 strains, but it is not uncommon to find more than one capsular type for strains of a single ST due to homologous recombination of the capsular locus (17). Plasmids of the ST273 strains were predicted using PlasmidFinder, but there was no common plasmid replicon type present in all of the ST273 strains.

In addition to the two carbapenemase genes, strain WCHKP020034 had 24 intact antimicrobial resistance genes mediating resistance to aminoglycosides [*aac(3)-IId*, *ant(3″)-Ih-aac(6′)-IId*, *aacA4*, *aadA1*, *aadA16*, *aph(3′)-Ia*, *strA*, and *strB*], β-lactams ($bla_{CTX-M-3}$ and $bla_{SHV-115}$), fosfomycin (*fosA*), macrolides [*mph*(A)], phenicol (*floR*), quinolones (*oqxA*, *oqxB*, *qnrB*, and *qnrS1*), rifampin (*arr3*), tetracycline [*tet*(A)], sulfonamides (*sul1* and *sul2*), and trimethoprim (*dfrA7*, *dfrA14*, and *dfrA27*) (Table 1).

Conjugation experiments were performed using filter- and broth-based methods at both 25°C and 37°C with the azide-resistant *Escherichia coli* strain J53 as the recipient. Transconjugants were screened using 1 μg/ml meropenem plus 150 μg/ml sodium azide, and the presence of bla_{NDM-1} or bla_{IMP-4} in transconjugants was screened by PCR. bla_{NDM-1} and bla_{IMP-4} were carried on two self-transmissible plasmids, designated pNDM1_LL34 and pIMP4_LL34, respectively. To obtain the complete sequence of the plasmids, strain WCHKP020034 was subjected to sequencing using the long-read MinION sequencer (Nanopore, Oxford, United Kingdom). The *de novo* hybrid assembly of both short Illumina reads and long MinION reads was performed using Unicycler under the conservative mode for increased accuracy (18). The complete circular contigs generated were then corrected using Plion with Illumina reads for several rounds until no change was detected (19). Plasmid replicon type and plasmid MLST were determined using the PlasmidFinder and pMLST tools (http://genomicepidemiology.org/). The hybrid assembly of Illumina and MinION reads revealed that strain WCHKP020034 has a 5,295,791-bp circular chromosome and three large plasmids, i.e., the 58,953-bp pNDM1_LL34 of IncN (ST7), a 260,974-bp pIMP4_LL34 carrying bla_{IMP-4} and $bla_{CTX-M-3}$ with replicon types unidentified by PlasmidFinder, and a 130,688-bp plasmid carrying *qnrB* that contains an IncFII(K) and an IncQ1 replicon (designated pQnrB_LL34) (Table 1).

To understand the distribution of ST7 IncN plasmids, sequences of three alleles to define ST7 were concatenated and then aligned against the nucleotide database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Plasmids of the ST7 IncN type were found in various species of *Enterobacteriaceae*, including *Citrobacter freundii*, *E. coli*,

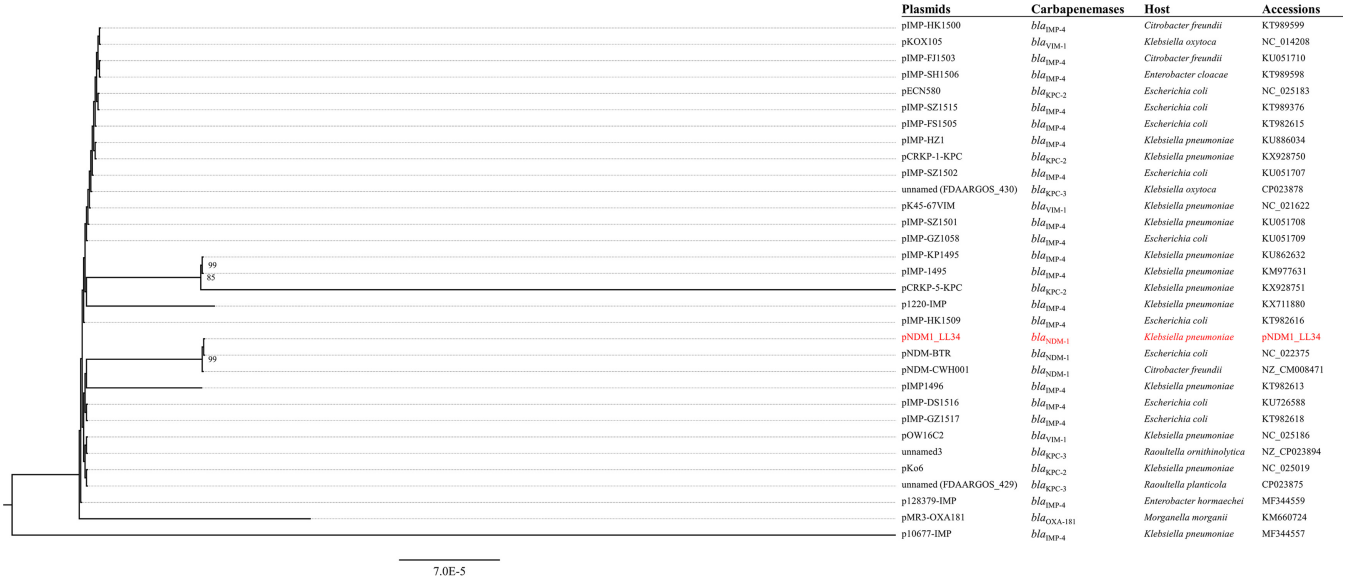

FIG 2 Phylogenetic tree of ST7 IncN plasmids. The names, host species, and accession numbers of the plasmids are shown. The tree was inferred using concatenated sequences of 26 genes belonging to the ST7 IncN backbone.

Enterobacter cloacae, *Enterobacter hormaechei*, *K. pneumoniae*, *Klebsiella oxytoca*, *Morganella morganii*, *Raoultella ornithinolytica*, and *Raoultella planticola* from different countries, suggesting that ST7 IncN plasmids are widely distributed. In addition, ST7 IncN plasmids have been found to mediate the dissemination of bla_{IMP-4} in *Enterobacteriaceae* in different regions of China (20). Sequences of all available ST7 IncN plasmids ($n = 32$) were retrieved from GenBank. Genes present on all ST7 IncN plasmids were considered backbone genes, which were identified using OrthoFinder (21). Sequences of backbone genes were concatenated and then aligned to infer a phylogenetic tree using RAxML with a 1,000-bootstrap test (16). pNDM1_LL34 is clustered with several plasmids from various species (Fig. 2), among which pNDM1_LL34 is closely related (99% coverage, 99% identity) to plasmid pNDM-BTR (GenBank accession no. KF534788), which is also an ST7 IncN plasmid carrying bla_{NDM-1} that was recovered from an *E. coli* isolate in Beijing, China, in 2013, as revealed by BLAST (blast.ncbi.nlm.nih.gov). The findings suggest interspecies spread of a common IncN plasmid. On pNDM1_LL34 and pNDM-BTR, bla_{NDM-1}, several genes that are commonly associated with bla_{NDM-1}, and the quinolone-resistant gene *qnrS1* were bracketed by IS*26* (Fig. 3). There were no 8-bp direct target repeats, which are characteristic of the insertion of IS*26*, flanking the two copies of IS*26*, suggesting that homologous recombination contributed to the formation of such a structure. Nonetheless, two copies of IS*26* have the potential to form a composite transposon to mediate the mobilization of the intervening genetic components, including bla_{NDM-1} and *qnrS1*. Outside of the two IS*26* copies, there was an interrupted Tn*3* family transposon, in which the transposase gene *tnpA* and both

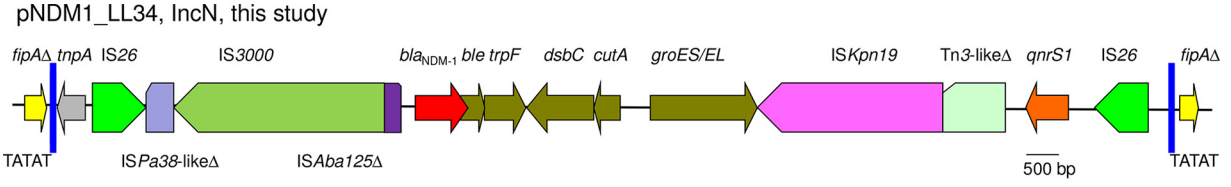

FIG 3 The genetic context of bla_{NDM-1} on pNDM1_LL34. Genes between bla_{NDM-1} and *qnrS1* are *ble* (mediating bleomycin resistance), *trpF* (encoding a phosphoribosyl anthranilate isomerase), *dsbC* (encoding an oxidoreductase), *cutA1* (encoding an ion-tolerant protein), and *groES*/*groEL* (encoding a chaperonin). The *tnpA* gene (encoding a transposase) and both inverted repeats (blue bars) of a Tn*3* family transposon are outside the region flanked by IS*26*. *fipA* (encoding a conjugal transfer inhibition protein) is interrupted by the insertion of the Tn*3* family transposon with characteristic 5-bp direct target repeats (TATAT). Δ, truncated genes or mobile genetic elements.

inverted repeats remained intact, but the resolvase gene *tnpR* was truncated. The *fipA* gene that encodes a conjugal transfer inhibition protein and belongs to the plasmid backbone was interrupted into two parts by the Tn*3* family transposon. The characteristic 5-bp direct target repeats flanked the Tn*3* family transposon, suggesting that the transposon inserted into *fipA*.

bla_{IMP-4} was carried by a class I integron in the bla_{IMP-4}-*qacG2-aacA4* cassette array on pIMP4_LL34. Chloramphenicol resistance gene *catB3* is usually seen together with bla_{IMP-4} in the bla_{IMP-4}-*qacG2-aacA4-catB3* cassette array but is absent from pIMP4_LL34. The integron is assigned In*1498* by INTEGRALL (http://integrall.bio.ua.pt/). By BLAST, the closest match of pIMP4_LL34 was p13190-VIM (88% coverage, 99% identity) (GenBank accession no. MF344563) from *K. pneumoniae* in Beijing China. pIMP4_LL34 has a replicon, which has been proposed as IncHI5 but has not been included in the database of PlasmidFinder (22). By BLAST, using the 885-bp replication protein-encoding gene of the IncHI5 replicon, we identified 15 additional IncHI5 plasmids in GenBank. These plasmids were found in *K. pneumoniae*, *K. oxytoca*, *K. michiganensis*, *R. ornithinolytica*, and *R. planticola*, and all but one were found at various locations in China. These findings suggest that IncHI5 plasmids have been circulated in China, which warrants further investigation. $bla_{CTX-M-3}$ was located downstream of IS*Ecp1* and upstream of a truncated *orf477* gene. The IS*Ecp1*-$bla_{CTX-M-3}$-orf477Δ unit was inserted in a gene encoding a protein of the *Hok/Gef* family with the presence of 5-bp direct target repeats, which is characteristic of the transposition of IS*Ecp1*. It became evident that IS*Ecp1* misrecognized a sequence in *orf477*, which has 8 out of 14 nucleotides matched with the right-hand inverted repeat (IRR), as its alternative IRR, and then realized the mobilization of $bla_{CTX-M-3}$ into the Hok/Gef family protein-encoding gene.

In conclusion, we identified an ST273 CRKP carrying the carbapenemase genes bla_{NDM-1} and bla_{IMP-4}. bla_{NDM-1} was carried by an ST7 IncN self-transmissible plasmid, and bla_{IMP-4} was located on an IncHI5 self-transmissible plasmid. This is yet another example of a clinical isolate containing multiple plasmids conferring resistance to carbapenems, as we described before (23). The coexistence of plasmids may generate new platforms to mediate further spread of carbapenem-resistant genes and questions our knowledge of the extent to which plasmids conferring multidrug resistance truly affect the fitness of host bacteria. The question arises as to why strains would possess multiple genes for the same resistance. The low diversity of ST273 isolates across continents and years suggests that the lineage merits further characterization.

Accession number(s). Complete sequences of the chromosome of strain WCHKP020034, pIMP4_LL34, and pNDM1_LL34 have been deposited into GenBank under accession numbers CP025963, CP025964, and CP025965.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00160-18.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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