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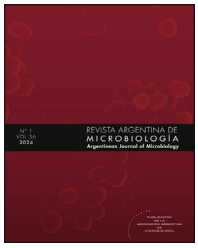

BRIEF REPORT

First report of KPC-35-producing *Klebsiella pneumoniae* ST258 isolated in Peru

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Abstract *Klebsiella pneumoniae* sequence type 258 (ST258) is the main cause of the global spread of KPC and a significant public health problem. In 2015, ceftazidime/avibactam (CZA) was introduced as a therapeutic alternative and since it has contributed to the development of new KPC variants. Here we present the identification of two consecutive isolations of *K. pneumoniae* ST258 (KP1 and KP2), from a patient with urinary tract infection. KP1 and KP2 harbored bla_{KPC-2} and bla_{KPC-35}, respectively. KP2 exhibited a modified susceptibility profile to carbapenems and resistance to CZA. To the best of our knowledge, this is the first report of *K. pneumoniae* ST258 in Peru, which highlights the increasing problem of CZA resistance.

PALABRAS CLAVE
Klebsiella pneumoniae;
Resistencia a los antimicrobianos;
Perú

Primer reporte de *Klebsiella pneumoniae* ST258 productora de KPC-35 en Perú

Resumen *Klebsiella pneumoniae* secuenciotipo 258 (ST258) es la principal causa de la propagación mundial del KPC, planteando un importante problema de salud pública. La introducción de ceftazidima/avibactam (CZA) ha contribuido al desarrollo de nuevas variantes de KPC. En 2021 se obtuvieron 2 aislamientos consecutivos de *K. pneumoniae* ST258 (KP1 y KP2), productores de KPC, de un paciente con infección del tracto urinario. KP1 y KP2 albergaron bla_{KPC-2} y bla_{KPC-35}, respectivamente. KP2 exhibió un perfil de susceptibilidad modificado a los carbapenémicos y resistencia a CZA. Hasta donde sabemos este es el primer informe de *K. pneumoniae* ST258 en Perú y destaca el creciente problema de resistencia a CZA.

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The rise of carbapenem resistance in *Enterobacterales* represents a significant public health problem as it severely restricts treatment alternatives and contributes to higher mortality rates[7]. Carbapenemase-like enzymes are the primary mechanism for gaining resistance[7]. The most prevalent carbapenemase types in *Enterobacterales* are the New Delhi Metallo-β-lactamase (NDM), *Klebisella pneumoniae* carbapenemase (KPC), and OXA-48[7,15].

KPC was first reported in 2001 in the United States and belongs to the family of class-A serine β-lactamase with a broad substrate profile, including penicillins, cephalosporins, aztreonam, carbapenems and β-lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam[13]. To date, 216 KPC variants have been described according to the Beta-Lactamase Database (http://www.bldb.eu/BLDB.php?prot=A#KPC). Being mostly identified in *K. pneumoniae*, which is considered the main agent of propagation.

K. pneumoniae sequence type 258 (ST258) represents a significant threat. ST258 is a successful clone[9], with numerous antimicrobial resistant genes, including aminoglycoside-modifying enzymes, chromosomal mutations that result in fluoroquinolone resistance and multiple β-lactamase genes[3].

Due to the increasing reports of resistance to carbapenems in *Enterobacterales*, diazabicyclooctane (DBO), a new β-lactamases family inhibitors, was developed. The first drug in this family to get approval by the FDA in 2015 was ceftazidime/avibactam (CZA)[11]. CZA has *in vitro* activity against class-A, class-C and class-D serine β-lactamase. Unfortunately, in recent years there have been increasing reports of KPC variants with resistance to CZA[5]. So the aim of this study was to describe two consecutive *K. pneumoniae* ST258 isolates, including the characterization of a unique CZA resistance profile.

In 2021, a carbapenem-resistant isolate of *K. pneumoniae* (KP1) was identified in a patient with urinary tract infection (UTI) in Lima, Peru. After 20 days of antibiotic treatment, a second strain of *K. pneumoniae* (KP2) was isolated with a similar susceptibility profile, just differing in MIC values for carbapenems and CZA.

The strain identification and the antimicrobial susceptibility testing were conducted using the Vitek®2 system (bioMérieux). The interpretation was performed following the guidelines by the CLSI M100-ED33 2023 (https://clsi.org/all-free-resources/). An immunochromatographic assay was conducted using RESIST-5 O. K. N. V. I (Coris BioConcept, Gembloux, Belgium) for the rapid detection of carbapenemase type.

Bacterial DNA was extracted using the GeneJetGenomic DNA Purification kit (ThermoScientific), following the manufacturer's instructions. The identification of bla_{KPC} and $bla_{CTX\text{-}M}$ genes were performed by PCR amplification[2,8]. The identification of $bla_{CTX\text{-}M\text{-}group1}$, $bla_{CTX\text{-}M\text{-}group2}$, $bla_{CTX\text{-}M\text{-}group9}$ groups were carried out by PCR using specific primers and protocols previously described[2].

Plasmid conjugation assays were performed with a mating-out assay, using *Escherichia coli* J53 sodium azide resistant (Az^r) as the recipient strain (ECJ53) and KP1 and KP2 as the donor strains. Transconjugants were chosen from an initial selection in LB agar containing ampicillin (50 μg/ml) and sodium azide (150 μg/ml), followed by a subsequent selection in LB agar supplemented with cefotaxime (2 μg g/ml) and sodium azide (150 μg/ml). Transconjugants (TCKP1 and TCKP2) were assessed based on the presence of bla_{KPC} gene and their antibiotic susceptibility profiles.

The genomes of KP1 (CP159934–CP159941) and KP2 (CP159926–CP159933) were fully sequenced via Ilumina and Nanopore technologies. A hybrid genome assembly was carried out with Unicycler v0.5.0, followed by genome annotation with Prokka v1.14.6 manually curated. Multi-locus sequence type (MLST) was determined with MLSTfinder v2.0, resistance genes and plasmid groups were analyzed with ResFinder v.1, CARD database and PlamidFinder v2.1. Genomic islands of resistance were predicted using IslandViewer 4 (https://www.pathogenomics.sfu.ca/islandviewer/). Illumina reads from KP1 were mapped to the assembled genome of KP2 using Bwa v0.7.17 and Snippy v4.6.0. Genetic relationships based on single-nucleotide polymorphisms (SNPs) were constructed using 443 genomes of *K. pneumoniae* ST258 obtained from a Pathogenwatch collection. Roary v3.13.0, SNP-sites v.2.5.1 and a maximum-likelihood were employed for the analysis. Cluster was inferred through IQ-TREE Phylogenomic v.1.5.5.3 utilizing the most suitable model determined with 1000 bootstrap value.

KP1 strain presented significant resistance to all β-lactams, amikacin, and ciprofloxacin, remaining only susceptible to tigecycline and CZA. KP2 was susceptible to all carbapenems, had decreased resistance to aztreonam (MIC, 16 μg/ml) and was resistant to CZA (MIC, ≥16 μg/ml) (Table 1). Both strains showed KPC production in an immunochromatographic test and PCR analysis revealed the presence of bla_{KPC} and $bla_{CTX\text{-}M\text{-}group1}$ gene in both isolates. Whole genome sequencing of KP1 and KP2 revealed that both isolates belong to ST258, serotype KL107/O1/O2v2. The chromosomal genomes of KP1 and KP2 contain 5 457 364 bp and 5 457 028 bp, respectively.

Additionally, we identified four circular plasmids, outlined in Table 2. Both strains revealed multiple resistance genes located analogously throughout the genome. The intrinsic $bla_{SHV\text{-}11}$ gene was identified, along with *fosA*, *oqxA* and *oqxB*, resistance determinants to fosfomycin and quinolone, respectively. Both genomes showed mutations in gyrA-83I and parC-80I, associated with quinolone resistance. The IncFIB(K)/FII plasmid which size is 203 225 bp, contained multiple aminoglycoside resistance genes, including: *aadA2*, *aph(3')-Ia* and *rmtB*. A resistance genomic island with 14 622 bp, contained most of *sul1*, *mph(A)*, *catA1* and *dfrA12* genes. Furthermore, IncC2 plasmid, which size is 173 048 bp, harbored the $bla_{CTX\text{-}M\text{-}14}$ and $bla_{TEM\text{-}1B}$ genes (the latest duplicated) and others resistance genes, like *aph(3'')-Ib*, *aph(6)-Id*, *aac(3')-IId* and *sul2*, *tet(G)*, *floR*, *erm(42)*. The IncX3/IncU, which size is 46 540 + /- 80 bp, harbors the $bla_{KPC\text{-}2}$ gene in KP1 and its allelic variant $bla_{KPC\text{-}35}$ in KP2. The two KPC variants differ by a single-nucleotide variant (T503C) that codes for L169P in the omega loop region. IncX3/IncU plasmids were almost indistinguishable from pKP64477d in a *K. pneumoniae* reported in Brazil (GenBank MF150120.1) (99% coverage with 99.96% identity and 100% coverage with 99.97% identity, respectively). The genetic context of $bla_{KPC\text{-}2/35}$ includes an upstream insertion sequence (IS*Kpn6*-like), as well as a resolvase. No inverted repeats related to Tn*4401* were detected, which is remarkable, since this transposon is frequently associated with the

Table 1 Antibiotic susceptibility profiles of *K. pneumoniae* strains and their transconjugants.

Strains	Minimum inhibitory concentration (μg/ml)[a]												
	SAM	TZP	CZA	C/T	CAZ	ATM	FEP	ETP	IMP	MER	AMK	CIP	TG
KP1	≥32	≥128	1	≥32	≥64	≥64	≥32	≥8	≥16	≥16	32	≥4	≤0.5
TCKP1[b]	≥32	≥128	≤0.12	1	≥64	≥64	0.5	≥8	8	≥16	≤1	≤0.06	≤0.5
KP2	≥32	≥128	≥16	≥32	≥64	16	≥32	0.25	0.5	≤0.25	32	≥4	≤0.5
TCKP2[c]	8	≤4	2	1	≥64	≤1	0.5	≤0.12	≤0.25	≤0.25	≤1	≤0.06	≤0.5
ECJ53	≤2	≤4	≤0.12	≤0.25	≤0.12	≤1	≤0.12	≤0.12	≤0.25	≤0.25	≤1	≤0.06	≤0.5

[a] SAM: ampicillin/sulbactam; TZP: piperacillin–tazobactam; CZA: ceftazidime–avibactam; C/T: ceftolozane–tazobactam; CAZ: ceftazidime; ATM: aztreonam; FEP: cefepime; ETP: ertapenem; IPM: imipenem; MEM: meropenem; AMK: amikacin; CIP: ciprofloxacin; TGC: tigecycline.
[b] *E. coli* J53 transconjugant with the bla_{KPC-2} harboring plasmid.
[c] *E. coli* J53 transconjugant with the bla_{KPC-35} harboring plasmid.

mobilization of the carbapenemase gene[6,17]. ColRNAI was also identified, with a size of 9294 bp. Surprisingly, the ColRNAI plasmid was not associated with resistance genes.

Furthermore, KP1 and KP2 showed several mutations in *OmpK35*, *OmpK36* and *OmpK37*, known for restriction of antimicrobial penetration into the periplasmatic space[11]. O*mpK35* was truncated in both isolates, as it is commonly found in ST258 and causing a dysfunctional pore[11]. In KP1, *OmpK36* contained an insGD116, which has been previously reported to restrict the penetration of meropenem[12]. This was not a finding for KP2.

Conjugation assays revealed that IncX3/IncU plasmid were conjugative in both strains. Two transconjugants were obtained, TCKP1 and TCKP2. In TCKP1, the bla_{KPC} gene was detected and showed the classical resistance profile of KPC: resistant to ceftazidime and carbapenems, and susceptible to CZA. In TCKP2, we identified the bla_{KPC} gene with significant resistance to ceftazidime and a mild MIC increase for CZA. However, there were no differences for carbapenems and aztreonam MICs values when compared to *E. coli* J53 (Table 1).

The phylogenetic relationship analysis indicates a close correlation, with 96.4% of shared genes differing in only 69 single-nucleotide polymorphisms (SNPs). Additionally, KP1 and KP2 present 10 mutations in coding genes: two hypothetical proteins, *uvrY*, *clpV1*, *betI*, *betB*, *hpcE*, *traJ*, and *flhC* genes.

A phylogenetic tree with 13 202 high-quality SNPs was reconstructed using the maximum-likelihood method in 443 genomes of *K. pneumoniae* ST258, serotype KL107/O1/O2v2. As predicted, KP2 and KP1 were found to be closely related (ΔSNP, 69). Two clades showed significant similarities: Clade I, included DRR076334, which was isolated from a sputum sample in Japan in 2016, ERR3518883, ERR3518876, and ERR3518858, which was isolated from Brazil in 2018; Clade II, included ERR2743754, which was isolated from a blood sample in Brazil in 2016. Among all the genomes, DRR076334 showed the closest relationship, differing in only 46 SNPs (Fig. 1).

K. pneumoniae ST258 has been shown to be globally disseminated and KPC described as one of the most prevalent carbapemase. However, in Peru, NDM is the most reported carbapenamase among *Enterobacterales*[14], including the *Enterobacterales* classes A, B and D. Furthermore, there are no previous reports in Peru of KPC-producing *K. pneumoniae*

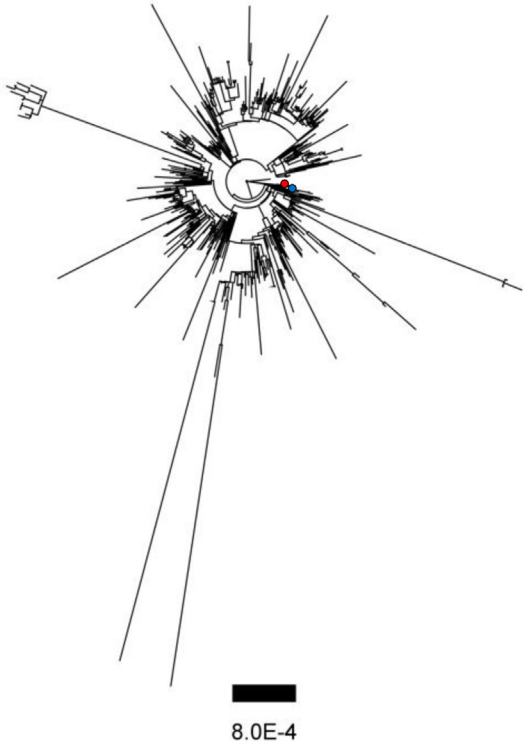

Figure 1 Phylogenetic tree with 445 genomes of *K. pneumoniae* ST258. Phylogenetic tree of 445 genomes of *K. pneumoniae* ST258 with 1000 bootstrap value. Four hundred forty-three were obtained from the Pathogenwatch collection. Blue circle: KP1; red circle: KP2.

ST258. The first report of a KPC-producing *K. pneumoniae* was presented in 2014; however, it was characterized as ST340 belonging to clonal group 258 (GC258) and carrying bla_{KPC-2} gene, located in a non-conjugative element[16].

Additionally to the isolates shown here, we found two other strains that belong to the ST258 in an analysis of 35 carbapenemase-producing *K. pneumoniae* (personal communication, unpublished data). The first report of KPC-35 was presented by Hemarajata and Humphries, showing a reversion of MICs for most β-lactams and a mild increase in MIC for CZA, which was attributed to an improved hydrolytic capacity for ceftazidime[4]. To our knowledge, there are no

Table 2 Genetic characteristics of *K. pneumoniae* strains.

ID	ST	Serotype	SNPs	Genetic material	Length (pb)	Resistance markers						Porins		
		O:KL				*bla*$_{KPC}$ allele	β-Lactam	Aminoglycoside	Fluoroquinolone	Sulfonamide	Macrolides	Others	*OmpK35*	*OmpK36*	*OmpK37*
KP1	258	O1/O2v2: KL107	0	Chromosome	5,457,364		*bla*$_{SHV-11}$		*GyrA-83I*, *ParC-80I*, *oqxA*, *oqxB*			*fofA6*	Deletion at 1-38aa	133insGD, 185insLSP, 311del NNFTGV	275delSSTNGG, 233delHYTH
				IncFIB(K)/ IncFII	203,225			*aph(3)-Ia*, *aadA2*, *rmtB*		*sul1*	*mph(A)*	*dfrA12*			
				IncC2	173,048		*bla*$_{CTX-M-14}$, *bla*$_{TEM-1B}$[a]	*aph(3″)-Ib*, *aph(6)-Id*, *aac(3)-IId*		*sul2*	*erm(42)*	*tet(G)*, *floR*			
				IncX3/ IncU ColRNAI	46,460 9,294	*bla*$_{KPC-2}$									
KP2	258	O1/O2v2: KL107	69	Chromosome	5,457,028		*bla*$_{SHV-11}$		*GyrA-83I*, *ParC-80I*, *oqxA*, *oqxB*			*fofA6*	Deletion at 1-38aa	185insLSP, 311del NNFTGV	275delSSTNGG, 233delHYTH
				IncFIB(K)/ IncFII	203,226			*aph(3)-Ia*, *aadA2*, *rmtB*		*sul1*	*mph(A)*	*dfrA12*			
				IncC2	173,048		*bla*$_{CTX-M-14}$, *bla*$_{TEM-1B}$[a]	*aph(3″)-Ib*, *aph(6)-Id*, *aac(3)-IId*		*sul2*	*erm(42)*	*tet(G)*, *floR*			
				IncX3/ IncU ColRNAI	46,620 9,294	*bla*$_{KPC-35}$									

[a] Duplicated.

other publication that characterizes the allelic variant of KPC.

KP1 shows the genetic traits of a multi-drug resistant strain: resistant to carbapenems and susceptible to CZA. KP2 showed a reversion of carbapenem resistance and a MIC increase of 5 dilutions (MIC $\geq$16) for CZA.

Furthermore, the conjugative assay indicates that IncX3/IncU plasmid, harboring bla_{KPC-35}, resulted in lower MICs for carbapenems, monobactams, and cephalosporins, except for ceftazidime. Although, IncX3/IncU contributed to the MIC value of CZA, its effect was mild (TCKP2 had two dilutions higher compared to KP2).

Nonetheless, the differences between KP1 and KP2 regarding MIC for CZA suggests the involvement of another mechanism. We discovered 10 genes with missense or frameshift variations between both strains (two of which were hypothetical proteins) that could explain these results. Additionally, KP1 presented an insGD116 mutation in *OmpK36*, which contributes to a 26% reduction in pore size and restricts antimicrobial entry[12]. Intriguingly, KP2 does not have this mutation. It has been hypothesized that a higher expression of bla_{KPC}, increases efflux activity (acrAB) and chromosomal modifications of the major outer membrane porins (*OmpK35* and *OmpK36*), decreasing the active concentration of CZA in its transpeptidase targets[11].

The phylogenetic analysis revealed close similarity between both strains; however, differences in 69 SNP suggest that the patient was colonized with both clones simultaneously, exceeding the proposed clonality cut-off[10]. Among all the genomes, *K. pneumoniae* producing KPC-2 (DRR076334), isolated from the sputum of a Japanese patient, showed greater similarity with KP1 and KP2, with minor differences in *rmtB* location or the absence of *catA*[1].

Since the clinical history is unknown, suggestions about CZA selection in KP2 are speculative. Nevertheless, this report on *K. pneumoniae* ST258 in Peru highlights the increasing epidemiological risks of antimicrobial resistance and reinforces the urgency for setting-up a well-funded antibiotic stewardship program in the region.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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