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Short Communication

Novel patterns in the molecular epidemiology of KPC-producing *Klebsiella pneumoniae* in Tucumán, Argentina

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ABSTRACT

Background: In Argentina, there has been an abrupt increase in KPC-2-producing *Klebsiella pneumoniae* (*K. pneumoniae*). Tucumán is a multi-border area, so the rapid dissemination of carbapenem-resistant *K. pneumoniae* is a clinically relevant problem for the region.
Objectives: This study aimed to investigate the epidemiological and molecular patterns of KPC-producing *K. pneumoniae* clinical isolates collected from different hospitals in Tucumán.
Methods: Carbapenem-resistant *K. pneumoniae* strains were sequentially and uniquely collected during two time periods. Antibiotic susceptibility was determined by the automated Vitex 2® system and using the standard agar dilution test. Multilocus sequence typing and pulsed-field electrophoresis were used for epidemiological analysis. The genetic structures around bla_{KPC} and the encoding genes of extended-spectrum β-lactamases were detected by polymerase chain reaction and sequencing. Plasmids were analysed by conjugation and using the plasmid relaxase gene-typing method.
Results: All 37 isolates were multidrug resistant, and thebla_{KPC-2} gene was confirmed in all of them. In 17 isolates (45.9%), the $bla_{CTX-M-2}$ gene was also amplified, as well as bla_{SHV-2} in five isolates (13.5%) and $bla_{CTX-M-2}$/bla_{SHV-2} in four isolates (10.8%). The molecular epidemiology of the bla_{KPC-2} gene has resulted in it being associated with an IncL/M transferable plasmid disseminating in various sequence types (STs) (ST17, ST556, ST342, ST147, ST461, ST65, ST15 and ST70), and in a new genetic environment with a 764-bp deletion in the IS*Kpn7*-bla_{KPC} region.
Conclusions: These findings contribute to the understanding of the great diversity of the bla_{KPC-2}-carrying genetic platforms.

1. Introduction

The emergence of carbapenem-resistant Gram-negative pathogens is a major clinical and public health concern [1]. As reported, bla_{KPC}, bla_{NDM} and bla_{OXA-48} are the most frequently observed carbapenemase genes among carbapenem-resistant Enterobacteriaceae clinical isolates [2]. In particular an increasing prevalence of *Klebsiella pneumoniae* (*K. pneumoniae*) carbapenemases (KPCs) has been reported worldwide and been the most frequently observed class A carbapenemases since their first description in eastern USA in1996 [3]. Of the many diff ;erent KPC family variants (KPC-1 to KPC-22), the most well-characterized variants are KPC-2 and KPC-3. KPCs are mostly plasmid-encoded enzymes, and bacteria producing these enzymes are only susceptible to a few antibiotics such as colistin and tigecycline. Most carbapenem-resistant Enterobacteriaceae are resistant to

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aminoglycosides, and blaKPC producers are susceptible to ceftazidime-avibactam [1].

The success of KPC is based on both gene and plasmid dissemination, and on the clonal spread of *K. pneumoniae* ST258 and its variants (e.g. ST11) [1]. The bla_{KPC} gene is located on a highly mobile Tn*3*-related transposon, Tn*4401*, that can be carried by different transferable plasmids of various incompatibility groups [3]. In addition to the transposase (*tnpA*) and resolvase (*tnpR*) genes, this transposon includes two insertion sequences (IS*Kpn6* and IS*Kpn7*) and is flanked by two 39-bp inverted repeat sequences. Moreover, five isoforms of Tn*4401* (a, b, c, d, e) have been characterized [4,5].

The epidemiology of *K. pneumoniae*-producing KPCs varies geographically; in South America, the endemic spread of KPCs was initially reported in 2006 in Colombia [6] and then in Brazil and Argentina [7,8]. The first KPC-2-producing salmonella in Argentina has previously been described [9]. In Brazil, Chile, Mexico and Colombia, the spread of KPCs has been associated with the ST258 and two major related clones (ST11 and ST437) carrying bla_{KPC-2} and bla_{KPC-3} [10–12].

In Argentina, previous studies have demonstrated that the emergence of bla_{KPC-2} is also associated with CC258 and was characterized by two patterns of dispersion: 1) irregular occurrence of diverse Enterobacteriaceae harboring blaKPC-2 in the IncL/M transferable plasmid in distant regions; 2) sudden clonal spread of *K. pneumoniae* ST258 harboring blaKPC-2 in Tn*4401a* [13]. The Pan American Health Organization reported that Argentina is one of the countries with the most 'pandrug-resistant' nosocomial isolations in Latin America [14]. Besides the numerous efforts made at local and national levels to control the spread of these species, the rapid dissemination of carbapenem-resistant *K. pneumoniae* has become a clinically relevant problem in the region. Tucumán is situated in the north of Argentine, within a multiborder area with Bolivia, Chile and Paraguay. Since 2006, active monitoring for carbapenem-resistant *K. pneumoniae* detection has been carried out at the current department.

The objective of this study was to determine the epidemiological and molecular patterns of KPC-producing *K. pneumoniae* clinical isolates collected from different hospitals in Tucumán, Argentina.

2. Materials and methods

2.1. Bacterial identification and antimicrobial susceptibility testing

Bacterial identification was confirmed by MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) (Microflex LT, Bruker Daltonics, Bremen, Germany). The antimicrobial susceptibility pattern was determined by the automated Vitex 2® system (BioMerieux, Merci l'Etoile, France) and using a standard agar dilution test including ampicillin (AMP), ampicillin/sulbactam (SAM), piperacillin/tazobactam (PTAZ), cephalothin (CEF), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), meropenem (MER), imipenem (IMP), gentamicin (GEN), amikacin (AKN), colistin (COL), trimethoprim/sulfamethoxazole (TMS) ciprofloxacin (CIP), tigecycline (TGC) and fosfomycin (FOS). Breakpoints were defined following the document M100-S24 of the Clinical and Laboratory Standards Institute [15] and according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org)

Synergy tests with boronic acid and EDTA disks close to the carbapenemes and the modified Hodge test (MHT) were performed to detect carbapenemases [16]. *Klebsiella pneumoniae* ATCC700603 and *Escherichia coli* ATCC 25922 were used as quality control strains for the antibiotic susceptibility tests.

2.2. Strain selection

Strains were sequentially and uniquely collected during two time periods: October–November 2013 at one hospital (Hospital Padilla) and May 2010–August 2012 in the other (Centro de Microbiología Médica, CMM). *Klebsiella pneumoniae* strains were selected on the basis of MIC values of $\geq$ 2 mg/L for any of the carbapenems IMP, meropenem (MER) or ertapenem, and synergy and Hodge tests positive, and were subsequently sent to the Bacteriology lab for reference testing.

β-lactamases molecular characterization

Multiplex polymerase chain reaction (PCR) targeting carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{VIM} and bla_{OXA-48}) and extended spectrum β-lactamases-ESBLs: SHV variants including SHV-2 (bla_{SHV-2}) and CTX-M variants including CTX-M-2 ($bla_{CTX-M-2}$) was performed [17]. The amplicons were sequenced with ABI3130CL (Applied Biosystems, USA) and the sequences were analyzed by the National Center for Biotechnology Information (NCBI) https://www.ncbi.nlm.nih.gov.

2.3. Population structure

Molecular typing was performed by pulsed-field electrophoresis (PFGE) and multilocus sequence typing (MLST). Isolates were typed by PFGE of *Spe*I-digested total genomic DNA (TaKaRa, Tokyo, Japan), and the DNA fragments were separated by electrophoresis on 1% SeaKeam Gold agarose (Lonza, Rockland, ME, United States) in 0.5X TBE (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA; ph 8.0) buffer using the CHEF Mapper XA PFGE system (Bio-Rad, United States) at 6 V/cm^2 and 14 °C, with alternating pulses at a 120º angle in a 5–20

Table 1a
Minimum inhibitory concentration (MIC) results of the 37 carbapenem-resistant Klebsiella pneumoniae isolates.

Antimicrobial agent	MIC range (mg/L)	MIC50 (mg/L)	MIC90 (mg/L)	Number of S (%)	Number of R (%)
Ampicillin	≥32	≥32	≥32	0 (0)	37 (100)
Ampicillin/Sulbactam	≥32	≥32	≥32	0 (0)	37 (100)
Piperacillin/Tazobactam	16-≥128	≥128	≥128	0 (0)	37 (100)
Cefalotin	≥64	≥64	≥64	0 (0)	37 (100)
Cefotaxime	≤1-≥64	≥64	≥64	0 (0)	37 (100)
Ceftazidime	4-≥64	≥16	≥16	0 (0)	37 (100)
Cefepime	≤1-≥64	≥16	≥16	0 (0)	37 (100)
Meropenem	≥16	≥16	≥16	0 (0)	37 (100)
Imipenem	8-≥16	≥16	≥16	0 (0)	37 (100)
Gentamicin	≤ 1-≥1 6	≥16	≥16	8 (21)	29 (79)
Amikacin	≤2–16	≤2	≤2	37 (100)	0 (0)
Colistin	≤0.5	≤0.5	≤0.5	37 (100)	0 (0)
Trimethoprim/sulfamethoxazole	≤ 20-≥320	≥320	≥320	12 (32)	25 (68)
Ciprofloxacin	≤0.25-32	1	1	0 (0)	37 (100)
Tigecycline	≤1–≥4	≤1	≤1	36 (97)	1 (3)
Fosfomycin	≤ 32–≥64	≤32	≤32	37 (100)	0 (0)

Table 1b
Comparison between Klebsiella pneumoniae donor isolates and their transconjugants (n = 11) with respect to non-susceptibility rates (%) and MICs of β-lactam antibiotics.

Antimicrobial agent	Non-susceptibility rates (%)		MICs (mg/L)		
	D	T	D (MIC 50, MIC 90)	T (MIC 50, MIC 90)	*Escherichia coli* strain J53 AzR (recipient)
Ampicillin	100	100	≥32; ≥32	≥32; ≥32	4
Ampicillin/Sulbactam	100	100	≥3 2; ≥ 2	≥16; ≥16	2
Piperacillin/Tazobactam	100	100	≥ 28; ≥128	≥128; ≥128	1
Cefotaxime	100	100	≥64; ≥64	≥64; ≥64	0.064
Ceftazidime	100	100	≥64; ≥64	≥64; ≥64	0.25
Cefepime	100	100	≥16; ≥16	≥16; ≥16	0.064
Meropenem	100	100	≥16; ≥16	≥16; ≥16	0.125
Imipenem	100	100	≥16; ≥16	≥16; ≥6	0.125

D: Klebsiella pneumoniae donors; T: Transconjugants (Escherichia coli strain J 53 Az R).

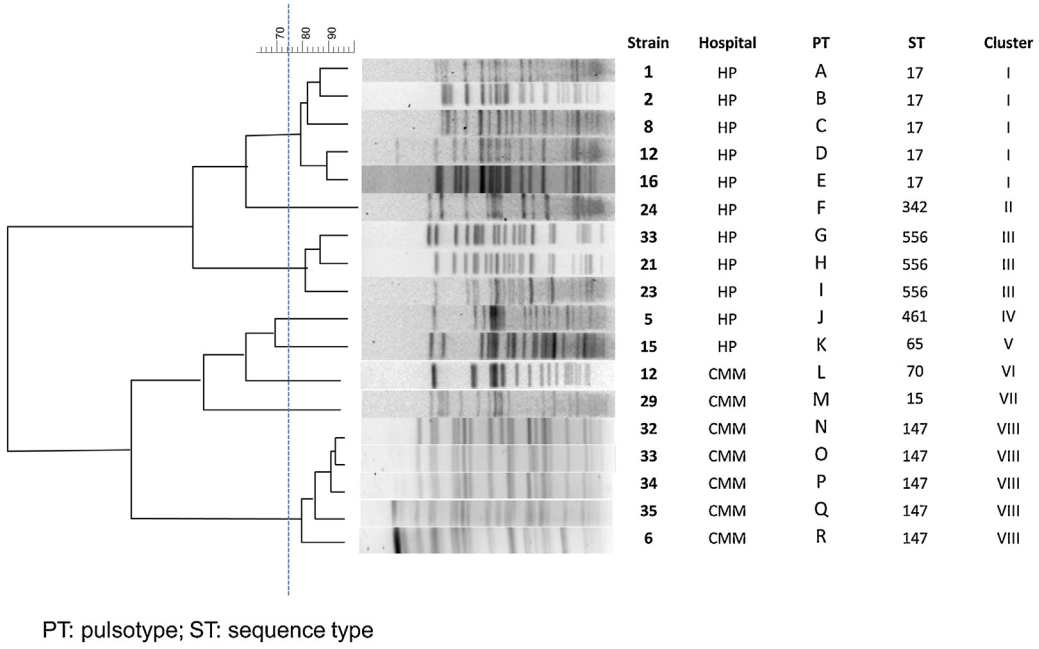


Fig. 1. DNA fingerprinting by pulsed-field electrophoresis (PFGE) and relation to sequence type (ST) in KPC-2 *Klebsiella pneumoniae* strains.

second pulse time gradients for 19 hours. DNA patterns were interpreted according to Tenover et al. [18]. Strains were considered to be the same clone (type) if they showed ≥ 75% genetic identity, or fewer than three fragment differences on the PFGE profiles. Subsequently, one isolate for each PFGE pulsotype was submitted to MLST technique following the *K. pneumoniae* MLST website guidelines http://bigsdb.pasteur.fr/.

2.4. PCR mapping of the Tn4401 flanking region

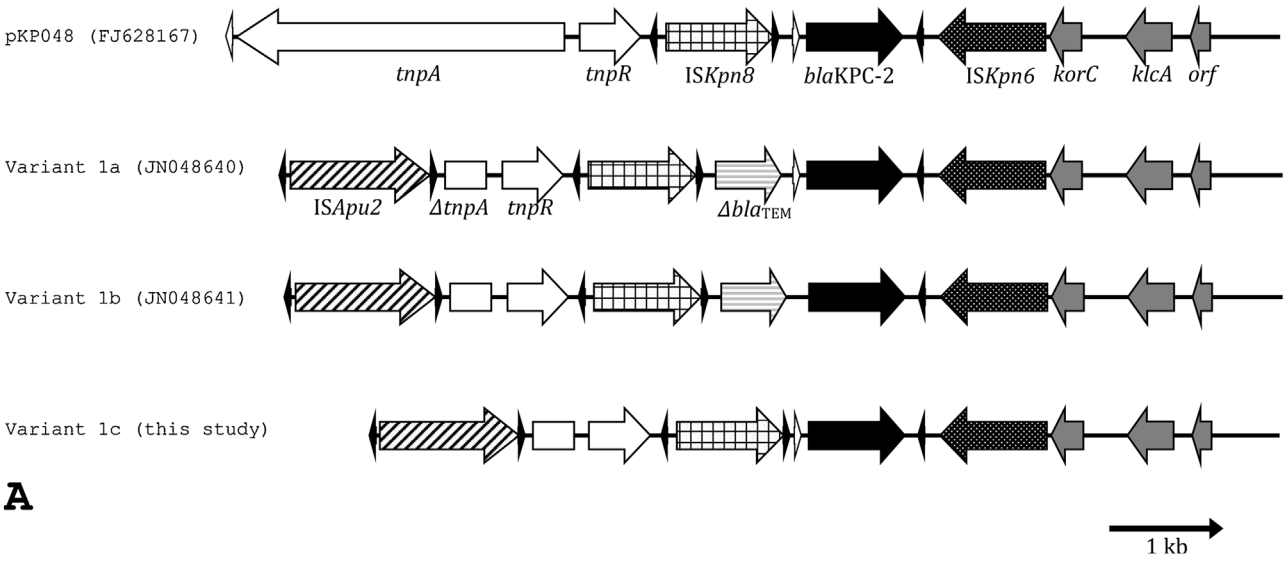
To complete the molecular analysis of KPC-producing *K. pneumoniae*, strains representative of different STs were further analyzed, the genetic surroundings of bla_{KPC2} were performed using primers reported elsewhere and primers designed in this study [13,19]. All PCR products were sequenced bidirectionally using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 3730xl capillary sequencer.

2.5. Plasmid analysis

Conjugation experiments were performed in all different ST strains, in Mueller Hinton broth (bio Merieux) at 37 °C using the azide-resistant Escherichia coli strain J53 as the receptor with a donor to recipient ratio of 1:1. Transconjugants were selected on Drigalski agar (Bio-Rad) containing 100 mg/L sodium azide and 2 mg/L meropenem. Plasmid typing was performed using the plasmid relaxase gene-typing method (PRaseT) [20]. Antimicrobial susceptibilities of the transconjugants were determined by standard agar dilution test including: AMP, SAM, PTAZ, CTX, CAZ, FEP, MER and IMP. MICs of the transconjugants were compared with those of the donors and *Escherichia coli* (J53 AzR) recipient strain. All the transconjugants were screened using PCR for the plasmid replicons and the bla_{KPC-2} gene.

3. Results and discussion

This study retrospectively characterized 37 carbapenem-resistant clinical strains of *K. pneumoniae*. The sample sources were the respiratory tract (n = 12, 32.4%), urinary tract (n = 12, 32.4%), soft tissue (n = 5, 13.6%), blood (n = 2, 5.4%), cerebrospinal fluid (n = 2, 5.4%), bone (n = 2, 5.4%), and abdominal fluid (n = 2, 5.4%). All 37 isolates were multidrug resistant and have similar susceptibility profiles (Table 1a); the bla_{KPC-2} gene was confirmed in all of them. In 17 isolates (45.9%), the $bla_{CTX-M-2}$ gene was also amplified, as



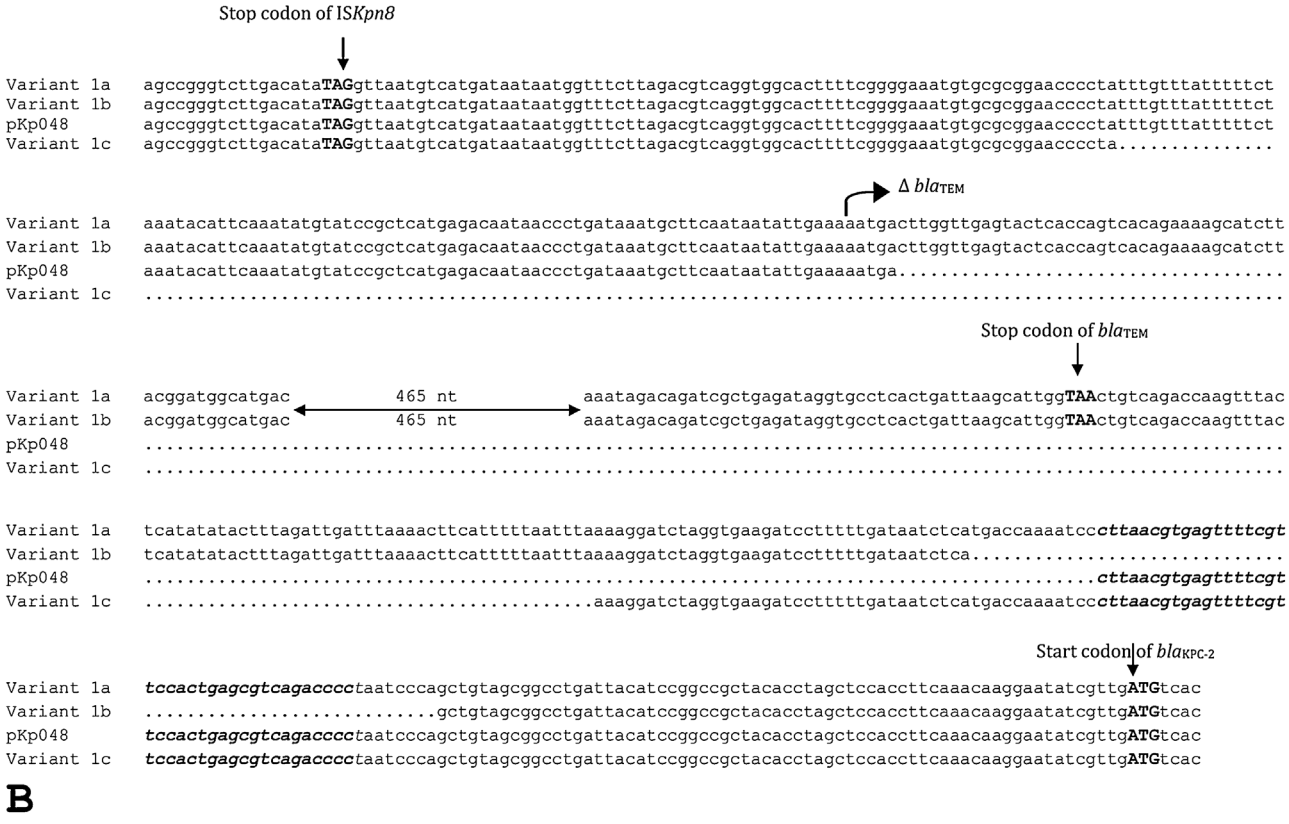


Fig. 2. Genetic environment of *bla*KPC-2 in *Klebsiella pneumoniae* from Tucumán, Argentina.
A. Genes (open reading frames) and their corresponding transcriptional orientations are indicated by horizontal broad arrows. Black and white triangles represent inverted repeats of insertion sequences (ISs) or of the Tn3-like transposon, respectively. The depicted region of the plasmid pKP048 corresponds to coordinates 15 736 to 25 596 (complement) in GenBank FJ628167 [19]. The variant 1a represents the sequence obtained from *Enterobacter cloacae* M11180 (GenBank JN048640) [13]. The variant 1b represents the sequence obtained from *Escherichia coli* M9884 (GenBank JN048641) [13].
B. Nucleotide alignment of the four variants showing the observed deletions. The depicted region corresponds to coordinates 4358 to 5330 in GenBank JN048640. The variant 1b differs from the variant 1a by a 58-bp deletion encompassing the Tn3-like right inverted repeat (in bold and italic).

well as bla_{SHV-2} in five isolates (13.5%) and $bla_{CTX-M-2}/bla_{SHV-2}$ in four isolates (10.8%).

PFGE analysis individualized 18 different pulsotypes (PT) (designated as A-R) grouped into eight different clusters, which corresponded to eight sequence types: ST17 (n = 5), ST 556 (n = 3), ST342 (n = 1), ST147 (n = 5), ST461 (n = 1), ST65 (n = 1), ST15 (n = 1), and ST70 (n = 1) (Fig. 1).

ST258, previously encountered in Latin American hospitals, was not detected in the present *K. pneumoniae* collection. This was somewhat surprising since bla_{KPC-2} has been reported in more than

115 distinct and heterogeneously distributed STs, with the vast majority of strains belonging to the CC258 that predominantly encompasses the related STs ST258 and ST11 [1]

In all the strains grouping in different STs (n = 18), the genetic environment of Tn*4401* was similar to that of the variant characterized on plasmid pKP48 found in a Chinese *K. pneumoniae* isolate [19]. This variant was reported in Argentina and named variant Ia, on the basis of two differences (a composite transposon and an additional 671-bp fragment containing a truncated bla_{TEM-1} gene) [13].

A similar variant has been observed in Chilean *K. pneumoniae* isolates [12]. However, with the primer pair, which hybridized to IS*Kpn8* and the bla_{KPC} gene, a shorter fragment was found upstream of the bla_{KPC} gene. Primers designed to target the IS*Kpn8*-bla_{KPC} region and sequencing confirmed a 764-bp deletion encompassing the bla_{TEM} gene (Fig. 2). The current variant was named variant 1c, which was different from the variant 1b also described in Argentina (Fig. 2) [13].

The bla_{KPC-2} gene was successfully transferred to *E. coli* in 11 of 18 strains, representative of the different STs. Plasmid typing showed all transconjugants to contain an IncL/M plasmid with high resistance rates against β-lactams (Table 1b). The bla_{KPC-2} gene was confirmed in all of them.

In conclusion, this study reports the molecular epidemiology of bla_{KPC-2} in *K. pneumoniae* isolated in Tucumán, Argentina. The gene was associated with an IncL/M transferable plasmid disseminating in various STs (ST17, ST556, ST342, ST147, ST461, ST65, ST15 and ST70). It was found to be part of a Tn*4401* entity similar to variant Ia. However, a new genetic environment with a 764-bp deletion in the IS*Kpn7*-bla_{KPC} region is reported. These findings contribute to the understanding of the high diversity of bla_{KPC-2}-carrying genetic platforms.

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Competing interests

None declared.

Ethical Approval

Not required.

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