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Short Communication

In vivo horizontal dissemination of the bla_{KPC-2} gene carried on diverse genetic platforms among clinical isolates of Enterobacteriaceae

M.S. Anchordoqui [a], D. De Belder [a,e], C. Lucero [a], M. Rapoport [a], D. Faccone [a,e], A. Rodriguez [b], A. Di Martino [c], F. Martino [a], I. Herrero [d], F. Pasteran [a], A. Corso [a], S.A. Gomez [a,e,*]

[a] Servicio Antimicrobianos, National Reference Laboratory in Antimicrobial Resistance, Instituto Nacional de Enfermedades Infecciosas ANLIS 'Dr Carlos G. Malbrán', Buenos Aires, Argentina
[b] Clínica y Maternidad Suizo Argentina, Buenos Aires, Argentina
[c] Sanatorio Mitre, Buenos Aires, Argentina
[d] Hospital Municipal de Urgencias, Córdoba, Argentina
[e] Research Career, CONICET (Consejo Nacional de Investigaciones Científicas y Tecnológicas)

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ABSTRACT

This study investigated the molecular characteristics of six bla_{KPC}-positive Enterobacteriaceae recovered from three patients in Argentina. Antimicrobial susceptibility testing was performed following Clinical and Laboratory Standards Institute (CLSI) 2014 recommendations. Molecular characterisation of the isolates was performed by biparental conjugation, PCR, sequencing, S1 nuclease restriction, and Southern blot hybridisation with a bla_{KPC} probe using standard protocols and conditions. The isolates studied were as follows. Case 1: *Escherichia coli* (ECO-P1) and *Klebsiella pneumoniae* (KPN-P1) isolated from a rectal swab harboured bla_{KPC-2} in transposon Tn*4401a* on non-typeable and non-conjugative plasmids. Case 2: *Enterobacter cloacae* (ECL-P2) and *K. pneumoniae* (KPN-P2) were isolated from two blood cultures. bla_{KPC-2} was found in a novel genetic variant of IS*Kpn8*–bla_{KPC-2}–IS*Kpn6*-like on conjugative plasmids of IncL/M type. Case 3, *Citrobacter freundii* (CFR-P3) and *Klebsiella oxytoca* (KOX-P3) were isolated from skin and skin-structure infection. The bla_{KPC} gene was detected on IS*Kpn8*–Δbla_{TEM}–bla_{KPC-2}–IS*Kpn6*-like located on an IncA/C conjugative plasmid. CFR-P3 and KOX-P3 harboured bla_{PER-2} in addition to the bla_{KPC} gene. In conclusion, we document the horizontal dissemination of bla_{KPC-2} from diverse Enterobacteriaceae clinical isolates with different genetic backgrounds. This is the first report of *E. coli* harbouring bla_{KPC} associated with Tn*4401a* in Argentina.

1. Introduction

Genes encoding KPC enzymes are predominantly identified among *Klebsiella pneumoniae* isolates and increasingly among other Enterobacteriaceae and non-fermenters. The global predominance of KPC-producing bacteria has been mainly associated with the successful and hyperepidemic clone of *K. pneumoniae* sequence type (ST) 258 and single and double-locus variants such as ST11 [1]. bla_{KPC} genes are typically transposon-encoded (Tn*4401* and its variants) and therefore have the potential to disseminate between plasmids and across bacterial species [2]. The spread of bla_{KPC} by horizontal genetic transfer has been previously documented in a simultaneous blood infection [3], in colonised patients possessing KPC-3, Tn*4401a* and pKpQIL-IT elements [4] and through the transmission of a promiscuous plasmid carrying the KPC gene in Tn*4401a* [5].

In Argentina, health institutions throughout the country refer clinical strains for characterisation to the National Reference Laboratory in Antimicrobial Resistance (LNRAR) following local guidelines. In this context, six Enterobacteriaceae isolates recovered from three patients with phenotypes indicative of KPC production were sent to LNRAR for confirmation and molecular characterisation.

2. Methods

2.1. Patients and clinical isolates

The epidemiological data and clinical characteristics of the patients are shown in Table 1. Patients 1, 2 and 3 were hospitalised

* Corresponding author. Present address: Servicio Antimicrobianos, Departamento de Bacteriología, Instituto Nacional de Enfermedades Infecciosas ANLIS 'Dr Carlos G. Malbrán', Av. Vélez Sarsfield 563 (C1282AFF), Ciudad Autónoma de Buenos Aires, Argentina. Tel.: +54 11 4303 2812; fax: +54 11 4303 2812.
E-mail address: sgomez@anlis.gov.ar (S.A. Gomez).

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Table 1
Epidemiological and clinical characteristics of patients infected or colonised with bla_{KPC}-positive isolates.

Patient	Isolate	Isolation date	Age (years)/sex	Hospital (city)	Reason for admission	Underlying conditions	KPC-positive specimen	LOS (days)	Antibiotic treatment (during hospital stay)	Outcome
1	ECO-P1 KPN-P1	24 Oct. 2010 25 Oct. 2010	94/F	Clínica y Maternidad Suizo Argentina (Buenos Aires)	Colostomy	Colon cancer	Rectal swab	45	UKN	UKN
2	ECL-P2 KPN-P2	14 March 2011 21 March 2011	87/M	Sanatorio Dupuytren (Buenos Aires)	UKN	Neurological patient with MV	Blood	>12	COL, VAN[a]	UKN
3	CFR-P3 KOX-P3	4 April 2012	52/M	Hospital Municipal de Urgencias (Córdoba)	Wound	DM, DVT, CD, bypass, supracondylar amputation	Wound	26	FLZ[a], CAZ, VAN[a], CLI[a], CIP, SXT + TZP	Died

LOS, length of stay; ECO, *Escherichia coli*; KPN, *Klebsiella pneumoniae*; ECL, *Enterobacter cloacae*; CFR, *Citrobacter freundii*; KOX, *Klebsiella oxytoca*; UKN, unknown; MV, mechanical ventilation; DM, diabetes mellitus; DVT, deep vein thrombosis; CD, cardiovascular disease; COL, colistin; VAN, vancomycin; FLZ, fluconazole; CAZ, ceftazidime; CLI, clindamycin; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; TZP, piperacillin/tazobactam.
[a] Chemotherapy used to treat fungal infections or Gram-positive bacteria.

in 2010, 2011 and 2012, respectively, in different institutions in two cities in Argentina. The isolates belonged to five species, including *Escherichia coli* ($n = 1$), *K. pneumoniae* ($n = 2$), *Enterobacter cloacae* ($n = 1$), *Klebsiella oxytoca* ($n = 1$) and *Citrobacter freundii* ($n = 1$) (Table 1).

2.2. Susceptibility testing and detection of resistance genes

Minimum inhibitory concentrations (MICs) to imipenem, meropenem, ertapenem, cefepime, cefotaxime, ceftazidime, gentamicin, amikacin, ciprofloxacin, minocycline, colistin, fosfomycin and tigecycline were determined by agar dilution using standard methods according to Clinical and Laboratory Standards Institute (CLSI) criteria [6]. MIC interpretation was according to CLSI guidelines (Table 2A in CLSI document M100-S24 [7]) for all antimicrobials except for fosfomycin (endovenous), colistin and tigecycline, which were interpreted following European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations. KPC production was detected phenotypically considering imipenem inhibition zones ≤22 mm and positive synergy between carbapenem disks and 3-aminophenil-boronic acid (APB) and negative synergy between carbapenem disks and ethylene diamine tetra-acetic acid/sodium mercaptoacetic acid (EDTA/SMA) [8,9]. Extended-spectrum β-lactamase (ESBL) production was suspected phenotypically by positive synergy with clavulanic acid. Locally prevalent ESBL genes were confirmed by specific PCRs using standard conditions (CTX-MU1, 5′-ATGTGC AGYACCAGTAARGT-3′; CTX-MU2, 5′-TGGGTRAARTARGTSACCAGA-3′; PER-2F, 5′-GTAGTATCAGCCCAATCCCC-3′; PER-2R, 5′-CCAATAAAGGCCGTCCAT CA-3′). All resistance genes and genetic contexts were sequenced using the above primers as well as others previously reported using BigDye™ Terminator methodology [10].

2.3. Conjugation experiments

Transfer of KPC-harbouring elements was tested by biparental conjugation with *E. coli* J53 (sodium azide-resistant) on Luria–Bertani (LB) agar plates in a 1:3 donor/recipient ratio. Transconjugants were selected on LB agar plates with sodium azide (150 μg/mL) and ampicillin (50 μg/mL) [10].

2.4. Genetic typing

For pulsed-field gel electrophoresis (PFGE), total DNA was digested with *Xba*I and the fragments were separated using 2.2 s and 54.2 s as initial and final pulse times over 20 h. The running was performed using a CHEF-DR® III Apparatus (Bio-Rad, Hercules, CA) and the gel was recorded and DNA patterns were analysed according to the criteria of Tenover et al. [10,11].

2.5. Plasmid analysis

Plasmid extraction was performed using a QIAGEN Midi Kit (QIAGEN, Hilden, Germany). Plasmids were classified by PCR-based replicon typing (PBRT) and S1 nuclease digestion of total DNA [12,13] and were analysed by Southern blot and hybridisation of the PFGE gels using a bla_{KPC-2}-specific probe (PCR DIG Probe Labeling Mix; Roche Applied Science, Barcelona, Spain).

2.6. Nucleotide sequence accession number

A 1554-bp sequence including the bla_{KPC-2} gene corresponding to isolate ECL-13354 (ECL-P2) and KPN-13355 (KPN-P2) has been submitted to the GenBank nucleotide sequence database under accession nos. KR108242 and KR108243, respectively.

3. Results and discussion

Epidemiological data for the patients is shown in Table 1. The three patients had prolonged hospitalisation and serious underlying conditions. Patients 2 and 3 had had previous antibiotic treatment due to multiple nosocomial bacterial and fungal infections. Isolate recovery was 1 day and 7 days apart for Cases 1 and 2, respectively, and simultaneously in Case 3.

The susceptibility patterns of all isolates are shown in Table 2. Susceptibility to carbapenems was variable, from fully susceptible to resistant, whilst the isolates were highly resistant to expanded-spectrum β-lactams. Resistance to aminoglycosides and fluoroquinolones was also variable, whereas colistin and fosfomycin remained susceptible. All studied isolates harboured bla_{KPC-2}, and the only ESBL detected was bla_{PER-2} in isolates from Patient 3 (Table 2).

The *K. pneumoniae* isolates from Patients 1 and 2 were not clonally related (>6 band difference); however, the isolate recovered from Patient 1 was genetically related to *K. pneumoniae* ST258 (data not shown).

Transconjugants were obtained for the isolates from Patient 2 (TC-ECL-P2 and TC-KPN-P2) and Patient 3 (TC-CFR-P3 and TC-KOX-P3). All transconjugants were shown by PCR to contain bla_{KPC-2}. Carbapenem MICs of transconjugants obtained from Patient 2 were at least three times higher than those of the recipient cells (*E. coli* J53) (Table 2). Moreover, bla_{PER-2} was

Table 2
Susceptibility testing and interpretation, genes detected, plasmid profile and genetic context of bla_{KPC-2}.

Patient	Isolate	MIC (μg/mL) [susceptibility interpretation]												*bla* gene	No. of plasmids and MW (kb)[a]	RT	Genetic element(s)
		IPM	MER	ETP	FEP	CAZ	CTX	MIN	COL	FOS	TIG	CIP	AMK	GEN			
1	ECO-P1	0.5 [S]	0.5 [S]	0.25 [S]	2 [S]	16 [I]	4 [R]	0.5 [S]	0.25 [S]	0.5 [S]	0.25 [S]	0.008 [S]	64 [R]	64 [R]	bla_{KPC-2}	<48.5, **70**	NT	Tn*4401a*
	KPN-P1	1 [S]	1 [S]	8 [S]	16 [R]	>64 [R]	16 [R]	16 [R]	0.5 [S]	4 [S]	1 [S]	≥16 [R]	64 [R]	32 [R]	bla_{KPC-2}	<48.5, 58, **70**, 211	NT	
2	ECL-P2	4 [R]	8 [R]	16 [R]	≥64 [R]	64 [R]	≥64 [R]	16 [R]	0.5 [S]	2 [S]	2 [I]	8 [R]	2 [S]	0.5 [S]	bla_{KPC-2}	**65.5**, 162	L/M	IS*Kpn8*–bla_{KPC-2}–IS*Kpn6*-like
	TC-ECL-P2	4 [R]	2 [I]	1 [I]	4 [I]	4 [S]	8 [R]	1 [S]	0.25 [S]	0.5 [S]	0.25 [S]	0.015 [S]	0.5 [S]	0.12 [S]	bla_{KPC-2}	**65.5**	L/M	
	KPN-P2	2 [I]	1 [S]	4 [R]	8 [I]	32 [R]	8 [R]	8 [I]	1 [S]	8 [S]	1 [S]	0.25 [S]	2 [S]	0.5 [S]	bla_{KPC-2}	**70**, 86	L/M	
	TC-KPN-P2	4 [R]	2 [I]	1 [I]	4 [I]	4 [S]	8 [R]	1 [S]	0.25 [S]	0.5 [S]	0.25 [S]	0.008 [S]	0.5 [S]	0.12 [S]	bla_{KPC-2}	**70**, 165	L/M	
3	CFR-P3	0.5 [S]	0.12 [S]	0.12 [S]	8 [I]	>64 [R]	16 [R]	32 [R]	0.25 [S]	0.25 [S]	4 [R]	8 [R]	32 [I]	8 [S]	bla_{KPC-2}, bla_{PER-2}	**70**, 178–291	A/C	IS*Kpn8*–Δbla_{TEM}–bla_{KPC-2}–IS*Kpn6*-like
	TC-CFR-P3	0.25 [S]	0.5 [S]	0.125 [S]	2 [S]	≥128 [R]	8 [R]	1 [S]	0.25 [S]	0.5 [S]	0.25 [S]	0.015 [S]	8 [S]	2 [S]	bla_{KPC-2}, bla_{PER-2}	70, 204	A/C	
	KOX-P3	0.5 [S]	0.12 [S]	0.5 [S]	8 [I]	>64 [R]	16 [R]	4 [S]	0.25 [S]	4 [S]	0.5 [S]	≥16 [IR	16 [S]	≥256 [R]	bla_{KPC-2}, bla_{PER-2}	**70**, 118, 168	A/C, L/M	
	TC-KOX-P3	0.5 [S]	0.5 [S]	0.25 [S]	2 [S]	64 [R]	8 [R]	1 [S]	0.25 [S]	0.5 [S]	0.25 [S]	1 [R]	8 [S]	4 [S]	bla_{KPC-2}, bla_{PER-2}	**76**	A/C, L/M	
	E. coli J53	0.25 [S]	0.03 [S]	0.008 [S]	0.06 [S]	0.25 [S]	0.12 [S]	N/D	N/D	N/D	N/D	0.015 [S]	2 [S]	0.25 [S]	N/A	N/A	N/A	N/A

MIC, minimum inhibitory concentration; IPM, imipenem; MEM, meropenem; ETP, ertapenem; FEP, cefepime; CAZ, ceftazidime; CTX, cefotaxime; MIN, minocycline; COL, colistin; FOS, fosfomycin; TIG, tigecycline; CIP, ciprofloxacin; AMK, amikacin; GEN, gentamicin; RT, replicon typing; MW, molecular weight; R, resistant; I, intermediate; S, susceptible; NT, non-typeable; N/D, not determined; N/A, not applicable.
[a] Numbers in bold represent positive hybridisation bands with the bla_{KPC-2} probe.

co-transferred to the transconjugants. The different MICs observed for β-lactams of TC-ECL-P2 compared with the parental strain ECL-P2 were possibly due to the presence of the chromosomal AmpC of *E. cloacae*.

Table 2 shows the plasmid profile obtained after S1 nuclease digestion of total DNA and PBRT [12,13]. The six clinical isolates carried multiple plasmids ranging from 45 kb to 300 kb. In Case 1, the plasmids carrying bla_{KPC-2} in ECO-P1 and KPN-P1 were non-typeable by PBRT and had an approximate size of 70 kb, suggesting that both strains may harbour the same plasmid. In Case 2, bla_{KPC-2} was detected on L/M-type plasmids, slightly different in size (ca. 65 kb in ECL-P2 and TC-ECL-P2 and ca. 70 kb in KPN-P2 and TC-KPN-P2) (Table 2). In Case 3, CFR-P3 and KOX-P3 carried bla_{KPC-2} on a ca. 70 kb A/C-type plasmid (Table 2). In this particular case, comparison of the sizes of the plasmids in the clinical strains differed with those of the transconjugants. The S1-PFGE gel of TC-CFR-P3 showed two bands of 70 kb and 204 kb, respectively, and no specific hybridisation was observed, possibly due to the low-copy number plasmid, below the sensitivity of the technique. In TC-KOX-P3, the specific hybridisation band observed was of ca. 76 kb. The differences observed could be explained by plasmid rearrangements or transposition events (e.g. Case 3, 70 kb vs. 76 kb).

Analysis of the genetic elements surrounding bla_{KPC-2} using PCR and sequencing revealed the presence of three different genetic elements and insertion sequences. In Case 1, ECO-P1 and KPN-P1 harboured the already described transposon Tn*4401a* [14]. To the best of our knowledge, this is the first report of Tn*4401* detected in *E. coli* in Argentina because this structure has always been associated with *K. pneumoniae* ST258 [10]. Isolates from Patient 2 and transconjugants shared a novel genetic context within the variable region between IS*Kpn-8* and bla_{KPC-2}, which we called Variant 3 as it differs from the Variant 2 published by Shen et al. [15] (GenBank FJ628167). These structures had a 114-bp deletion that included 24 bp of the inverted repeat right of the Tn3-like transposon (GenBank KM403446). Finally, isolates from Patient 3 harboured bla_{KPC-2} in the genetic context Variant 1a: IS*Kpn8*–Δbla_{TEM}–bla_{KPC-2}–IS*Kpn6*-like (GenBank JN048639) already reported in Argentina [10].

In conclusion, this work documents three cases of in vivo horizontal transfer of bla_{KPC}. The results describe the variety of genetic elements harbouring bla_{KPC} that are circulating in Argentina as well as the first description of bla_{KPC}-producing *E. coli* associated with Tn*4401a*.

The broad dissemination of KPC-producing *K. pneumoniae* increases the likelihood of interspecies transfer of the antibiotic resistance determinants into highly fit clones of other Enterobacteriaceae. Therefore, early detection, characterisation and surveillance of these resistance elements are extremely important to avoid their dissemination and consequent treatment failures.

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Competing interests

None declared.

Ethical approval

Not required.

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