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BRIEF REPORT

First characterization of *K. pneumoniae* ST11 clinical isolates harboring bla_{KPC-3} in Latin America

Virginia Garcia-Fulgueiras[a,1], Yuliana Zapata[b], Romina Papa-Ezdra[a], Pablo Ávila[a], Leticia Caiata[a], Verónica Seija[c], Ana E. Rojas Rodriguez[b], Carmen Magallanes[d], Carolina Márquez Villalba[d], Rafael Vignoli[a,*,1]

[a] *Departamento de Bacteriología y Virología, Instituto de Higiene, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay*
[b] *Grupo de Investigación en Enfermedades Infecciosas, Universidad Católica de Manizales, Caldas, Colombia*
[c] *Departamento de Laboratorio Clínico, Área Microbiología, Hospital de Clínicas, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay*
[d] *Cátedra de Microbiología, Instituto de Química Biológica, Facultad de Ciencias y de Química, Universidad de la República, Montevideo, Uruguay*

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Abstract Antimicrobial resistance due to carbapenemase production in *Enterobacteriaceae* clinical isolates is a global threat. *Klebsiella pneumoniae* harboring the bla_{KPC} gene is one of the major concerns in hospital settings in Latin America.

The aim of this study was to characterize the antibiotic resistance mechanisms and to typify four carbapenem-resistant *K. pneumoniae* clinical isolates from the city of Manizales, Colombia.

We identified bla_{KPC-3} in all four isolates by polymerase chain reaction and subsequent sequencing. The plasmid-mediated quinolone resistance genes *qnrB19-like* and *aac(6′)Ib-cr*; fosfomycin resistance gene *fosA* and an insertion sequence IS*5*-like in *mgrB* (colistin resistance) were also detected. Sequence types ST11 with capsular type *wzi*75, and ST258 with *wzi*154, were characterized. The bla_{KPC-3} gene was mobilized in a 100-kb IncFIB conjugative plasmid with *vagCD* toxin–antitoxin system.

This work reports multiple resistance genes in bla_{KPC}-producing *K. pneumoniae* and the first occurrence of ST11 clinical isolates harboring bla_{KPC-3} in Latin America.

* Corresponding author.
E-mail address: rvignoli@higiene.edu.uy (R. Vignoli).
[1] These two authors share the senior authorship of this work.

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PALABRAS CLAVE
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Resistencia a fosfomicina

Primera caracterización de aislamientos clínicos de *K. pneumoniae* ST11 portadores de bla_{KPC-3} en América Latina

Resumen La resistencia a antibióticos mediada por la producción de carbapenemasas en aislamientos clínicos de *Enterobacteriaceae* es una amenaza mundial. *Klebsiella pneumoniae* portador de bla_{KPC} es uno de los mayores problemas a nivel hospitalario en Latinoamérica.

El objetivo de este estudio fue caracterizar los mecanismos de resistencia antibiótica y tipificar cuatro aislamientos clínicos de *K. pneumoniae* resistentes a carbapenems obtenidos en la ciudad de Manizales, Colombia.

Se identificó bla_{KPC-3} en todos los aislamientos mediante reacción en cadena de polimerasa y secuenciación. También se detectaron los genes de resistencia transferible a quinolonas *qnrB19-like y aac(6')Ib-cr* y a fosfomicina *fosA*, y la secuencia de inserción IS*5-like* en *mgrB* (asociada a la resistencia a colistina). Se caracterizaron los secuenciotipos ST11 (cápsula *wzi*75) y ST258 (cápsula *wzi*154). Se comprobó que bla_{KPC-3} fue movilizado por un plásmido conjugativo IncFIB-*vagCD* de 100 kb.

En este trabajo se reportan múltiples genes de resistencia en *K. pneumoniae* productor de bla_{KPC} y se describen por primera vez aislamientos clínicos ST11 productores de bla_{KPC-3} en Latinoamérica.

Antimicrobial resistance due to carbapenemase production in *Enterobacteriaceae* clinical isolates is a global threat[4]. Recently, the WHO established a list of microorganisms to be prioritized in the research and development of new antimicrobials, placing carbapenem and/or third-generation cephalosporin-resistant enterobacteria within the microorganisms of maximum interest[17].

Enterobacterial clinical isolates usually present not only β-lactam resistance due to either extended spectrum β-lactamase (ESBL) or carbapenemase production, but also resistance to both aminoglycosides and fluoroquinolones[4]. In this sense, plasmid-mediated quinolone resistance (PMQR) mechanisms could be present in these isolates; e.g. *qnr* alleles, the efflux pump *oqxAB* or the *aac(6')Ib-cr* allele, which confer resistance to quinolones and the latter also to aminoglycosides (amikacin, kanamycin, tobramycin)[10,19].

Occasionally, other antibiotics such as colistin or fosfomycin are necessary therapeutic options to treat clinical infections caused by these multiresistant microorganisms, particularly in carbapenemase-producers[8]. However, transferable fosfomycin and colistin resistance genes like *fosA*/*fosB* and different *mcr* alleles respectively have been detected in enterobacterial clinical isolates[3,16].

All the aforementioned resistance determinants are mainly codified in conjugative plasmids considered epidemic. ''Epidemic resistance plasmids'' (ERP) play an important role in antibiotic resistance gene dissemination. Different features are described for those ERP, such as incompatibility groups, e.g. IncFII, IncA/C, IncL/M, IncN and IncI1, plasmid size larger than 50 kb, conjugation properties, toxin–antitoxin systems related to plasmid maintenance in daughter cells, among others[6].

In relation to enterobacterial clinical isolates, *Klebsiella pneumoniae* is represented by the letter ''K'' in the acronym ESKAPE, among the six most significant and dangerous causes of hospital infections by antimicrobial-resistant microorganisms[15].

Within the *K. pneumoniae* isolates, those who are KPC-producers are the main problem in hospital settings in Latin America[9].

In particular, *K. pneumoniae* isolates belonging to clonal group CG258 (ST258, ST11, their single-locus variants and other closely related sequence types) are the most widely distributed in the world. ST258 was recognized as a successful clone, playing an important role in emergence and dissemination of bla_{KPC}[19], and ST11 is also significantly associated with the worldwide dissemination of this resistance mechanism, being similarly considered a successful clone[1].

Identification of capsular types by amplification and sequencing of the *wzi* gene (Wzi protein anchors capsular polysaccharide to the cell surface) is often used to characterize these sequence types. In this sense, there are different *wzi* alleles that correlate with different capsule locus (KL)[20], for example capsular type *wzi*154 (KL107), has been related to KPC-producing ST258 isolates[16].

K. pneumoniae isolates belonging to ST258 were first reported in Latin America in Medellin, Colombia, carrying either bla_{KPC-2} carbapenemase in 2005[18] and bla_{KPC-3} in 2008[11]. Subsequently, both enzymes have spread to other cities in Colombia and to other clonal complexes[16].

Although there are reports of KPC-producing isolates recovered from the departments of Cundinamarca and Antioquia, where the cities of Bogotá and Medellin are respectively located[16], there are so far no reports of this type of isolates from the Department of Caldas, geographically located between those previously mentioned departments.

In 72 h, three carbapenem-resistant clinical isolates of *K. pneumoniae* were obtained from two hospitals located in the city of Manizales (capital of Caldas Department,

Colombia) and a fourth isolate was obtained 30 days later, also displaying colistin resistance.

The aim of this study was to characterize the antibiotic resistance mechanisms and to typify four carbapenem-resistant *K. pneumoniae* clinical isolates from Manizales city.

Four *K. pneumoniae* isolates (Kpn1–4) were collected from the microbiology laboratories of two hospitals (A and B) in the city of Manizales between May and June 2016. Identification was determined by MALDI-TOF MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry). Susceptibility testing was evaluated by the automatized system MicroScan and the agar dilution test was performed to determine fosfomycin susceptibil -ity. All the results were interpreted in accordance with the EUCAST guidelines (www.eucast.org/clinical_breakpoints). Carbapenemase production was suspected by the phenotypic test KPC + MBL Confirm ID Kit (Rosco, Taastrup, Denmark). Resistance mechanisms were investigated by polymerase chain reaction (PCR) using primers to amplify the following determinants (Table S1): ESBL ($bla_{\text{CTX-M-group-1}}$, $bla_{\text{CTX-M-group-2}}$, $bla_{\text{CTX-M-group-3}}$, $bla_{\text{CTX-M-group-4}}$, $bla_{\text{CTX-M-group-25}}$, bla_{TEM}, bla_{SHV}, $bla_{\text{OXA-1}}$, $bla_{\text{OXA-2}}$, $bla_{\text{PER-2}}$); carbapenemases (bla_{KPC}, $bla_{\text{OXA-48}}$, bla_{GES}); transferable-resistance determinants for: aminoglycosides (*aac(6′)Ib*, methylases: *armA*, *rmtA-D*, *npmA*), quinolones (*aac(6′)Ib-cr*, *qnrA*, *B*, *C*, *D*, *S*, *VC*, *qepA*), fosfomycin (*fosA*, *fosA3*) and colistin (*mcr-1* to *3*). Positive results were confirmed by Sanger sequencing, and sequences were analyzed using BLASTn (https://blast.ncbi.nlm.nih.gov).

Capsular type was determined by PCR and sequencing for *wzi* gene, alleles were identified in the *Klebsiella* sequence typing database (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html). *mgrB* alterations were searched by both PCR and sequencing in order to determine other mechanisms involved in colistin resistance (Table S1).

Conjugation assays were carried out using tetracycline-resistant *E. coli* CAG12177 [F^- λ^- *zej-298*::Tn*10*(Tet^r) *gyrA261*(Nal^r) *rph-1*] (*E. coli* Genetic Stock Center) as recipient. Transconjugants were selected on Luria–Bertani agar plates supplemented with tetracycline (32 mg/l) and ceftazidime (2 mg/l)[10].

Incompatibility groups and toxin–antitoxin systems were detected by PCR using transconjugant genomic DNA as template[5,13].

XbaI-pulsed-field gel electrophoresis (PFGE) analysis was performed and results were analyzed using the unweighted pair-group method with an arithmetic mean (UPGMA) as previously reported[10].

Multi locus sequence typing characterization of *K. pneumoniae* isolates was conducted according to the *K. pneumoniae* MLST database. (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

Plasmid size was estimated for transconjugants by treatment with S1 nuclease followed by PFGE[10].

This study was approved by the Direction Board of the involved hospitals, and the patients' data were obtained from clinical records.

Kpn1 and Kpn2 isolates were recovered from urine cultures of catheterized patients in intermediate care units from Hospital A (39-year-old woman, May 13th; and 72-year-old man in May 15th, respectively); meanwhile Kpn3 was obtained from a midstream urine sample from a 70-year-old patient in Hospital B, May 14th. Finally, Kpn4 was recovered from a catheter tip from an adult patient in Hospital A, June 17th. Antibiotic susceptibility results are shown in Table 1.

All isolates showed positive synergy between boronic acid and meropenem in the phenotypic test. We confirmed the presence of $bla_{\text{KPC-3}}$ in all four isolates. Additionally, we found: $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-1}}$ in both Kpn1 and Kpn2, $bla_{\text{TEM-1}}$ in Kpn4, and the ESBL coding gene $bla_{\text{SHV-12}}$ in Kpn3 (Table 1).

The *aac(6′)Ib* gene responsible for amikacin resistance was detected in Kpn3 and Kpn4. With regard to plasmid-mediated quinolone resistance genes, the combination *qnrB19-like* and *aac(6′)Ib-cr* was detected in both Kpn1 and Kpn2. The fosfomycin-resistance gene *fosA* was characterized in Kpn3, which displayed *in vitro* resistance to this antibiotic. Colistin resistance was observed in Kpn4 due to the presence of the insertion sequence IS*5*-like in the *mgrB* gene at position 74–75 (Table 1).

Three pulsotypes (PT) were identified by PFGE: Kpn1 and Kpn2 belonged to PT-A, Kpn3 to PT-B and Kpn4 to PT-C. Additionally, PT-A belonged to sequence type ST-11 and both PT-B and PT-C belonged to ST-258 (Fig. 1).

Capsular typing by *wzi* sequences showed different alleles: *wzi*75 (KL105) was identified in isolates belonging to ST11 and *wzi*154 (KL107) in the ST258 isolates (Table 1).

Conjugation assays were positive to Kpn1-2, TcKpn1 and TcKpn2 presented $bla_{\text{KPC-3}}/bla_{\text{TEM-1}}$. Incompatibility group IncFIB and the toxin–antitoxin system *vagCD* were characterized in transconjugants. The transferred plasmid size was approximately 100- kb in both transconjugants (Table 1).

In this work, we describe the first *K. pneumoniae* ST11 isolates harboring the $bla_{\text{KPC-3}}$ gene in Latin America. Sequence types characterized in this study are closely related, since it is believed that *K. pneumoniae* ST258 was originated by a recombination process between ST11 and ST442 clones. In this sense, ST11 is a single locus variant from ST258 and belongs to the same clonal complex (CC258)[7].

There are different reports highlighting the relevance of *K. pneumoniae* from ST258 or ST11 with $bla_{\text{KPC-2}}$ or extended spectrum β-lactamases in Latin America[1,9,10,12].

Andrade et al. recently described in Brazil the circulation of *K. pneumoniae* ST11 harboring $bla_{\text{KPC-2}}$ or $bla_{\text{CTX-M-2}}$, with different capsular types (K27, K64 and KL202) from those reported in this work[1]. Taking these results into consideration, the strains reported in our work would not be related to those circulating in Brazil.

With regard to publications on this topic from Colombia, Rojas et al. described ST11 in *K. pneumoniae* by whole genome sequencing. However, those strains were non-carbapenemase producers and their capsular types were not available. Interestingly, those isolates did present the combination of transferable quinolone resistance genes *aac(6′)Ib-cr* and *qnrB*[16].

Although information concerning the capsular type of these KPC-producing isolates is not available, it is possible to hypothesize that ST11 isolates reported by Rojas et al. could have acquired the conjugative IncFIB-$bla_{\text{KPC-3}}$-*vagCD* plasmid, becoming resistant to carbapenems.

This incompatibility group was previously associated with $bla_{\text{KPC-3}}$ plasmids, but principally IncFIB(K) type[16]. In regard to toxin–antitoxin systems, information in databases is scarce and we were not able to find any results about *vagCD*

Table 1 Characteristics of *K. pneumoniae* isolates.

Strain	Genotype	PT	ST	MIC (mg/l)											*wzi* (K-locus)	*mgrB* (bp and insertion)	Tc genotype	Inc Tc	TA Tc	S1 Tc
				CTX	CAZ	FEP	MEM	IMP	CIP	GN	AK	COL	SXT	FOS						
Kpn1	bla_{KPC-3}, bla_{TEM-1}, bla_{OXA-1}, *qnrB-19-like*, *aac(6')Ib-cr*	A	11	≥64	≥64	≥64	≥16	≥16	≥4	≤1	8	≤0.5	40	2	75 (KL105)	nd	bla_{KPC-3}, bla_{TEM-1}	FIB	*vagCD*	~100 kb
Kpn2	bla_{KPC-3}, bla_{TEM-1}, bla_{OXA-1}, *qnrB-19-like*, *aac(6')Ib-cr*	A	11	≥64	≥64	8	≥16	≥16	≥4	≤1	8	≤0.5	≤20	1	75 (KL105)	nd	bla_{KPC-3}, bla_{TEM-1}	FIB	*vagCD*	~100 kb
Kpn3	bla_{KPC-3}, bla_{SHV-12}, *fosA*, *aac(6')Ib*	B	258	8	≥64	2	≥16	8	≥4	≤1	≥64	≤0.5	160	256	154 (KL107)	nd	-	nd	nd	nd
Kpn4	bla_{KPC-3}, bla_{TEM-1}, *aac(6')Ib*	C	258	≥64	≥64	≥64	≥16	≥16	≥4	≥16	≥64	≥16	80	4	154 (KL107)	1500-IS*5*-like	-	nd	nd	nd

PT: pulsotype, ST: sequence type, MIC: minimum inhibitory concentration CTX: cefotaxime, CAZ: ceftazidime, FEP: cefepime, MEM: meropenem, IMP: imipenem, CIP: ciprofloxacin, GN: gentamicin, AK: amikacin, COL: colistin, SXT: trimethoprim-sulfamethoxazole, FOS: fosfomycin, wzi: capsular gene and K-locus, *mgrB*: colistin resistance gene, Tc: transconjugant (−: negative results), Inc: incompatibility group, TA: toxin–antitoxin systems, S1: plasmid size in bla_{KPC} positive transconjugants (kb), nd: not determined

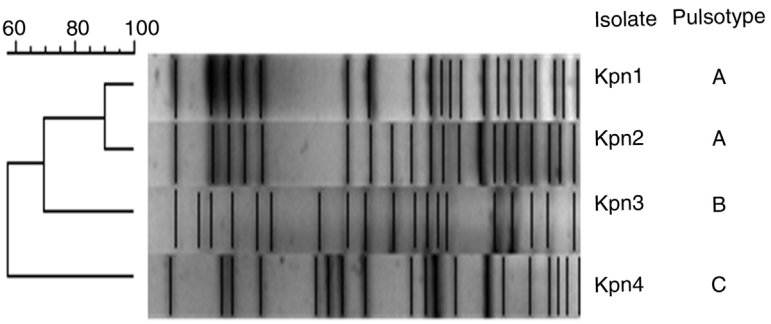

Figure 1 *K. pneumoniae* pulsed-field gel electrophoresis.

presence in this kind of plasmids. We believe that the report of IncFIB-bla_{KPC-3}-*vagCD* plasmids is a novel contribution.

Kpn1 and Kpn2 belonged to the same pulsotype and were recovered (only two days apart) from patients with urinary catheters, hospitalized in the same nosocomial unit. For these reasons, we believe that horizontal transmission of the strain could have occurred between these patients, probably through inadequate urine catheter manipulation.

In relation to ST258, the strains belonging to this sequence type corresponded to different pulsotypes and were isolated from different hospitals. Although the identified capsular type was *wzi*154, such as the capsular type reported in Rojas et al. in ST258 strains[16], the resistance profiles of the two isolates were dissimilar.

With regard to KL107 (*wzi*154) isolates, Arena F et al. reported bla_{KPC-3}-producing ST258 *K. pneumoniae* as not hypermucoviscous or hypervirulent clones[2]. It is remarkable that there is scarce information about the capsule locus KL105 (*wzi*75) identified in ST11 isolates in the databases visited to date.

On the other hand, fosfomycin resistance was detected in Kpn3 due to the presence of *fosA*, while, colistin resistance was identified in Kpn4 due to the insertion of IS*5*-like in *mgrB*. The presence of *fosA* and mutations in *mgrB* was recently reported in ST258 *K. pneumoniae* strains from Colombia. However, in the work by Poirel L. et al., bla_{KPC-2} was the carbapenemase detected in ST258 isolates, and the strain that harbored bla_{KPC-3} was from France and not from Colombia[14,16].

It has been extensively reported that bla_{KPC} dissemination is a growing problem worldwide. This phenomenon occurs in different *K. pneumoniae* lineages, due to a wide variety of plasmids, and is intimately linked to CC258 as we previously mentioned[19].

As far as we know, ST11 belonging to CC258 and harboring bla_{KPC-3} has not already been reported in Latin America.

In this sense, we believe that new studies are required to verify if the ST11 clone harboring bla_{KPC-3} had already been circulating or if this is actually the first occurrence of a clone which could become successful in Colombia.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ram.2019.10.003

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