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Changing Epidemiology of KPC Producing *Klebsiella pneumoniae* in Argentina: Emergence of Hypermucoviscous ST25 and High Risk Clone ST307

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Highlights

- Change in the epidemiology of KPC- *K. pneumoniae* isolates in Argentina
- Dissemination of resistant and hypermucoviscous clone: KPC- *K. pneumoniae* ST25
- First detection of *K. pneumoniae* ST307 and bla_{KPC-3} in Argentina
- *mgrB* inactivation in colistin resistant KPC- *K. pneumoniae* isolates

Abstract:

Objectives: To assess the epidemiological features of 76 KPC producing *K. pneumoniae* isolates (KPC-Kp) recovered in 3 hospitals of Buenos Aires, Argentina, during 2015-2017.

Methods: Antimicrobial susceptibilities were determined according to CLSI. Molecular typing of KPC-Kp was performed by PFGE-X*ba*I and MLST. Plasmid encoded genes involved in carbapenem, fosfomycin and colistin resistance were detected by PCR and sequencing. Also *mgrB* inactivation was investigated in those colistin resistance isolates. Genetic platforms involved in horizontal spread of bla_{KPC} were investigated by PCR mapping.

Results: Besides β-lactams, high resistance rates were observed for gentamycin, quinolones and trimethoprim-sulfamethoxazole. KPC-Kp ST258 corresponded to

26% of the isolates while 42% corresponded to ST25. The other isolates were distributed in a diversity of lineages such as ST11 (10.5%), ST392 (10.5%), ST307, ST13, ST101, ST15 and ST551. *bla*$_{KPC-2}$ was detected in 75/76 isolates, and one ST307 isolate harbored *bla*$_{KPC-3.}$ Tn*4401* was identified as the genetic platform for *bla*$_{KPC}$ in epidemic lineages such as ST258 and ST307. However in ST25 and ST392, usually no related to *bla*$_{KPC}$, it was identified *bla*$_{KPC}$ -bearing non- Tn*4401* element. Alterations in *mgrB* were detected in 7 out of 11 colistin resistant isolates.

Conclusions: Despite previous reports in Argentina, ST258 is no longer the absolute clone among KPC-Kp isolates. In the present study, the dissemination of more virulent lineages such as the hypermucoviscous ST25 was detected. Even more, it was noticed for the first time in our region the emergence of the high risk clone ST307 and also the occurrence of *bla*$_{KPC-3}$.

Keywords: KPC- *K. pneumoniae*; ST307, ST25, ST11 and ST392; *bla*$_{KPC-3}$, *mgrB* inactivation

1. Introduction

Carbapenem resistant isolates, mainly those producing *K. pneumoniae* carbapenemase (KPC), are worldwide spread and are associated with high morbidity and mortality rates [1]. KPC- *K. pneumoniae* global expansion has been associated with the dissemination of epidemic clones, especially the dominant strain *K. pneumoniae* sequence type (ST) 258 and related strains of clonal complex (CC) 258 [2]. This ST is a hybrid clone which derived from large recombination events

between ST11 and ST442 [3-5]. This clone emerged in Argentina in 2010 becoming the prevalent ST and achieving epidemic status in many settings [6, 7]. CC258 includes multidrug resistant microorganisms, displaying resistance to all β-lactams, aminoglycosides, quinolones, trimethoprim and sulfonamides [2]. Singularly, ST258 is reported to be mildly virulent. The high mortality rates attributed to this clone may be explained, at least in part, by the low efficacy of the antimicrobials used against KPC-*K. pneumoniae* infections and the severity of the underlying conditions of the patients [3]. It is highly susceptible to serum killing and phagocytosis, and lacks typical *K. pneumoniae* virulence factors such as aerobactin, K1, K2 and K5 capsular genes, and the regulator gene of the mucoid phenotype, *rmpA* [5, 8].

However, in the recent years new strains of KPC-*K. pneumoniae* have emerged internationally, which correspond to hypervirulent clones that have acquired extensively drug resistance markers [9, 10].

Transposon Tn*4401* constitutes the most frequent genetic context of $bla_{KPC,}$ and is considered the origin of acquisition and dissemination of this marker. Tn*4401* is approximately 10 kb in size, delimited by two 39-bp imperfect inverted repeat sequences. It harbors two insertion sequences flanking bla_{KPC}, IS*Kpn6* and IS*Kpn7*, in addition to a transposase and resolvase genes [5, 11]. Moreover, bla_{KPC}-bearing non-Tn*4401* elements have been recognized including the integration of a Tn*3*-based transposon and a partial Tn*4401* structure (ORF order: Tn*3*-transposase, Tn*3*-resolvase, IS*Kpn8*, bla_{KPC} and IS*Kpn6*). Even, variants in the upstream bla_{KPC} region, lacking the Tn*3*-transposase and its resolvase and displaying a partial fragment of bla_{TEM}, have been reported [5, 12].

The aim of this study was to assess the epidemiological features of KPC-*K. pneumoniae* in order to understand the ongoing evolution of carbapenem resistance in our region.

2. Material and Methods

2.1 Bacterial isolates and antimicrobial susceptibility testing

Seventy six non repetitive *K. pneumoniae* isolates, positive in the double-disk synergy test using phenyl boronic acid (300 µg) and carbapenem containing disks were included in this study. These isolates were recovered from inpatients at 3 hospitals in Buenos Aires during 2015-2017. A total of 374 *K. pneumoiae* isolates were recovered from inpatients during the studied period, being 81(22 %) of them carbapenem resistant and 76 (20 %) KPC producers. These resistant isolates were delivered to the Laboratorio de Resistencia Bacteriana in an encrypted way to preserve the identity of the patients.

Susceptibilities to ampicillin, ampicillin-sulbactam, piperacillin-tazobactam, ceftazime, ceftriaxone, imipenem, meropenem, gentamycin, amikacin, ciprofloxacin, levofloxacin and trimethoprim-sulfamethoxazole were assessed by automated systems. Colistin and tygecycline minimum inhibitory concentrations (MICs) were determined by broth microdilution method and agar dilution method was performed to determine the fosfomycin susceptibility. Antimicrobial susceptibilities were interpreted using the CLSI breakpoints, except for colistin and tigecycline where EUCAST and U.S. Food and Drug Administration (FDA) breakpoints were used, respectively [13].

2.2 Molecular detection of antimicrobial resistance genes

The presence of the most prevalent carbapenemase genes (*bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$-like) was investigated by multiplex-PCR using specific primers [14]. Plasmidic *mcr-1* and *mcr-2*, and *fosA3*, *fosA4* and *fosC2* were screened by PCR in those isolates which displayed resistance to colistin and fosfomycin, respectively [15, 16]. Colistin resistance mediated by *mgrB* inactivation was studied according to Cannatelli *et al* [17]. Complete *bla*$_{KPC}$ was amplified as previously described [18]. All amplicons were sequenced at external facilities (Macrogen Inc., Seoul, Korea) and analyzed using the BLAST program at NCBI (http://www.ncbi.nlm.nih.gov/BLAST).

2.3 Molecular typing of K. pneumoniae isolates harboring bla$_{KPC}$

Clonal relationship was analyzed, for the 76 isolates, by PFGE after digestion of genomic DNA with Xba*I* and interpreted according to van Belkum *et al*. [19, 20]. MLST analysis was performed in representative isolates of each different pulsotype. STs were assigned amplifying and sequencing the following 7 housekeeping genes: *gapA, infB, mdh, pgi, phoE, rpoB, tonB* in accordance with http://bigsdb.web.pasteur.fr/klebsiella/.

2.4 Analysis of genetic platforms carrying bla$_{KPC-2}$

Natural plasmids were extracted using the Kado & Liu extraction method [21]. The genetic context of *bla*$_{KPC}$ was studied by a PCR mapping approach in order to detect the Tn*4401* structure and *bla*$_{KPC}$-bearing non- Tn*4401* elements, as previously described [11, 12].

3. Results

Clinical features of KPC-*K. pneumoniae* isolates and main demographic data are shown in table 1. The incidence rate of KPC-*K. pneumoniae* was about 7.3 per 1000 discharges, in these hospitals. Most of the 76 isolates were recovered from urine and blood samples. Patients age ranged from 20 to 94 years (median age was 72 and 70 for females and males, respectively), and the male to female ratio was 1.76. Although all patients presented underlying conditions, cancer and urinary tract diseases were the most common primary cause; 43.5 % of them were hospitalized in intensive care units and 87 % had received previous antibiotic treatment (Table 1).

High resistance rates were detected for β-lactams, gentamycin, quinolones and trimethoprim-sulfamethoxazole (TMS) (Figure 1). All isolates were resistant to ampicillin, ampicillin-sulbactam, ceftazidime, ceftriaxone, 8/76 were intermediate and 68/76 were resistant to imipenem, while 4/76 and 72/76 isolates were intermediate and resistant to meropenem, respectively. $bla_{\text{KPC-2}}$ carbapenemase gene was detected in 75 isolates while $bla_{\text{KPC-3}}$ was harbored in the remaining. Fifteen % of the isolates (11/76) were resistant to colistin while 18% (14/76) were resistant to fosfomycin. The plasmidic resistance genes for colistin and fosfomycin were not detected. Alterations in *mgrB* locus were identified in 7/11 colistin resistant isolates: 4 displayed Δ*mgrB* and 3 exhibit mutations which were not previously described. Two isolates presented g85t mutation (D29Y), and the remaining presented g59a, rendering a premature stop (Table 2).

The PFGE-Xba*I* analysis showed the presence of 18 different pulsotypes which were related to different STs, corresponding to: ST25 (n:32), ST258 (n:20), ST11 (n:8),

ST392 (n:8), ST307 (n:2), ST13 (n:1), ST101 (n:1), ST15 (n:1) and ST551 (n:1). Those isolates which belonged to ST258, ST307, ST101 and 4/8 ST11 presented the complete Tn*4401* while those isolates which corresponded to ST25, ST392, ST13, ST15, ST551 and 1/8 ST11 presented IS*Kpn8* upstream $bla_{KPC,}$ as part of Tn*3* (Tn3-IS*Kpn8*), and IS*Kpn6* downstream (Figure 2). Presence of Δbla_{TEM} upstream to bla_{KPC} was not detected in any isolate.

4. Discussion

Even if previous studies performed in Argentina showed that KPC-*K. pneumoniae* dissemination corresponded to the propagation of a unique clone, ST258 [6, 7], today CC258 accounted for only 37% of the isolates (ST258 (26 %), ST11 (11%)). New lineages of KPC-*K. pneumoniae* were detected, and among them, a high proportion of isolates (42%) belonged to the emerging ST25, a virulent and hypermucoviscous clone which presents the capsular serotype K2. Interestingly, almost all ST25 isolates displayed susceptibility to amikacin. Reports on this ST from clinical samples are scarce; in China, Yao *et al* reported 1 clinical isolate of *K. pneumoniae* ST25 recovered from a tracheal secretion, but this one was susceptible to carbapenems [10]. Also carbapenem susceptible *K. pneumoniae* ST25 has been described as cause of septicemia in neonate pigs, in England and Australia, being responsible for a total of 19 outbreaks in 16 piggeries and 2 outbreaks in 2 pig farms, respectively [22]. One NDM-1 producing *K. pneumoniae* ST25 isolate was recovered in Serbia in 2011 and 5 KPC-2 producing *K. pneumoniae* ST25 isolates, causing invasive human infections, were only previously described in Ecuador, in 2016 [23, 24].

Furthermore, 10.5 % of the isolates corresponded to ST392, a lineage related to the dissemination of OXA-48 in Europe. Even though the emergence and spread of KPC-2-*K. pneumoniae* ST392 was described in 2013, in China, this strain has been sporadically reported [25]. More recently, in 2017, KPC-3-*K. pneumoniae* ST392 was reported in Italy [26].

The remaining isolates were distributed in a diversity of lineages as ST13, ST15, ST101, ST551 and ST307. Singularly KPC-*K. pneumoniae* ST307 is recognized as a high risk clone which is being increasingly documented in several countries and is considered a candidate to become the worldwide epidemic clone [27-31]. Compared with the low virulent ST258, capsulated ST307 isolates show higher resistance to complement-mediated killing. Even more, ST307 clone harbors a cluster for glycogen synthesis which could provide an advantage allowing long term survival and growth in environments outside the host [31]. This high-risk clone has not been previously recognized in Argentina. The 2 isolates which belonged to this ST were recovered from different hospitals in 2017 and presented different non β-lactam susceptibility profiles. One of them presented $bla_{\text{KPC-2}}$, and was susceptible to colistin and resistant to TMS. The other one harbored $bla_{\text{KPC-3}}$, and was resistant to colistin and susceptible to TMS. This bla_{KPC} allele has not been previously described in Argentina, it is worth highlighting that mutations in $bla_{\text{KPC-3}}$ can conduce to KPC-3 variants with significantly reduce ceftazidime-avibactam susceptibility [32].

Two main bla_{KPC} genetic platforms were identified. Except for ST11, each context could be associated with a particular ST. In accordance with the literature, the well-established Tn*4401* was identified in those lineages recognized as epidemics (ST258

and ST307), while Tn*3*-IS*Kpn8* was detected in those STs not usually related to *bla*$_{KPC}$, as ST25 and ST392 [4].

The colistin plasmid encoded mechanisms do not seem to have an epidemiological impact in *K. pneumoniae*. Although in this study, mutations in *mgrB* locus were detected in many of the colistin resistant isolates, changes in other loci involved in upregulation of the Pmr system, which is responsible for modification of the lipopolysaccharide polymyxin target, could be also present [17].

5. Conclusions

This study describes the changing epidemiology of KPC-*K. pneumoniae* lineages in clinical settings. The almost absolute prevalence of ST258 in our region is being replaced by the dissemination of more virulent lineages such as ST25 and ST11, and the worrisome emergence of the high-risk clone ST307. Even more, we report for the first time in Argentina, the detection of ST307- bla_{KPC-3}-*K. pneumoniae*. The dichotomy between carbapenem resistant *K. pneumoniae* and hypervirulent *K. pneumoniae* has been blurred by the emergence of carbapenem resistant-hypervirulent clones; their dissemination may mark an evolutionary step toward their establishment as major nosocomial pathogens.

Declarations

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Ethical Approval: Not required

Competing Interests: None

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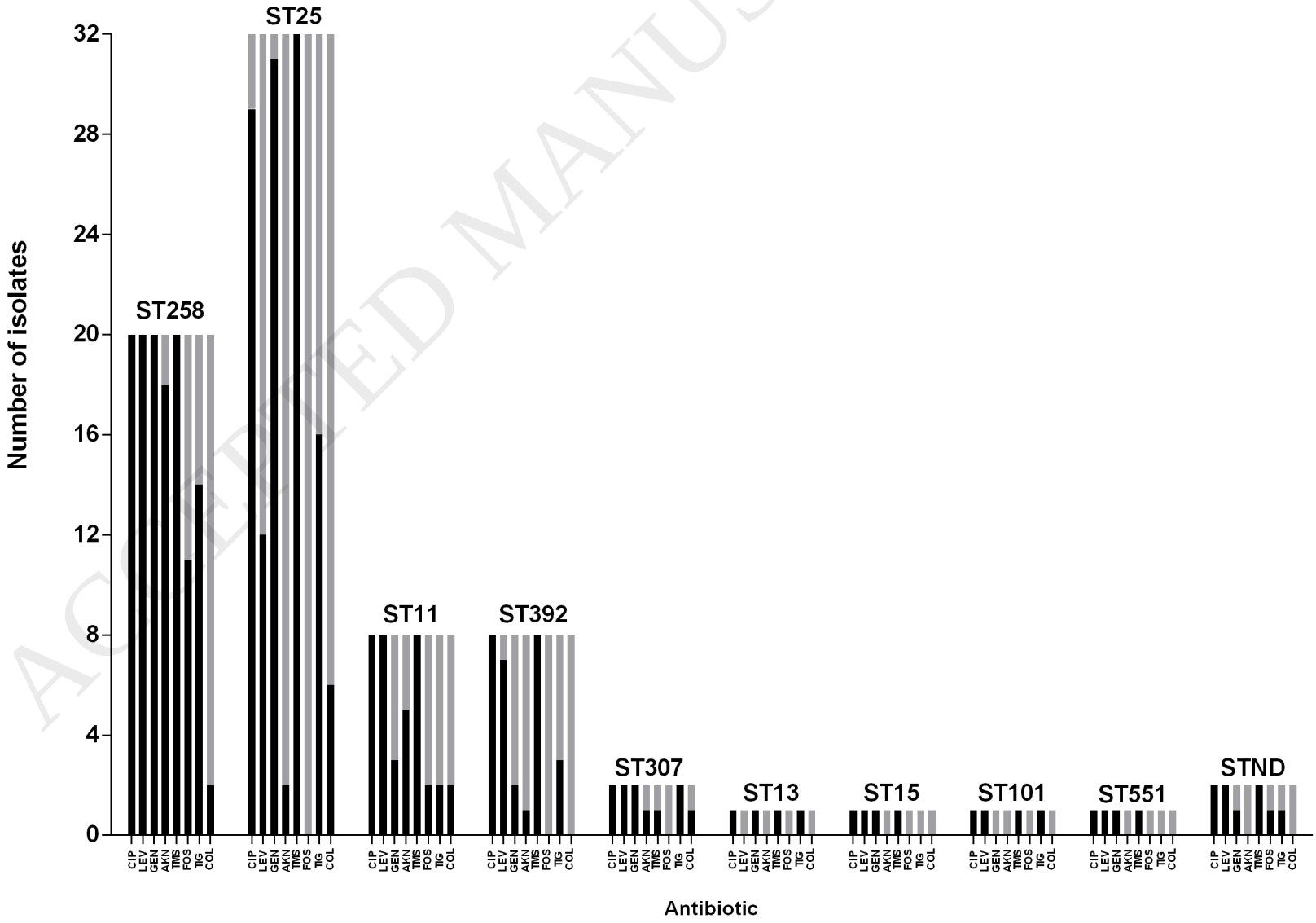
Figure 1: Accompanying resistance in KPC producing *K. pneumoniae* isolates.



Legend figure 1: In each column, resistant isolates are shown in black and susceptible ones in grey. ND: ST not determined. CIP: ciprofloxacin, LEV: levofloxacin, GEN: gentamycin, AKN: amikacin, TMS: timethoprim-sulfamethoxazole, FOS: fosfomycin, TIG: tigecycline, COL: colistin.

Figure 2: Genetic contexts of *bla*$_{KPC}$

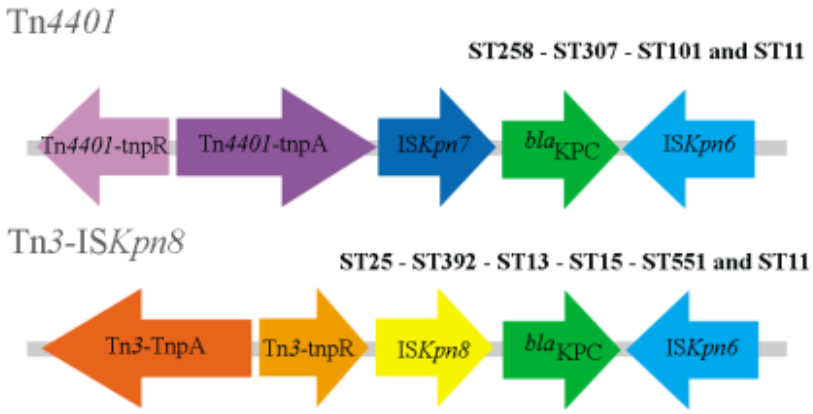


Table 1: Sites of isolation of KPC- *K. pneumoniae* isolates and clinical features of patients

Total number of isolates: 76		n
Isolation Sites	Urine	31
	Blood	20
	Lower Respiratory Tract	8
	Rectal swab	8
	Skin and soft tissue	3
	Abdominal	3
	Cerebrospinal	2
	Stool	1
Total number of inpatients: 69		n
Underlying diseases [a]	Gastrointestinal	3
	Oncology	15
	Cardiovascular	11
	Kidney transplant	11
	Lung	8
	Urinary Tract	15
	Traumatism	3
	Diabetes	4
	Other	6
Wards	Intensive care unit	30
	Medical wards	39
Prior use of antibiotics	Yes	60
	No	9
Female Male	(n)/Age median (range)	25/72 (40 -94) 44/70 (20-93)

[a]: some patients presented more than one underlying disease

Table 2: Molecular characterization of KPC producing *K pneumoniae* isolates

ST (%)	PFGE Type	Total No. of isolates	*bla*$_{KPC}$	*bla*$_{KPC}$ genetic context	Colistin resistance	
					No. of resistant isolates	*mgrB* status
258 (26%)	A	20	*bla*$_{KPC-2}$	Tn*4401*	2	Δ*mgrB* locus (n:2)
25 (42%)	B	32	*bla*$_{KPC-2}$	Tn*3*-IS*Kpn8*	6	g85t (D29Y) (n:2) WT (n:3) Δ*mgrB* locus (n:1)
11 (10.5%)	C_1 C_2 C_3 C_4 C_5	1 1 1 4 1	*bla*$_{KPC-2}$	Tn*4401*, Tn*3*-IS*Kpn8* and unknown	2	Δ*mgrB* locus (n:1) WT (n:1)
392 (10.5%)	D_1 D_2 D_3 D_4	5 1 1 1	*bla*$_{KPC-2}$	Tn*3*-IS*Kpn8*	0	-
307	E	2	*bla*$_{KPC-2}$	Tn*4401*	1	g59a (nonsense,

(2.6%)			*bla*$_{KPC-3}$			premature termination) (n:1)
13 (1.3 %)	F	1	*bla*$_{KPC-2}$	Tn*3*-IS*Kpn8*	0	-
15 (1.3%)	G	1	*bla*$_{KPC-2}$	Tn*3*-IS*Kpn8*	0	-
101 (1.3%)	H	1	*bla*$_{KPC-2}$	Tn*4401*	0	-
551 (1.3%)	I	1	*bla*$_{KPC-2}$	Tn*3*-IS*Kpn8*	0	-
ND (2.6%)	J K	1 1	*bla*$_{KPC-2}$	Tn*3*-IS*Kpn8* Tn*4401*	0	-

WT: Wild type; ND: Not determined