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Update of the molecular epidemiology of KPC-2-producing *Klebsiella pneumoniae* in Brazil: spread of clonal complex 11 (ST11, ST437 and ST340)

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Objectives: To perform molecular epidemiology for 113 KPC-producing *Klebsiella pneumoniae* isolated in 2010 from 12 Brazilian states.

Methods: The resistance profile was determined by disc diffusion and Etest. Genetic polymorphism was analysed by PFGE and multilocus sequence typing. The genetic environment of the bla_{KPC} gene was determined by PCR and identification of the carrier plasmid was determined by hybridization.

Results: Most of the isolates were multidrug resistant, with 15% and 49% being resistant to polymyxin and tigecycline, respectively. Twenty-two sequence types (STs) were observed, with ST11, ST437 and ST340 [clonal complex 11 (CC11)] being the most prevalent (75% of isolates) observed in 10 states. bla_{KPC-2} was associated with transposon Tn*4401* 'b' and in 36% this gene was found in IncN plasmids of 40 kb.

Conclusions: In Brazil, the spread of bla_{KPC-2} is occurring due to dispersion of Tn*4401* 'b', carried by IncN plasmids of 40 kb, and mainly the dissemination of CC11, with ST437 and ST11 playing an important role.

Keywords: KPC, *K. pneumoniae*, CC11, Tn*4401*, IncN plasmids

Introduction

The most important mechanism of resistance against carbapenems in Enterobacteriaceae is the production of carbapenemases such as *Klebsiella pneumoniae* carbapenemase (KPC). This enzyme was first described in a *K. pneumoniae* strain isolate from the USA in 1996[1] and has been responsible for many outbreaks worldwide.[2] In addition to being resistant to all β-lactams available, this carbapenemase has a high capacity to spread, since its gene has been described in transferable plasmids and transposons such as Tn*4401* associated with insertion sequence (IS) elements.[3]

KPC-producing *K. pneumoniae* was first described as occurring in Brazil in 2006[4] and since then its incidence has greatly increased.[5] Seki *et al.*[5] observed the presence of the bla_{KPC-2} gene in *K. pneumoniae* strains isolated in five states from 2006 through to 2009. In 2010, however, a great dispersion of this gene was observed in this country, with the spread of carbapenem-resistant *K. pneumoniae* observed in several hospitals in different Brazilian cities and states.

Thus, the aim of this study was to perform molecular typing, determine the antimicrobial resistance profile and identify the carrier plasmid and the flanking region of the bla_{KPC} gene of 113 KPC-producing *K. pneumoniae* from 32 hospitals located in 12 Brazilian states belonging to the five different geographical regions of this country in 2010.

Materials and methods

Bacterial isolates and phenotypic tests

Our laboratory (LAPIH), located at Instituto Oswaldo Cruz—FIOCRUZ, Ministry of Health, Brazil, routinely receives clinical bacterial isolates from hospitals and LACENs (Central Laboratory of Public Health) from different states. According to the clinical specimens (such as blood, urine, abdominal fluid and lower respiratory tract secretions) and locality [south (SC), south-east (ES, RJ and MG), north-east (PE, AL, CE, PI and MA), north (AM) and mid-west (GO and DF)], we selected 113 non-duplicate *K. pneumoniae* isolates among the 500 KPC-producing strains received in 2010. Bacteria were identified by conventional techniques[6] and identification of KPC allele variants was performed by PCR and sequencing.[5] Antimicrobial susceptibility testing was performed by the disc diffusion method[7] and Etest (AB Biodisk, Solna, Sweden).[8]

Molecular investigations

For molecular typing and epidemiological analysis we used PFGE[9] and multilocus sequence typing (MLST).[10]

The genetic environment of the bla_{KPC-2} gene was investigated by PCR as described previously by Naas *et al.*,[3] except for amplification of ISK*pn*7, in which the primers described by Kitchel *et al.*[11] were used.

Using multiplex PCR, we analysed the plasmid incompatibility groups present in the different MLST types according to the scheme proposed by Carattoli *et al.*[12]

The plasmids were extracted by alkaline lysis,[13] and the Southern blot hybridizations[14] were performed with digoxigenin-labelled bla_{KPC-2} and plasmid incompatibility group probes generated by the PCR DIG detection system (Roche Diagnostics).

Results and discussion

The majority of the 113 isolates were recovered from patients from the south-east ($n=46$) and mid-west ($n=42$) regions, mainly the Federal District (38/113, 34%) and Rio de Janeiro state (28/113, 25%) (Figure 1). Most of the isolates were recovered from blood (46/113, 41%) and urine (37/113, 33%). All isolates possessed the KPC-2 allele variant.

According to CLSI 2011 breakpoints,[7] most of the isolates were resistant to β-lactams, such as cefotaxime (93.8%), ceftazidime (87.6%) and piperacillin/tazobactam (100%). All isolates were non-susceptible to ertapenem (MIC_{50} >32 mg/L, MIC range 2–>32 mg/L), 99.1% to imipenem (MIC_{50} >32 mg/L, MIC range 1–>32 mg/L) and 99.1% to meropenem (MIC_{50} >32 mg/L, MIC range 0.5–>32 mg/L). We observed elevated resistance to ciprofloxacin (94.6%) and sulfamethoxazole/trimethoprim (85.4%), whereas 68/113 (60.2%), 47/113 (41.6%) and 6/113 (5.3%) were resistant to gentamicin, amikacin and fosfomycin trometamol, respectively. According to EUCAST 2011 breakpoints,[8] 11/113 (9.7%) of the strains were resistant to polymyxin B ($MIC_{50}=0.75$ mg/L, $MIC_{90}=2$ mg/L, MIC range 0.25–32 mg/L), whereas 43/113 (38.1%) were resistant to tigecycline ($MIC_{50}=2$ mg/L, $MIC_{90}=6$ mg/L, MIC range 0.38–24 mg/L).

A high level of resistance to different antimicrobial classes has frequently been observed in KPC-producing isolates worldwide.[2] Resistance to polymyxin and tigecycline is very worrisome, because these antimicrobials have been the last option for treatment of severe infections caused by KPC-producing organisms. According to Endimiani *et al.*,[15] fosfomycin demonstrates *in vitro* activity against KPC-producing *K. pneumoniae* isolates and represents a possible alternative to polymyxin and tigecycline as a salvage therapy.

Figure 1 shows the distribution of the 22 PFGE clones observed in the 113 KPC-producing *K. pneumoniae* for each Brazilian state. Despite the clonal diversity observed, the most prevalent profiles were A/Kp-RJ (28%), C (29%) and Q (13%). About 70% of polymyxin- and tigecycline-resistant strains belonged to clones A/Kp-RJ and C (polymyxin: A/Kp-RJ=4, C=4; tigecycline: A/Kp-RJ=20, C=10).

MLST performed on 39 isolates representing all PFGE clones, including isolates from each state, also found 22 sequence types (STs), showing great consistency between the two methodologies used (Figure 1). Thirteen new STs were first described in our study and were deposited in the *K. pneumoniae* MLST

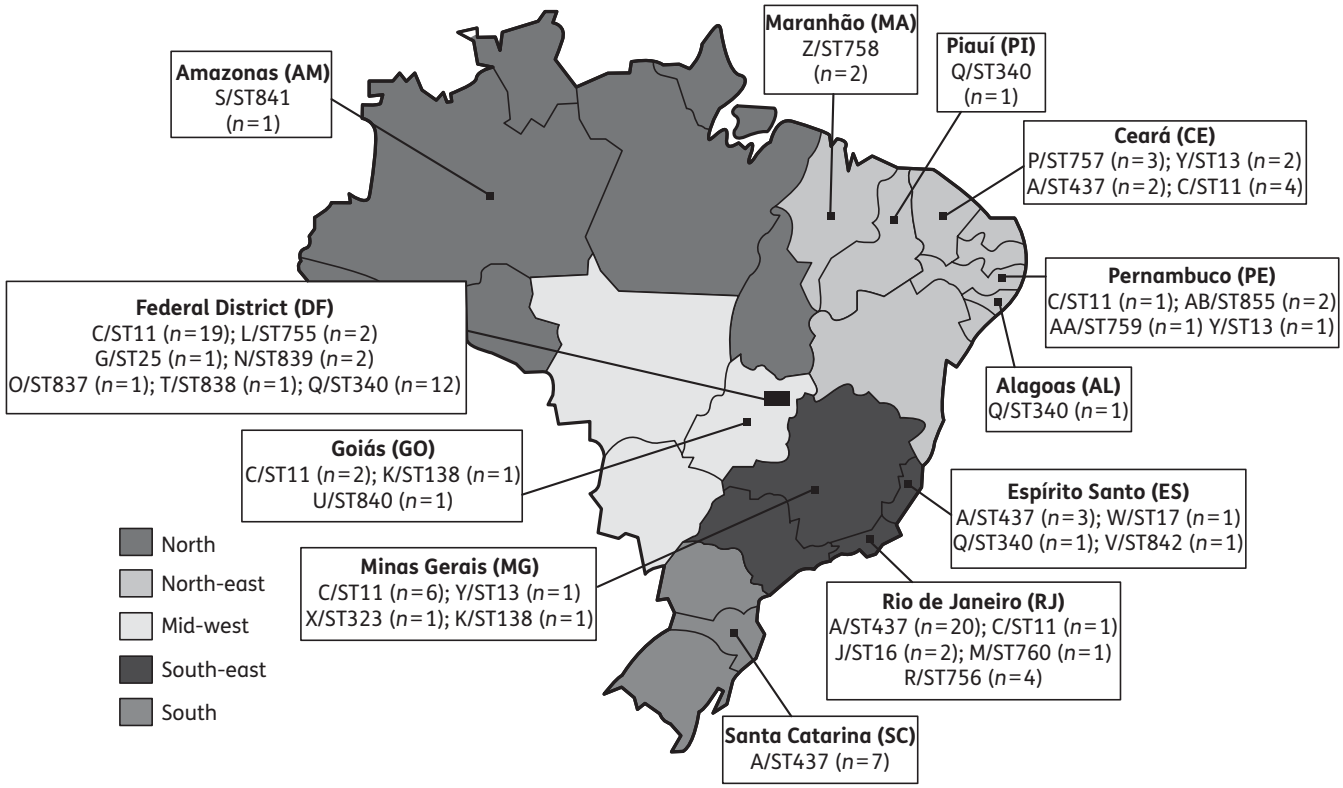

Figure 1. Distribution of the 22 PFGE clones and STs according to state and geographical region of 113 KPC-producing *K. pneumoniae* from Brazil.

Table 1. MLST analysis, genetic environment of the bla_{KPC-2} gene and plasmid analysis of 39 KPC-producing *K. pneumoniae* from 12 states in Brazil

Isolate number	PFGE profile[a]	ST	CC	State	Specimen	Tn*4401*	bla_{KPC}-containing plasmid size (kb)	Incompatibility group
CCBH 5658	C	11	CC11	Goiás	blood	'b' without *ISKpn7*	60	UT
CCBH 5982	C	11	CC11	Distrito Federal	blood	'b'	40	IncN
CCBH 6370	C	11	CC11	Ceará	blood	'b'	48–25–3	UT
CCBH 6375	C	11	CC11	Ceará	blood	'b' without *ISKpn7*	43–16–7	UT
CCBH 6429	C	11	CC11	Minas Gerais	urine	'b'	16–7	UT
CCBH 6633	C	11	CC11	Distrito Federal	blood	'b' without *ISKpn7*	43	UT
CCBH 6740	C	11	CC11	Pernambuco	urine	'b'	15–7–5	UT
CCBH 6798	C	11	CC11	Rio de Janeiro	blood	'b'	5	UT
CCBH 5788	Y	13	—	Minas Gerais	urine	'b'	5	UT
CCBH 6372	Y	13	—	Ceará	blood	'b'	39	UT
CCBH 6530	Y	13	—	Pernambuco	blood	'b'	39	UT
CCBH 6106	J	16	CC16-17	Rio de Janeiro	blood	'b'	40	IncN
CCBH 6030	W	17	CC16-17	Espírito Santo	urine	'b'	ND	ND
CCBH 6707	G	25	—	Distrito Federal	urine	'b'	33	UT
CCBH 5659	K	138	—	Goiás	urine	'b' without *ISKpn7*	60	UT
CCBH 5683	K	138	—	Minas Gerais	blood	'b' without *ISKpn7*	2	UT
CCBH 6428	X	323	—	Minas Gerais	urine	'b'	58–6	UT
CCBH 5641	Q	340	CC11	Espírito Santo	abdominal fluid	'b'	20–13	UT
CCBH 5745	Q	340	CC11	Distrito Federal	urine	'b'	40	IncN
CCBH 6018	Q	340	CC11	Distrito Federal	catheter	'b' without *ISKpn7*	32	UT
CCBH 6556	Q	340	CC11	Alagoas	ND	'b'	50–15–6	UT
CCBH 6805	Q	340	CC11	Piauí	LRTS	'b'	17	UT
CCBH 5623	A-KpRj	437	CC11	Santa Catarina	catheter	'b'	40	IncN
CCBH 5631	A-KpRj	437	CC11	Espírito Santo	urine	'b'	40	IncN
CCBH 6366	A-KpRj	437	CC11	Ceará	urine	'b'	40	IncN
CCBH 6540	A-KpRj	437	CC11	Rio de Janeiro	catheter	'b'	40	IncN
CCBH 5625	L	755	—	Distrito Federal	blood	'b'	40	IncN
CCBH 6091	R	756	—	Rio de Janeiro	catheter	'b'	40	IncN
CCBH 6354	P	757	CC11	Ceará	blood	'b'	39	UT
CCBH 6524	Z	758	CC758-840	Maranhão	catheter	'b'	40	IncN
CCBH 6742	AA	759	—	Pernambuco	blood	'b'	46–35–8	UT
CCBH 6806	M	760	—	Rio de Janeiro	urine	'b'	40	IncN
CCBH 6010	O	837	—	Distrito Federal	catheter	'b'	44	UT
CCBH 6298	T	838	—	Distrito Federal	blood	'b'	40	IncN
CCBH 6306	N	839	—	Distrito Federal	catheter	'b'	40	IncN
CCBH 6511	U	840	CC758-840	Goiás	LRTS	'b'	10	UT
CCBH 6527	AB	841	—	Pernambuco	blood	'b'	21–7–3	UT
CCBH 6566	S	842	—	Amazonas	blood	'b'	6	UT
CCBH 6603	V	855	CC11	Espírito Santo	blood	'b'	40	IncN

LRTS, lower respiratory tract secretion; ND, not determined; UT, untypeable.
[a]Assignment of profiles followed the numbering used by Seki *et al.*[5]

database (ST755, ST756, ST757, ST758, ST759, ST760, ST837, ST838, ST839, ST840, ST841, ST842 and ST855).

The most prevalent PFGE profiles were designated by MLST as A/Kp-RJ/ST437, C/ST11 and Q/ST340. ST11 and ST340 were reported in Greece.[16] In our previous report that considered isolates during the years 2006–09,[5] we found ST437 in the south-east region (RJ and ES) and ST11 in the north-east and south-east (PE and MG). The current study reports the dispersion of these clones to different regions, with ST437 now being found in the south (SC), south-east (ES and RJ) and north-east (CE) regions. The ST11 and ST340 clones were found in the mid-west, south-east and north-east regions (ST11 in CE, DF, GO, MG, PE, RJ and ST340 in AL, DF, ES, PI). Andrade *et al.*[17] also found ST11 and ST437 in the south-east region: São Paulo and Rio de Janeiro, respectively.

Phylogenetic analysis showed that all these STs belong to the CC11 (where CC stands for clonal complex), which is widespread worldwide and includes the epidemic clone ST258. Two other clones first described in our study also belong to this CC: ST757 and ST855. All these clones were considered single locus variants, displaying differences in the *tonB* gene. Therefore, we found CC11 strains dispersed in 10 of the 12 Brazilian states studied (AL, CE, DF, ES, GO, PE, RJ and SC), comprising 75% of isolates. This study confirms that CC11 should be considered the most important CC related to bla_{KPC} gene dispersion worldwide.[18]

Two other CCs were found in our isolates: CC16-17 (ST16 and ST17) and CC758-840 (ST758 and ST840). ST16 was previously described in Canada, in strains producing NDM-1.[19] ST17 has already been observed in KPC-2-producing *K. pneumoniae* from Greece.[16]

We found the bla_{KPC-2} gene is associated with Tn*4401*, variant 'b', in all strains tested ($n=39$) (Table 1). This isoform has already been observed in the USA,[11] Greece[16] and Colombia.[20] In Brazil, previous reports observed the Tn*4401* variants 'a' and 'b' associated with KPC-2-producing organisms of ST11, ST258 and ST437.[17,20] The inverted repeat sequences of the flanking region were not amplified, suggesting that the insertion site found in our isolates may be different from that of *K. pneumoniae* YC of Naas *et al.*[3] ISK*pn*7 was observed in 84.6% (33/39) of the isolates, being absent from three isolates of ST11, two of ST138 and one of ST340. The lack of this IS suggests that another transposition event might have occurred.

The size of the plasmids carrying the bla_{KPC-2} gene varied between 3 and 60 kb, with the most prevalent being ~40 kb (24%). In just one isolate of ST17 it was not possible to observe the bla_{KPC-2} gene associated with plasmids. Incompatibility group analysis of these plasmids revealed that in 14 isolates (36%) belonging to different STs (ST11, ST16, ST340, ST437, ST756, ST758, ST760, ST838, ST839, ST855), the bla_{KPC-2} gene was associated with 40 kb IncN plasmids; in the other 24 isolates we could not identify the incompatibility group of the bla_{KPC}-carrying plasmids (Table 1). Andrade *et al.*[17] found IncN (40 kb), IncL/M (50–60 kb) and IncFII (130 kb) plasmids associated with the bla_{KPC-2} gene of ST11, ST437 and ST258 in Brazil. In one global study that included six countries, the bla_{KPC-2} gene was also found associated with plasmids of IncN (35, 70, 75 and 80 kb), IncL/M (12, 35 and 75 kb) and IncFII (80 kb).[20]

Conclusions

In Brazil, we believe the spread of the bla_{KPC-2} gene is occurring due to the dispersion of Tn*4401* 'b', carried and spread by strains of different STs. We described 13 new STs, however, a great dispersion of CC11 isolates, mainly A-KpRJ/ST437 and C/ST11, carrying IncN plasmids of 40 kb were observed. Thus, the spread of KPC carbapenemase may be facilitated by its localization on plasmids and transposons and also by efficient clones.

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Transparency declarations

None to declare.

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