The importance of clonal complex 258 and IncF$_{K2\text{-like}}$ plasmids among a global collection of *Klebsiella pneumoniae* with *bla*$_{KPCs}$

Gisele Peirano[1,2], Patricia A. Bradford[3], Krystyna M. Kazmierczak[4], Liang Chen[5], Barry N. Kreiswirth[5] and Johann D. D. Pitout[1,2,6,7*]

Departments of Pathology & Laboratory Medicine, University of Calgary, Calgary, Alberta, Canada[1]; Division of Microbiology, Calgary Laboratory Services, Calgary, Alberta, Canada[2]; AstraZeneca Pharmaceuticals LP, Waltham, MA, USA[3]; International Health Management Associates, Schaumburg, Illinois, USA[4]; Public Health Research Institute TB Center, New Jersey Medical School, Rutgers University, Newark, NJ, USA[5]; Department of Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada[6]; University of Pretoria, Pretoria, South Africa[7]

Keywords**:** *Klebsiella pneumoniae*, ST258, IncF$_K$ plasmids

Running Title: *K. pneumoniae* ST258 with *bla*$_{KPCs}$ and IncF$_{K2}$ plasmids

[*]Corresponding Author: Johann D.D. Pitout

Calgary Laboratory Services, #9, 3535 Research Road NW

Calgary, Alberta, CANADA, T2L 2K8

Tel: +1 (403) 770 3309; Fax: +1(403) 770 3347

Email: johann.pitout@cls.ab.ca

Abstract

This study was designed to determine the global distribution of clonal complex (CC) 258 and $IncFII_{K2\text{-}like}$ plasmids with bla_{KPC} among 522 global KPC-producing *K. pneumoniae*. CC258 (i.e. ST258 [clades I and II], ST11, ST512) and ST147 were statistically associated with $IncFII_{K2\text{-}like}$ KPC-containing plasmids and may possess an epidemiological advantage over isolates that harboured non-IncF KPC-harboring plasmids.

The class A *Klebsiella pneumoniae* carbapenemases (KPC) ß-lactamases have been extensively reported in *Klebsiella pneumoniae* (1). KPC are present in more than 100 different *K. pneumoniae* sequence types (STs), but the KPC pandemic is primarily driven by the spread of members of clonal complex (CC) 258 namely sequence types (STs) 258 (clades I, II), ST11, ST340 and ST512 (1).

Several different KPC-containing plasmids (i.e. IncF, IncI2, IncX, IncA/C, IncR, and ColE1) have been identified in CC258 (2), however, the most predominant plasmid type is IncF with FIIk replicons i.e. $IncFII_{K1}$ ($FIB_{pKPN\text{-}like}$) and $IncFII_{K2}$ ($FIB_{pKPQIL\text{-}like}$) (3). pKpQIL was the prototype of the $IncFII_{K2}$ group and one of the most common bla_{KPC}-harboring plasmids, reported in Israel, USA, UK, Colombia and Italy (2). pKPN-3 was the prototype of the $IncFII_{K1}$ and was not initially associated with bla_{KPC} but was as a virulence plasmid and co-resident with pKpQIL within ST258 (2). In the current study, we set out to determine the presence and global distribution of CC258 among a defined population consisting of 522 KPC-producing *K. pneumoniae* from the AstraZeneca's (AZ) international surveillance study on antimicrobial resistance (2012–14). We also investigated the association of $IncFII_{K2\text{-}like}$ plasmids containing bla_{KPC} with CC258 strains in comparison to non-CC258 strains.

The AstraZeneca global surveillance program was initiated in 2012 and includes a wide representation of microbiology laboratories among the various continents (4). Up to 100 consecutive non-selected Gram-negative aerobic and facultative bacilli from each of participating countries/hospital were included. All organisms were deemed clinically significant based upon the criteria of the local investigators and were obtained from the urinary tract, skin structures, intra-abdominal, and lower respiratory tract specimens. The countries that participated in the surveillance include the following: *Africa*: Egypt, Kenya, Nigeria, South Africa. *Asia*:

China, South Korea, Taiwan, Thailand. *Europe:* Austria, Belgium, Bulgaria, Greece, Czech Republic, Denmark, France, Germany, Hungary, Italy, Macedonia, Portugal, Poland, Russia, Romania, Slovakia, Spain, Turkey, United Kingdom. *Latin America*: Argentina, Brazil, Chile, Colombia, Mexico, Uruguay, Venezuela. *Middle East:* Lebanon, Israel, Syria, Kuwait. *North America*: United States. *South Pacific*: Australia, Philippines, Japan.

The AstraZeneca surveillance program does have some drawbacks; it only includes 100 consecutive non-selected Gram-negative aerobic and facultative bacilli from each of participating countries/hospital/year. It is therefore possible that the program will miss outbreaks of a particular resistance mechanism. The program is also biased towards sites that have AstraZeneca representation within that region/city/country (i.e. hospitals that can afford AstraZeneca products). Moreover, it only includes isolates from urinary tract, skin structures, intra-abdominal, and lower respiratory tract specimens. However, the program does have some significant advantages; it includes a wide representation of microbiology laboratories among the various continents, although Africa and Asia are underrepresented. It only includes clinically significant bacteria and is not biased towards a certain resistance phenotype. Therefore, it provides a "snapshot" view on what types of resistance determinants are endemic in the specific hospital/region.

Molecular screening for bla_{KPC} was performed on carbapenem resistant *K. pneumoniae* as described previously (5). Genetic relatedness between the isolates was initially determined using pulsed-field gel electrophoresis **(**PFGE) (6) and the major pulsotypes (i.e. those with more than 10 isolates pre pulsotype) also underwent multilocus sequencing typing (MLST) (7). PCR typing was used to determine the presence of bla_{KPCs} on $IncFII_{K2\text{-}like}$ types of plasmids and to also identify the different Tn*4401* isotypes (8, 9). Table 1 illustrates the PCR primers and their

Table 1. Primers and target sizes for the characterization of IncFII$_{K2\text{-like}}$ plasmids that contain *bla*$_{KPC}$

PCR number	Name	Sequence	Size (bp)	Target	Reference
PCR-I	FIIK-repA-F1	CTTCACGTCCCGTTTTGATT	657	IncFII *repA* gene	3
	FIIK-repA-R1	CGCTTCAGCGCTTCTTTATC			
PCR-II	QIL-F1	ACAGGGAGTGCCAGGAAAG	2,001	Junction between Tn*4401 tnpR* and upstream IncFII$_{K2}$backbone gene	8
	QIL-R1	TGTATTTGCATGGCGATGAG			
PCR-III	Tn4401v-F(3098U)	TGACCCTGAGCGGCGAAAGC	604	pKpQIL-associated Tn*4401a* isoform	9
	Tn4401v-R1	GCAAGCCGCTCCCTCTCCAG			
	Tn4401v-R(3781L)	CACAGCGGCAGCAAGAAAGC			
PCR-IV	QIL-F2	GCCTCAGATAGATGCGGTAGC	1,831	Junction between Tn*4401* IS*Kpn6* and	9

PCR number	Name	Sequence	Size (bp)	Target	Reference
	QIL-R2	AAGCTGGAGACATGGAATGG		downstream gene	
PCR-V	QIL-hsdR-F1	GGGTCGTTCACAAAGTCGAT	498	$IncFII_{K2}$-associated type I restriction modification system *hsdR* gene	9
	QIL-hsdR-R1	CGTTGAGCACTTCACCAAAA			
PCR-VI	K2-repB-F1	CCATTCCGATCCTTTTCTGA	395	$IncFII_{K2}$ *repFIB* gene	9
	K2-repB-R1	AACGCTACTGTCCAGCCTGT			

respective targets used in this study. IncFII$_{K2\text{-like}}$ plasmids were identified with amplifications with all the primer sets namely I II, III, IV, V and VI.

PFGE identified four major pulsotypes (i.e. more than 10 isolates per pulsotype) among 412 (79%) of isolates, that were designated as follows; cluster A (n = 290), B (n=80), C (n=27), and D (n=15). We also recognized 3 minor pulsotypes (i.e. less than 10 isolates per pulsotype) among 23 (4%) of isolates, designated as follows; cluster E (n=9), F (n=7) and G (n=7). The remaining isolates (n=87 [17%]) were not clonally related, i.e. exhibited <60% similar PFGE profiles and did not show patterns similar to those from clusters A to G. MLST identified the different pulsotypes as follows: cluster A as ST258, B as ST11, C as ST147, D as ST512, E as ST189, F as ST15 and cluster G as ST437. ST258 was further differentiated into clades I and II (10). The geographical distribution of the different *bla*$_{KPCs}$, STs and IncFII$_{K2\text{-like}}$ plasmids associated with *bla*$_{KPC}$ are shown in Table 2.

K. pneumoniae ST258 is a prototype of a high-risk clone and has been largely responsible for the global spread of carbapenem resistance among the Enterobactericeae (11). Kreiswirth and colleagues performed whole genome sequencing on a global collection of *K. pneumoniae* ST258 and showed that this ST belonged to two well defined lineages namely clade I and clade II: clade I was associated with KPC-2 and clade II was associated with KPC-3 (10). The majority of *K. pneumoniae* (n=290 [56%]) from our global collection belonged to ST258; clade I (n=165 [32%]) was associated with *bla*$_{KPC\text{-}2}$ on IncFII$_{K2\text{-like}}$ plasmids. This clade was mostly present in Argentina and Greece, and to a lesser extent in Belgium, China, Italy, Romania and USA (Table 2). ST258 clade II (n=125 [24%]) was associated with *bla*$_{KPC\text{-}3}$ on IncFII$_{K2\text{-like}}$ plasmids. This clade was mainly identified in Italy and USA and to a lesser extent in Austria, Belgium, Brazil, Chile, Colombia, Germany, Greece, Israel, Mexico and Venezuela (Table 2). There was

Table 2. Sequence types, global distribution, presence of IncFII$_{K2\text{-like}}$ plasmids among *Klebsiella pneumoniae* with *bla*$_{KPC}$

Sequence Type	Country of Isolation (no)	KPC (no)	IncFII$_{K2\text{-like}}$ plasmids (no)
ST258-I (n=165)	**Argentina (33),** Belgium (2), China (1), **Greece (104),** Italy (11), Romania (4), USA (10)	KPC-2 (154), KPC-3 (9), KPC-9 (2)	103
ST258-II (n=125)	Austria (1), Belgium (1), Brazil (7), Chile (1), Colombia (2), Germany (2), Greece (1), Israel (7), **Italy (72),** Mexico (1), **USA (28)**, Venezuela (1)	KPC-2 (22), KPC-3 (103)	85
ST11 (n=80)	Argentina (7), Austria (1), **China (19),** Brazil **(45),** Colombia (1), Israel (1), Taiwan (1), USA 3), Venezuela (1)	KPC-2 (73), KPC-3 (6), KPC-12 (1)	33
ST147 (n=27)	Argentina (2), **Greece (20),** Italy (1), Philippines (1), Romania (1), Venezuela (2)	KPC-2 (26), KPC-3 (1)	19
ST512 (n=15)	**Colombia (9),** Israel (2), Italy (2), USA (2)	KPC-2 (4), KPC-3 (11)	5
ST189 (n=9)	**Colombia (8),** Venezuela (1)	KPC-2 (9)	0
ST15 (n=7)	**Portugal (6),** Colombia (1)	KPC-2 (1), KPC-3 (6)	0

ST437 (n=7)	**Brazil (7)**	KPC-2 (7)	0
Other STs (n=87)	Argentina (12), Austria (1), Belgium (2), Brazil (4), China (1), **Colombia (17),** Czech Rep (1), Greece (5), Italy (10), Israel (5), Philippines (1), **Portugal (13),** Romania (1), UK (1), USA (9), Venezuela (4)	KPC-2 (56), KPC-3 (31)	19

not a specific association of *bla*$_{KPC-2}$ or *bla*$_{KPC-3}$ with IncFII$_{K2-like}$ plasmids (i.e. 40% of KPC-2 and 54% of KPC-3 were harboured on IncFII$_{K2-like}$ plasmids (Table 2).

ST11, which is closely related to ST258, is the major ST among *K. pneumoniae* harboring *bla*$_{KPC}$ from Asia (especially China) (12), Latin America (13) and sometimes contain other carbapenemases (14, 15). ST11 was the second most common ST in our study (n=80 [15%]), and was associated with *bla*$_{KPC-2}$ on IncFII$_{K2-like}$ plasmids. ST11 was present in Brazil and China and to a lesser extend in Argentina, Colombia, Israel, Taiwan, USA, and Venezuela (Table 2).

Other STs that belong to CC258 with *bla*$_{KPC}$ have been reported from Colombia (i.e. ST512), Italy (i.e. ST512), Israel (i.e. ST512), Brazil (i.e. ST340, ST437) and Greece (i.e. ST340) (13). The remaining members of CC258 from our study included ST512 and ST437. ST512, mainly from Colombia, was the fourth most common ST (n=15 [3%]) in our collection and was also identified in Israel, Italy and the USA (Table 2). The ST was associated with *bla*$_{KPC-3}$ on IncFII$_{K2-like}$ plasmids. ST437 (n=7) was identified in Brazil and did not contain IncF plasmids.

K. pneumoniae ST147 is an emerging high risk clone that was first identified in Greece and has been associated with *bla*$_{VIM}$ and *bla*$_{KPC}$ in that country (16). NDM (18) and OXA-181 (6) carbapenemases have also been described in ST147 from various countries, such as Switzerland, Iraq, Canada, UK, India and Italy (1). ST147, mainly from Greece, was the third most common ST (n=27 [5%]) in our study and was associated with *bla*$_{KPC-2}$ on IncFII$_{K2-like}$ plasmids. ST147 was also present in Argentina, Italy, Philippines, Romania and Venezuela (Table 2).

The geographical distribution of the other minor STs was as follows: ST189 (n=9) in Colombia and ST15 (n=7) in Portugal (Table 2). The isolates that did not belong to major or minor STs (n=87) showed a global distribution. The IncFII (none k2-like) plasmids (n=141) contained the following Tn*4401* isotypes: a (66[47%]), b (34[24%]) and d (5[4%]); $IncFII_{K2\text{-}like}$ plasmids (n=264) only contained isotype a.

It has been postulated recently that the presence of IncF plasmids with FIIk replicons harboring bla_{KPC} is central to the global success of CC258 and that they have significantly contributed to the evolutionary dominance of ST258 (11). Our molecular epidemiological data supports this hypothesis. The majority of CC258 from our study harboured $IncFII_{K2\text{-}like}$ plasmids containing bla_{KPC} when compared to non-CC258 STs (i.e. 226/392 [58%] vs 38/130 [23%] p<0.0001) {95% confidence interval 25.37 to 43.50}). This is especially true for ST258 in that 188/290 [65%] of plasmids with bla_{KPC} from this ST belonged to $IncFII_{K2\text{-}like}$ (Table 2). *K. pneumoniae* ST147 was also associated with IncF plasmids, especially $IncFII_{K2\text{-}like}$ (Table 2). Our data suggests that certain successful high-risk *K. pneumoniae* clones (i.e. CC258 and ST147) are linked to specific narrow host range IncF plasmids with bla_{KPC}, and this association may possess epidemiological advantages over other clones that carry non-IncF KPC plasmids. It is possible that the maintenance and co-evolution of GC258 with $IncF_{K2\text{-}like}$ plasmids have provided rapid and continual adaptation opportunities for this CC providing them with the additional ability to outcompete other *K. pneumoniae* clones. This scenario is consistent with both the macro- and micro-evolutionary versions of the Red Queen hypothesis of co-evolution (11). However, this might be a very simplistic view regarding the role of $IncF_{K2\text{-}like}$ plasmids in the success of CC258 and this CC frequently harbors non-F antimicrobial resistance plasmids (1).

This study was not designed to specifically address the epidemiological advantage attributed to $IncFII_{K2\text{-}like}$ plasmids as compared to other features of CC258. The $IncFII_{K2\text{-}like}$ plasmids are clearly the most common KPC containing plasmids disseminating in *K. pneumoniae* but are not necessarily restricted to CC258 (i.e. 23% of non-CC258 STs also contained $IncFII_{K2\text{-}like}$ plasmids). To the best of our knowledge, this is the first study that provided a comprehensive overview on the global distribution of different STs with $bla_{KPC,}$ and their association with $IncFII_{K2\text{-}like}$ plasmids in a defined population.

Funding

This work was supported in part by a research grant from the Calgary Laboratory Services (#10015169).

Transparency declaration

JDDP had previously received research funds from Merck and Astra Zeneca.

References

1. **Pitout JD, Nordmann P, Poirel L.** 2015. Carbapenemase-Producing *Klebsiella pneumoniae*, a Key Pathogen Set for Global Nosocomial Dominance. Antimicrob Agents Chemother **59:**5873-5884.

2. **Chen L, Mathema B, Chavda KD, DeLeo FR, Bonomo RA, Kreiswirth BN.** 2014. Carbapenemase-producing *Klebsiella pneumoniae*: molecular and genetic decoding. Trends Microbiol **22:**686-696.

3. **Chen L, Chavda KD, Melano RG, Jacobs MR, Levi MH, Bonomo RA, Kreiswirth BN.** 2013. Complete sequence of a $bla_{(KPC-2)}$-harboring $IncFII_{(K1)}$ plasmid from a *Klebsiella pneumoniae* sequence type 258 strain. Antimicrob Agents Chemother **57:**1542-1545.

4. **Kazmierczak KM, Rabine S, Hackel M, McLaughlin RE, Biedenbach DJ, Bouchillon SK, Sahm DF, Bradford PA.** 2016. Multiyear, Multinational Survey of the Incidence and Global Distribution of Metallo-β-Lactamase-Producing Enterobacteriaceae and *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **60:**1067-1078.

5. **Peirano G, Bradford PA, Kazmierczak KM, Badal RE, Hackel M, Hoban DJ, Pitout JD.** 2014. Global incidence of carbapenemase-producing *Escherichia coli* ST131. Emerg Infect Dis **20:**1928-1931.

6. **Lascols C, Peirano G, Hackel M, Laupland KB, Pitout JD.** 2013. Surveillance and molecular epidemiology of *Klebsiella pneumoniae* isolates that produce carbapenemases: first report of OXA-48-like enzymes in North America. Antimicrob Agents Chemother **57:**130-136.

7. **Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S.** 2005. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. J Clin Microbiol **43:**4178-4182.

8. **Chen L, Chavda KD, Melano RG, Hong T, Rojtman AD, Jacobs MR, Bonomo RA, Kreiswirth BN.** 2014. Molecular survey of the dissemination of two *bla*$_{KPC}$-harboring IncFIA plasmids in New Jersey and New York hospitals. Antimicrob Agents Chemother **58:**2289-2294.

9. **Chen L, Chavda KD, Melano RG, Jacobs MR, Koll B, Hong T, Rojtman AD, Levi MH, Bonomo RA, Kreiswirth BN.** 2014. Comparative genomic analysis of KPC-encoding pKpQIL-like plasmids and their distribution in New Jersey and New York Hospitals. Antimicrob Agents Chemother **58:**2871-2877.

10. **Deleo FR, Chen L, Porcella SF, Martens CA, Kobayashi SD, Porter AR, Chavda KD, Jacobs MR, Mathema B, Olsen RJ, Bonomo RA, Musser JM, Kreiswirth BN.** 2014. Molecular dissection of the evolution of carbapenem-resistant multilocus sequence type 258 *Klebsiella pneumoniae*. Proc Natl Acad Sci U S A **111:**4988-4993.

11. **Mathers AJ, Peirano G, Pitout JD.** 2015. The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant Enterobacteriaceae. Clin Microbiol Rev **28:**565-591.

12. **Yang J, Ye L, Guo L, Zhao Q, Chen R, Luo Y, Chen Y, Tian S, Zhao J, Shen D, Han L.** 2013. A nosocomial outbreak of KPC-2-producing *Klebsiella pneumoniae* in a Chinese hospital: dissemination of ST11 and emergence of ST37, ST392 and ST395. Clin Microbiol Infect **19:**E509-515.

13. **Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, Cornaglia G, Garau J, Gniadkowski M, Hayden MK, Kumarasamy K, Livermore DM, Maya JJ, Nordmann P, Patel JB, Paterson DL, Pitout J, Villegas MV, Wang H, Woodford N, Quinn JP.** 2013. Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. Lancet Infect Dis **13:**785-796.

14. **Giske CG, Froding I, Hasan CM, Turlej-Rogacka A, Toleman M, Livermore D, Woodford N, Walsh TR.** 2012 Diverse sequence types of *Klebsiella pneumoniae* contribute to the dissemination of $bla_{\text{NDM-1}}$ in India, Sweden, and the United Kingdom. Antimicrob Agents Chemother **56:**2735-2738.

15. **Williamson DA, Sidjabat HE, Freeman JT, Roberts SA, Silvey A, Woodhouse R, Mowat E, Dyet K, Paterson DL, Blackmore T, Burns A, Heffernan H.** 2012. Identification and molecular characterisation of New Delhi metallo-β-lactamase-1

(NDM-1)- and NDM-6-producing Enterobacteriaceae from New Zealand hospitals. Int J Antimicrob Agents **39:**529-533.

16. **Papagiannitsis CC, Kotsakis SD, Petinaki E, Vatopoulos AC, Tzelepi E, Miriagou V, Tzouvelekis LS.** 2011. Characterization of metallo-beta-lactamase VIM-27, an A57S mutant of VIM-1 associated with *Klebsiella pneumoniae* ST147. Antimicrob Agents Chemother **55:**3570-3572.

17. **Giakkoupi P, Papagiannitsis CC, Miriagou V, Pappa O, Polemis M, Tryfinopoulou K, Tzouvelekis LS, Vatopoulos AC.** 2011. An update of the evolving epidemic of bla_{KPC-2}-carrying *Klebsiella pneumoniae* in Greece (2009-10). J Antimicrob Chemother **66:**1510-1513.

18. **Peirano G, Pillai DR, Pitondo-Silva A, Richardson D, Pitout JD.** 2011. The characteristics of NDM-producing *Klebsiella pneumoniae* from Canada. Diagn Microbiol Infect Dis **71:**106-109.