Clonal dissemination of *Klebsiella pneumoniae* ST258 harbouring KPC-2 in Argentina

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Abstract

The present work describes the abrupt emergence of *Klebsiella pneumoniae* carbapenemase (KPC) and characterizes the first 79 KPC-producing enterobacteria from Argentina (isolated from 2006 to 2010). The emergence of bla_{KPC-2} was characterized by two patterns of dispersion: the first was the sporadic occurrence in diverse enterobacteria from distant geographical regions, harbouring plasmids of different incompatibility groups and bla_{KPC-2} in an unusual genetic environment flanked by IS*Kpn8*-Δbla_{TEM-1} and IS*Kpn6-like*. bla_{KPC-2} was associated with IncL/M transferable plasmids; the second was the abrupt clonal spread of *K. pneumoniae* ST258 harbouring bla_{KPC-2} in Tn*4401a*.

Keywords: bla_{KPC-2}, *Enterobacteriaceae*, ST11, ST258, Tn*4401a*

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The emergence and rapid dissemination of *Klebsiella pneumoniae* carbapenemase (KPC)-producing Gram-negative bacilli has become an important therapeutic and infection control problem in the healthcare setting [1]. The worldwide spread of KPC-producing *K. pneumoniae* strains has revealed the successful dissemination of a major clone defined as sequence type (ST)258 [2–4]. bla_{KPC} has been commonly associated with Tn*4401*, which is possibly responsible for its acquisition [5,6]. A different bla_{KPC} environment was described in plasmids from Chinese enterobacteria, where bla_{KPC-2} was associated with a Tn*801*-like transposon [7]. Moreover, ST11 was demonstrated to be the dominant clone of KPC-producing *K. pneumoniae* in China, where ST258 was not detected [8].

We previously reported the emergence of bla_{KPC} in *Enterobacteriaceae* from Argentina (2006), and designed an algorithm to detect class A carbapenemases and metallo-β-lactamases at the level of the clinical microbiology laboratory [9,10]. Since 2008, as the National Reference Laboratory in Antimicrobial Resistance (NRLAR), we have used the algorithm to implement active surveillance of such carbapenemases among 432 laboratories across Argentina that participate in the National Quality Control Programme in Bacteriology (Argentinean Ministry of Health). All enterobacteria were screened through the algorithm: isolates that showed (i) imipenem inhibition zones ≤21 mm, (ii) resistance or intermediate resistance to expanded-spectrum cephalosporins and (iii) positive synergy between the disks of carbapenems and 3-aminophenyl-boronic acid were suspected of KPC production and sent to the NRLAR. Possible interference by AmpC was overcome by using the oxacillin-based modified Hodge test [11].

In this study, we included the first two KPC producers detected in Argentina in 2006 [9], plus the 77 consecutive isolates (one per patient) that were confirmed at the NRLAR by PCR as KPC producers (subsequently characterized as bla_{KPC-2} by sequencing). These 77 isolates were referred between January 2008 and July 2010 from 30 hospitals from five distant geographical regions (no. isolates/no. hospitals): Buenos Aires City (54/19), Buenos Aires Province (16/8), Mendoza (4/1), Neuquén (2/1), and Formosa (1/1).

From January 2008 to July 2009, isolates of *Citrobacter freundii* (n = 2), *K. pneumoniae* (n = 5), *Serratia marcescens* (n = 1), *Enterobacter cloacae* (n = 2) and *Escherichia coli* (n = 1) were recovered from six hospitals (four regions). However, from August 2009, the number of bla_{KPC-2} isolates and the number of hospitals affected increased dramatically, mostly owing to *K. pneumoniae* recovered from Buenos Aires City and Province (Fig. 1).

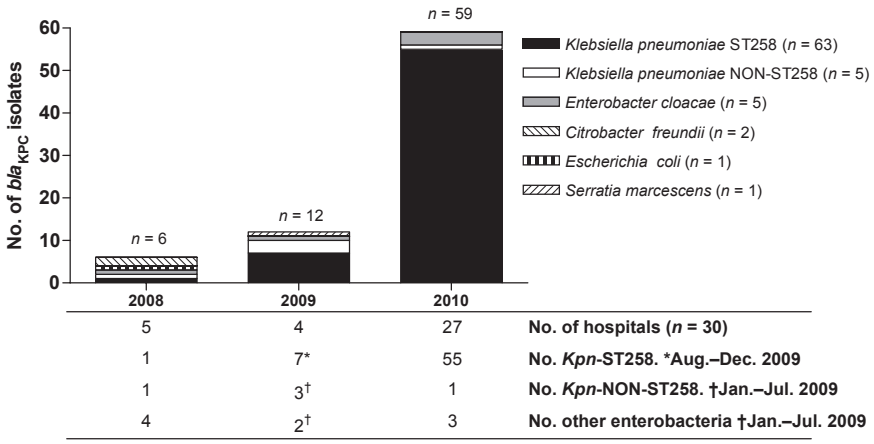

FIG. 1. Species distribution of the 77 bla_{KPC-2}-producing isolates in the different time periods. The first two KPC-producing isolates collected in 2006 (*Citrobacter freundii* and *Klebsiella pneumoniae* NON-ST258) were excluded. For each time period, the number of hospitals where bla_{KPC-2}-producing isolates were recovered, and the numbers of *Kpn*-ST258, *Kpn*-NON-ST258 and other enterobacteria, are shown. *Kpn*, *K. pneumoniae*.

Pulsed-field gel electrophoresis (PFGE) was performed as previously described [12], and the results were analysed according to the van Belkum guidelines [13]. Five types were observed among the 69 *K. pneumoniae* isolates: K1 (63 isolates); K2 (three isolates); and K3–K5 (one isolate each). No genotypic relationship was found among the three *C. freundii* or the five *E. cloacae* isolates.

Multilocus sequence typing (MLST) [14] was performed according to the MLST Database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/). Six representative strains of *K. pneumoniae* PFGE type K1 (different hospitals, isolated in 2008–2010) were identified as ST258. The minor PFGE types belonged to three STs: K2 and K5 to ST11, a single-locus variant of ST258; K3 to ST476; and K4 to the new ST526 described here.

The genetic surroundings of bla_{KPC-2} were studied by PCR mapping and sequencing (see primers used in the Supporting Information). Two different genetic environments were found (Fig. 2). All of the 63 *K. pneumoniae* clone K1/ST258 isolates harboured bla_{KPC-2} in the Tn*4401a* isoform [5]. To analyse the remaining 16 clinical isolates that did not harbour Tn*4401*, we constructed *Eco*RI-based DNA libraries [15] from *C. freundii* M9169 [9] and *E. cloacae* M11180. The bla_{KPC-2} environment found was very similar to the so-called Variant 1 (the accession number was not provided), a variant of the region found in plasmid pKP048 from a Chinese *K. pneumoniae* isolate [7]. Therefore, we named the Argentinean bla_{KPC-2} environment Variant 1a (Fig. 2a). However, we found two key differences: a composite transposon (partial sequence) from plasmid pFBAOT6 of *Aeromonas punctata* [16], which disrupts *tnpA* of the Tn*801*-like transposon, and an additional 671-bp fragment between IS*Kpn8* and bla_{KPC-2}, which contains a truncated bla_{TEM-1}.

By PCR mapping (Fig. 2b), Variant 1a was detected in 14 of 16 isolates, whereas the remaining two showed the new Variant 1b (Fig. 2a). As a whole, Variants 1a and 1b were found in five enterobacterial species, including *K. pneumoniae* ST11, ST476, and ST526. Interestingly, ST11 was also described in two KPC-producing PFGE clones from Brazil, but these harboured a Tn*4401* [17], in contrast to the ST11 Argentinean and Chinese [8] isolates. As essentially the same bla_{KPC-2} environment (Variants 1a and 1b) was found in different species, we speculated that a unique plasmid could be responsible for such interspecies dissemination. However, a plasmid content analysis [18] of the 16 isolates harbouring Variants 1a and 1b showed a range of plasmid sizes (7–80 kb), with several isolates harbouring more than one plasmid (data not shown). In addition, a Southern blot analysis with a bla_{KPC} probe showed that bla_{KPC-2} was associated with plasmids of different sizes (data not shown). Therefore, PCR-based replicon typing [19] was performed on the 16 clinical isolates. We detected a single Inc group in 11 isolates: IncHI2 (n = 3), IncL/M (n = 3), and IncA/C (n = 5). Two isolates produced amplicons for three Inc groups (*Escherichia coli*, IncFIA–IncFIB–IncFrepB; *K. pneumoniae*, K5/ST11, IncL/M–IncA/C–IncFIIS) and three isolates gave negative results with all of the Inc primers tested (see Supporting Information).

Biparental conjugation [15] of six representative ST258 isolates (from different hospitals) did not yield transconjugants. Interestingly, three of 11 selected isolates (different hospitals and species) harbouring Variants 1a and 1b yielded transconjugants: two *C. freundii* (Variants 1a and 1b) and one *S. marcescens* (Variant 1a). β-Lactam MICs [20] of the transconjugants were lower than those of the parental strains (MIC range, mg/L): cefotaxime, 1–16 vs. 8–64; ceftazidime,

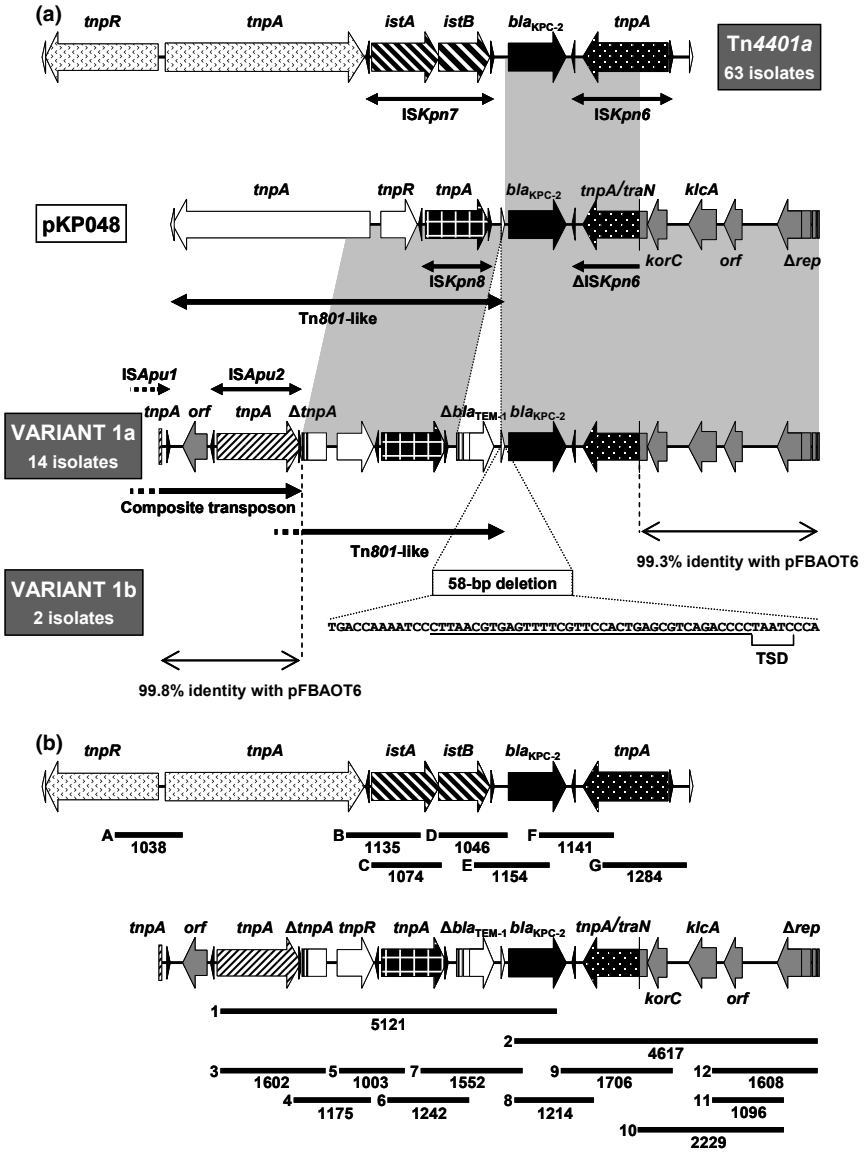

FIG. 2. Genetic environment of bla_{KPC-2} in enterobacteria from Argentina. Genes (open reading frames) and their corresponding transcriptional orientations are indicated by horizontal broad arrows. Filled and empty triangles represent inverted repeats of insertion sequences (ISs) or Tn*3*-group transposons, respectively. (a) The Tn*4401a* sequence was from plasmid pNYC (GenBank accession number EU176011) and the region of the plasmid pKP048 depicted corresponds to coordinates 15 736 to 25 596 (complement) in GenBank FJ628167. Variant 1a represents the sequence obtained from *Citrobacter freundii* M9169 and *Enterobacter cloacae* M11180 [9] (10 047 bp, GenBank JN048639 and JN048640, respectively). Δbla_{TEM-1} indicates a deletion of 10 bp upstream of the bla_{TEM-1} start plus the first 291 nucleotides of the encoding gene for the Tn*3* sequence. Only the 58-bp deleted fragment in Variant 1b is shown, indicating the Tn*801*-like inverted repeat right (underlined text) and its A/T-rich pentameric target site duplication (TSD). The grey-shaded areas show identical regions among the compared structures. Thick lines with single or double arrowheads indicate the different ISs or transposons found, and thin lines with double arrowheads indicate the identity with plasmid pFBAOT6 from *Aeromonas punctata* (GenBank CR376602). (b) PCR mapping used for characterizing Tn*4401a*-harbouring (upper) or Variant 1a/b-harbouring isolates (lower). The amplicons obtained in the different PCR assays and the lengths (bp) of the different amplicons are represented by thick lines. The letters and numbers at the left of each line indicate the different PCR used (primers are listed in the Supporting Information). Amplicons 7 and 8 were sequenced in all isolates to discriminate between Variants 1a and 1b.

2–16 vs. 4–64; imipenem, 0.5–2 vs. 2–16; meropenem, 0.125–0.5 vs. 2–16; and ertapenem 0.25–1 vs. 2–8 mg/L. Replicon typing analysis allowed us to associate bla_{KPC-2} with IncL/M transferable plasmids in two transconjugants, whereas the remaining one was negative for all of the Inc primers tested, as was its parental strain.

The present work shows the results of a 3-year study (2008–2010) conducted in Argentina by the NRLAR to detect, characterize and warn of KPC emergence. The dissemination of bla_{KPC-2} occurred in two phases (Fig. 1): (i) from January 2008 to July 2009, with the sporadic occurrence of bla_{KPC-2} in diverse enterobacteria obtained from distant locations; and (ii) since August 2009, with a six-fold increase in the number of bla_{KPC-2} isolates, 94% of which were ST258. Therefore, the abrupt dissemination of KPC in Argentina could be explained by the surge of a unique clone of the international ST258. Moreover, the fact that Tn*4401a* was only found among these isolates reinforces the notion of clonal expansion in our hospital settings. Conversely, phase I of the local KPC emergence was driven by a diversity of enterobacterial species harbouring plasmids of several incompatibility groups. However, these isolates harboured bla_{KPC-2} in a unique and unusual genetic environment (Variants 1a and 1b) that was transferable by conjugation. These facts strongly suggest that not only plasmid transferability but also bla_{KPC-2} mobilization among different plasmids (i.e. transposition) could have played a major role in phase I of the Argentinean KPC emergence, leading to dissemination of Variants 1a and 1b in diverse clones and species.

To conclude, the actual KPC epidemiology in Argentina is complex and diverse. Cooperative efforts and strict infection control policies are still required in all institutions afflicted by dissemination of KPC.

The nucleotide sequences obtained here have been assigned GenBank accession numbers JN048639 and JN048640 (Variant 1a), and JN048641 and JN048642 (Variant 1b).

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Transparency Declaration

The authors have no conflicts of interest to declare.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Pulse field gel electrophoresis patterns of bla_{KPC-2} isolates.

Table S1. Primers used in PCR mapping of bla_{KPC-2} genetic environments.

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