Double copies of Tn*4401*a:*bla*$_{KPC-3}$ on an IncX3 plasmid in *Klebsiella pneumoniae* successful clone ST512 from Italy

Daniela Fortini[1], Laura Villa[1], Claudia Feudi[1], João Pires[2], Celestino Bonura[3], Caterina Mammina[3], Andrea Endimiani[2], and Alessandra Carattoli[1,2]*

[1]Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy; [2] Institute for Infectious Diseases, University of Bern, Switzerland; [3]Department of Sciences for Health Promotion and Mother-Child Care 'G. D'Alessandro', University of Palermo, Palermo, Italy

Running Title: IncX3 plasmid carrying two Tn*4401*a:*bla*$_{KPC-3}$

Key words: KPC, ST258, ST512, *Klebsiella*, Tn*4401*, XDR, carbapenemase, carbapenem

***Corresponding author:**

Department of Infectious, Parasitic and Immune-Mediated Diseases,

Istituto Superiore di Sanità, Viale Regina Elena 299 00161 Rome, Italy

Tel +39-06-4990-3128

Fax. +39-06-4938-7112

Email: alecara@iss.it

Abstract

A carbapenem-resistant sequence type (ST) 512 KPC-3-producing *Klebsiella pneumoniae* strain showing a novel variant plasmid content was isolated in Palermo, Italy, in 2014. ST512 is a worldwide successful clone associated with the spread of bla_{KPC} genes located on the IncFIIk pKpQIL plasmid. In our ST512 strain, the bla_{KPC-3} was exceptionally located on an IncX3 plasmid whose complete sequence was determined. Two copies of the Tn*44011*a:bla_{KPC-3} transposon due to a intramolecular transposition events were detected in the plasmid.

Extensively drug-resistant (XDR) and pandrug-resistant KPC-producing *Klebsiella pneumoniae* (KPC-*Kp*) of the hyperepidemic clonal complex 258 (CC258) are detected worldwide as hospital-acquired pathogens and frequently responsible for outbreaks. In particular, sequence type 258 (ST258), ST512, ST11 and ST340 are the most frequently detected variants of KPC-*Kp* isolates (1, 2).

During May-June 2011, a countrywide Italian survey focusing on the diffusion of carbapenem nonsusceptible *K. pneumoniae* isolates showed that the most frequent lineages belonged to CC258 (ST258 or ST512) (3). The epidemiology of KPC-*Kp* in Palermo, Italy, also confirmed the emergence of ST258 beginning in 2008 (4). More recently, a six-month surveillance performed in Sicily suggested that a major epidemiological change is likely ongoing in this geographic area, with ST258 being still prevalent, but circulating along with several additional STs, including ST307 and ST273 (4). In particular, only one isolate of ST512 (i.e., strain 45) was identified on a total of 94 KPC-*Kp* strains (4). This unique isolate of ST512 has been further investigated and described in this study.

As shown in Table 1, the ST512 *K. pneumoniae* 45 showed an XDR phenotype (4). It was screened by PCR for the following plasmid-mediated quinolone resistance and β-lactamase genes: *qnrA, qnrB, qnrC, qnrD, qnrS, aac(6')-Ib*-cr, *qepA, oqxAB, bla*$_{\text{KPC}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{LAP}}$, *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{CMY}}$ (5-8). Positive amplicons underwent Sanger DNA sequencing for identification of the variant genes. *K. pneumoniae* 45 resulted positive for *bla*$_{\text{KPC-3}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-1}}$, *aac(6')-Ib*-cr, *bla*$_{\text{SHV-11}}$, and *oqxAB* genes. The implementation of the PCR-based replicon typing (PBRT) kit (Diatheva) indicated that plasmids carried by strain 45 were not typable. Therefore, the *bla*$_{\text{KPC-3}}$-carrying plasmid was transformed in *Escherichia coli* DH5α competent cells (Invitrogen), selecting on Luria–Bertani

agar plates (Sigma), containing ampicillin (50 µg/ml). Transformants were then screened by PCR for the presence of bla_{KPC}. Plasmid DNA of one representative bla_{KPC-3}-positive transformant (named p45) was purified using a Invitrogen Plasmid Midi Kit (Invitrogen) and fully sequenced. A shot-gun library was obtained and sequencing was performed with the 454-GS Junior platform following the standard sequencing procedure (Roche Diagnostics). Plasmid coverage was >80x. Reads were aligned and assembled using the Newbler assembler software version 2.0.01.14 (Roche Diagnostics). As result, plasmid p45 was split in three contigs and the complete sequence was reconstructed by PCR-based gap closure method. Open reading frames (ORFs) were predicted and annotated using the Artemis software (Welcome Trust Sanger Institute, UK). Each predicted protein was compared against protein database using BlastP (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Gene sequences were compared and aligned with GenBank data using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The IncX3 plasmids pKpS90, and pIncX-SHV (GenBank: JX461340, and JN247852, respectively) were used as reference for annotation and comparative analyses. GenBank file was compiled using Sequin (http://www.ncbi.nlm.nih.gov/Sequin/), and deposited at the NCBI GenBank under accession no. KT362706.

As shown in Fig. 1, our results indicated that p45 is 63,203 bp in size and shows the typical IncX3 scaffold, including the replicase gene, *tax,* and *pilX* gene clusters (9). The bla_{KPC-3} is located in the Tn*3*-like element Tn*44011*a (10). However, we observed that two copies of Tn*44011*a were present at a distance of 8,658 bp within the IncX3 plasmid scaffold of p45.

One copy of Tn*44011*a was integrated within an IS*3000*-a1 element which resulted truncated by the insertion of the transposon. The duplication of five base pairs sequence was identified at the site of integration, immediately flanking the IRR and IRL of Tn*4401*a. The bla_{SHV-11} was

identified at 4 kb from the Tn*4401*a-*bla*$_{KPC-3}$ integration site, followed by an IS*26* element. The same structure carrying IS*26*-*bla*$_{SHV-11}$-IS*3000* was previously detected in plasmid pIncX-SHV (11; GenBank: JN247852). In a similar plasmid (i.e., pKpS90), the Tn*4401:bla*$_{KPC-2}$ was integrated in the same region as p45 but within a different site, causing the interruption of the *ygbK* gene (12). The progenitor of p45 was therefore a pIncX-SHV-like plasmid, carrying the *bla*$_{SHV-11}$ (11).

The second copy of the Tn*4401*a in p45 was integrated in *topB* (a gene constantly present in all IncX3 plasmids) and in opposite orientation compared to the first transposon (Fig. 1). The target site duplication was also identified in this site, adjacent to the inverted repeats of the transposon. It is plausible that the acquisition of the second Tn*4401*a in p45 occurred by intramolecular transposition. In the literature, the presence of two *bla*$_{KPC}$ copies located *in trans* on different plasmids simultaneously present in the same bacterial host were previously reported in several collections (13-16). However, only two examples of *in cis bla*$_{KPC}$ genes were previously described and differed from the arrangement described in p45. Two Tn*4401*a-*bla*$_{KPC-3}$ copies were identified in the MNCRE44 strain, an extraintestinal pathogenic *E. coli* belonging to the ST131 H*30R* subclade and found in the US (13). This plasmid (pMNCRE44_5) was a 116 kb hybrid of IncX3 and IncFIA(HI1) plasmids and the two transposon copies were located one copy for each of the two fused plasmid portions (13). The duplication of the similar transposon Tn*4401*b::*bla*$_{KPC-2}$ was described on the IncN plasmid S9 from *K. pneumoniae* in the US (17).

MICs for several antibiotics for KPC-*Kp* strains of ST512 and ST258 carrying *bla*$_{KPC-3}$ on pKpQIL (18), those of KPC-*Kp* 45 (ST512 and with IncX3), as well as those of their corresponding *E. coli* DH5α transformants, were determined implementing the microdilution ESB1F and GNX2F plates (Trek Diagnostics) (Table 1). As result, we noted that the *E. coli*

DH5α transformant carrying the IncX3 plasmid with two copies of Tn*4401*a-bla_{KPC-3} showed significantly increased MICs for carbapenems, cephalosporins, and β-lactam/β-lactamase inhibitor combinations compared to the transformant carrying the classical pKpQIL plasmid. This phenomenon was not observed for the original *K. pneumoniae* strains of different ST and possessing different bla_{KPC} genetic background. The MIC difference may be due not only to the double copy of the bla_{KPC} and their level of gene expression (14), but also to IncX3 and pKpQIL different copy numbers.

In conclusion, our study describes the change of the typical plasmid content of the ST512 *K. pneumoniae*: the worldwide described pKpQIL plasmid carrying bla_{KPC} (19; GenBank: GU595196) was substituted by an IncX3 plasmids carrying two copies of the Tn*4401*a-bla_{KPC-3}. The change of plasmid type in *K. pneumoniae* strains 45 could represent an important evolution of the ST512 lineage.

Acknowledgments

This work was partially supported by the Swiss National Science Foundation (SNF; grant number 32003B_153377 due to AE) and by the Italian National Center for Disease Prevention and Control of the Minister of Health (fund CCM 2014 to AC and CM)

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Legend to Figure 1. **Major structural features of plasmids p45, pKpS90, and pIncX-SHV.** Predicted open reading frames (ORFs) on plasmids are represented by white arrows. The ORFs of p45 were identified in this study; the ORFs of pKpS90, and pIncX-SHV were deduced from GenBank files JX461340 and JN247852, respectively. Transposase genes of the Tn*4401 1*a transposons flanked by IRR and IRL inverted repeats (thick black lines) are indicated by grey arrows; the bla_{KPC-3} genes inside Tn*44011*a are indicated by black arrows. The DNA sequences of duplicated target sites are indicated above IRR and IRL. The *ygbK*, IS*3000* and *topB* genes targeted by Tn*4401-a* transposition events occurred in the different plasmids are indicated by arrows filled with stripes, dot and black squares, respectively.