

1 **Double copies of Tn4401a:bla<sub>KPC-3</sub> on an IncX3 plasmid in *Klebsiella pneumoniae* successful**  
2 **clone ST512 from Italy**

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13 **Running Title:** IncX3 plasmid carrying two Tn4401a:bla<sub>KPC-3</sub>

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24 **Abstract**

25 A carbapenem-resistant sequence type (ST) 512 KPC-3-producing *Klebsiella pneumoniae* strain  
26 showing a novel variant plasmid content was isolated in Palermo, Italy, in 2014. ST512 is a  
27 worldwide successful clone associated with the spread of *bla*<sub>KPC</sub> genes located on the IncFIIk  
28 pKpQIL plasmid. In our ST512 strain, the *bla*<sub>KPC-3</sub> was exceptionally located on an IncX3  
29 plasmid whose complete sequence was determined. Two copies of the Tn44011a:*bla*<sub>KPC-3</sub>  
30 transposon due to a intramolecular transposition events were detected in the plasmid.

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

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

46 As shown in Table 1, the ST512 *K. pneumoniae* 45 showed an XDR phenotype (4). It  
47 was screened by PCR for the following plasmid-mediated quinolone resistance and  $\beta$ -lactamase  
48 genes: *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib-cr*, *qepA*, *oqxAB*, *bla<sub>KPC</sub>*, *bla<sub>VIM</sub>*, *bla<sub>NDM</sub>*, *bla<sub>OXA</sub>-*  
49 *48*, *bla<sub>OXA</sub>*, *bla<sub>SHV</sub>*, *bla<sub>TEM</sub>*, *bla<sub>LAP</sub>*, *bla<sub>CTX-M</sub>* and *bla<sub>CMY</sub>* (5-8). Positive amplicons underwent  
50 Sanger DNA sequencing for identification of the variant genes. *K. pneumoniae* 45 resulted  
51 positive for *bla<sub>KPC-3</sub>*, *bla<sub>OXA-1</sub>*, *bla<sub>CTX-M-15</sub>*, *bla<sub>TEM-1</sub>*, *aac(6')-Ib-cr*, *bla<sub>SHV-11</sub>*, and *oqxAB* genes.  
52 The implementation of the PCR-based replicon typing (PBRT) kit (Diatheva) indicated that  
53 plasmids carried by strain 45 were not typable. Therefore, the *bla<sub>KPC-3</sub>*-carrying plasmid was  
54 transformed in *Escherichia coli* DH5 $\alpha$  competent cells (Invitrogen), selecting on Luria-Bertani

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

71 As shown in Fig. 1, our results indicated that p45 is 63,203 bp in size and shows the  
72 typical IncX3 scaffold, including the replicase gene, *tax*, and *pilX* gene clusters (9). The *bla*<sub>KPC-3</sub>  
73 is located in the Tn3-like element Tn44011a (10). However, we observed that two copies of  
74 Tn44011a were present at a distance of 8,658 bp within the IncX3 plasmid scaffold of p45.  
75 One copy of Tn44011a was integrated within an IS3000-a1 element which resulted truncated by  
76 the insertion of the transposon. The duplication of five base pairs sequence was identified at the  
77 site of integration, immediately flanking the IRR and IRL of Tn4401a. The *bla*<sub>SHV-11</sub> was

78 identified at 4 kb from the Tn4401a-*bla*<sub>KPC-3</sub> integration site, followed by an IS26 element. The  
79 same structure carrying IS26-*bla*<sub>SHV-11</sub>-IS3000 was previously detected in plasmid pIncX-SHV  
80 (11; GenBank: JN247852). In a similar plasmid (i.e., pKpS90), the Tn4401:*bla*<sub>KPC-2</sub> was  
81 integrated in the same region as p45 but within a different site, causing the interruption of the  
82 *ygbK* gene (12). The progenitor of p45 was therefore a pIncX-SHV-like plasmid, carrying the  
83 *bla*<sub>SHV-11</sub> (11).

84 The second copy of the Tn4401a in p45 was integrated in *topB* (a gene constantly present in all  
85 IncX3 plasmids) and in opposite orientation compared to the first transposon (Fig. 1). The target  
86 site duplication was also identified in this site, adjacent to the inverted repeats of the transposon.  
87 It is plausible that the acquisition of the second Tn4401a in p45 occurred by intramolecular  
88 transposition. In the literature, the presence of two *bla*<sub>KPC</sub> copies located *in trans* on different  
89 plasmids simultaneously present in the same bacterial host were previously reported in several  
90 collections (13-16). However, only two examples of *in cis* *bla*<sub>KPC</sub> genes were previously  
91 described and differed from the arrangement described in p45. Two Tn4401a-*bla*<sub>KPC-3</sub> copies  
92 were identified in the MNCRE44 strain, an extraintestinal pathogenic *E. coli* belonging to the  
93 ST131 H30R subclade and found in the US (13). This plasmid (pMNCRE44\_5) was a 116 kb  
94 hybrid of IncX3 and IncFIA(HI1) plasmids and the two transposon copies were located one copy  
95 for each of the two fused plasmid portions (13). The duplication of the similar transposon  
96 Tn4401b::*bla*<sub>KPC-2</sub> was described on the IncN plasmid S9 from *K. pneumoniae* in the US (17).

97 MICs for several antibiotics for KPC-*Kp* strains of ST512 and ST258 carrying *bla*<sub>KPC-3</sub> on  
98 pKpQIL (18), those of KPC-*Kp* 45 (ST512 and with IncX3), as well as those of their  
99 corresponding *E. coli* DH5 $\alpha$  transformants, were determined implementing the microdilution  
100 ESB1F and GNX2F plates (Trek Diagnostics) (Table 1). As result, we noted that the *E. coli*

101 DH5 $\alpha$  transformant carrying the IncX3 plasmid with two copies of Tn4401a-*bla*<sub>KPC-3</sub> showed  
102 significantly increased MICs for carbapenems, cephalosporins, and  $\beta$ -lactam/ $\beta$ -lactamase  
103 inhibitor combinations compared to the transformant carrying the classical pKpQIL plasmid.  
104 This phenomenon was not observed for the original *K. pneumoniae* strains of different ST and  
105 possessing different *bla*<sub>KPC</sub> genetic background. The MIC difference may be due not only to the  
106 double copy of the *bla*<sub>KPC</sub> and their level of gene expression (14), but also to IncX3 and pKpQIL  
107 different copy numbers.

108 In conclusion, our study describes the change of the typical plasmid content of the ST512  
109 *K. pneumoniae*: the worldwide described pKpQIL plasmid carrying *bla*<sub>KPC</sub> (19; GenBank:  
110 GU595196) was substituted by an IncX3 plasmids carrying two copies of the Tn4401a-*bla*<sub>KPC-3</sub>.  
111 The change of plasmid type in *K. pneumoniae* strains 45 could represent an important evolution  
112 of the ST512 lineage.

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196 **Legend to Figure 1. Major structural features of plasmids p45, pKpS90, and pIncX-SHV.**

197 Predicted open reading frames (ORFs) on plasmids are represented by white arrows. The ORFs  
198 of p45 were identified in this study; the ORFs of pKpS90, and pIncX-SHV were deduced from  
199 GenBank files JX461340 and JN247852, respectively. Transposase genes of the *Tn4401 Ia*  
200 transposons flanked by IRR and IRL inverted repeats (thick black lines) are indicated by grey  
201 arrows; the *bla*<sub>KPC-3</sub> genes inside *Tn4401 Ia* are indicated by black arrows. The DNA sequences  
202 of duplicated target sites are indicated above IRR and IRL. The *ygbK*, *IS3000* and *topB* genes  
203 targeted by *Tn4401-a* transposition events occurred in the different plasmids are indicated by  
204 arrows filled with stripes, dot and black squares, respectively.

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