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Genomic evolution of the globally disseminated multidrug-resistant

***Klebsiella pneumoniae* clonal group 147**

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Keywords: phylogenomics, pandrug resistance, last-resort antibiotics, high-risk clone, NDM-5, CRISPR/Cas systems, IncF plasmids, genomic epidemiology

Repositories: Reads and genomic sequences of the isolates analysed in this study were deposited at the European Nucleotide Archive (accession number PRJEB41234).

24 **ABSTRACT**

25 The rapid emergence of multidrug-resistant *Klebsiella pneumoniae* (Kp) is largely
26 driven by the spread of specific clonal groups (CG). Of these, CG147 includes 7-gene MLST
27 sequence types ST147, ST273 and ST392. CG147 has caused nosocomial outbreaks across
28 the world, but its global population dynamics remain unknown. Here, we report a pandrug-
29 resistant ST147 clinical isolate from India (strain DJ) and define the evolution and global
30 emergence of CG147. Antimicrobial susceptibility testing (EUCAST guidelines) and genome
31 sequencing (Illumina and Oxford Nanopore Technologies, Unicycler assembly) were
32 performed on strain DJ. Additionally, we collated 217 publicly available CG147 genomes
33 (NCBI, May 2019). CG147 evolution was inferred within a temporal phylogenetic
34 framework (BEAST) based on a recombination-free sequence alignment (Roary/Gubbins).
35 Comparative genomic analyses focused on resistance and virulence genes and other genetic
36 elements (BIGSdb, Kleborate, PlasmidFinder, PHASTER, ICEFinder and
37 CRISPRCasFinder).

38 Strain DJ had a pandrug resistance phenotype. Its genome comprised 7 plasmids and
39 1 linear phage-plasmid. Four carbapenemase genes were detected: *bla*_{NDM-5} and 2 copies
40 of *bla*_{OXA-181} in the chromosome, and a second copy of *bla*_{NDM-5} on an 84 kb IncFII
41 plasmid. CG147 genomes carried a mean of 13 acquired resistance genes or mutations; 63%
42 carried a carbapenemase gene and 83% harbored *bla*_{CTX-M}. All CG147 genomes presented
43 GyrA and ParC mutations and a common subtype IV-E CRISPR-Cas system. ST392 and
44 ST273 emerged in 2005 and 1995, respectively. ST147, the most represented phylogenetic
45 branch, was itself divided into two main clades with distinct capsular loci: KL64 (74%, DJ
46 included, emerged in 1994 and disseminated worldwide, with carbapenemases varying
47 among world regions) and KL10 (20%, 2002, predominantly found in Asian countries,
48 associated with carbapenemases NDM and OXA-48-like). Further, subclades within ST147-

49 KL64 differed at the yersiniabactin locus, OmpK35/K36 mutations, plasmid replicons and
50 prophages. The absence of IncF plasmids in some subclades was associated with a possible
51 activity of a CRISPR-Cas system.

52 *K. pneumoniae* clonal group CG147 comprises pandrug- or extensively-resistant
53 isolates and carries multiple and diverse resistance genes and mobile genetic elements,
54 including chromosomal *bla*_{NDM-5}. Its emergence is driven by the spread of several
55 phylogenetic clades marked by their own genomic features and specific temporo-spatial
56 dynamics. These findings highlight the need for precision surveillance strategies to limit the
57 spread of particularly concerning CG147 subsets.

58

59 **Data Summary**

60 Sequence read files and complete genome assembly of DJ have been deposited in European
61 Nucleotide Archive under the BioProject number PRJEB41234.

62 INTRODUCTION

63 The increasing number of antimicrobial resistant infections by *Klebsiella pneumoniae*
64 (Kp), especially by extended-spectrum beta-lactamases (ESBL)- and carbapenemase-
65 producing Kp, led to the declaration of Kp as an ‘urgent threat’ and ‘priority pathogen’ by
66 public health agencies (1,2). Molecular analyses of Kp isolates has evidenced that the rapid
67 emergence of multidrug resistant (MDR) Kp is largely driven by the geographic spread of
68 successful clonal groups (CG; e.g., CG15, CG101, CG147, CG258, CG307) (3), some of
69 them carrying epidemic resistance plasmids (4). In order to treat MDR Kp infections, last-
70 resort drugs such as polymyxins (especially colistin) and tigecycline are used (3,5).
71 Consequently, resistance is also observed to these last resort drugs, especially to colistin, and
72 may culminate in the emergence and spread of pandrug-resistant strains (5,6). Pandrug-
73 resistant Kp strains leave few or no therapeutic options and are associated with high mortality
74 rates (7–11).

75 The 7-gene MLST sequence type (ST) ST147 has been recognized as a globally
76 distributed antimicrobial resistance clone (12), and is closely related to ST273 and ST392,
77 which themselves comprise MDR isolates. Based on genomic classifications, these three STs
78 are grouped into the CG147 clonal group (13,14). The earliest studies of CG147 date from
79 2008-2010 in Hungary, and correspond to ciprofloxacin-resistant CTX-M-15-producing
80 ST147 isolates, which had been disseminating in the country since 2005 (15,16). Between
81 2010 and 2014, CG147 (mainly ST147) was described worldwide in association with several
82 carbapenemases (17). Most reported CG147 isolates are from clinical samples, although
83 some were found in companion animals, river waters, chimpanzees, poultry animals and
84 poultry environment (18–24). The above studies were locally restricted and so far, no study
85 of the global spread and genome dynamics of this clone was performed.

86 Here, we report a pandrug-resistant clinical isolate from India belonging to ST147,
87 and investigate the genomic evolution and antimicrobial resistance gene dynamics in the
88 global CG147 population. We also analyze the phylogenetic context of virulence-associated
89 genomic features, CRISPR loci and mobile genetic elements in this successful Kp sublineage.
90

91 **MATERIAL AND METHODS**

92 **Isolation and phenotypic and genomic characterization of strain DJ**

93 Isolate DJ was recovered from the urine of a 45-years old female patient diagnosed
94 with a urinary tract infection (UTI) in Vadodara (Gujarat, India) in October 2016. The isolate
95 was confirmed to be Kp by biochemical tests and 16S *rRNA* sequencing. Antibiotic
96 susceptibility tests performed using a semi-automated commercial system (Vitek,
97 BioMérieux) revealed resistance to all antibiotics tested. Confirmatory antimicrobial
98 susceptibility tests were carried out for colistin and tigecycline using broth dilution; and for
99 fosfomycin (agar supplemented with 25 mg/L glucose 6-phosphate sodium salt) using agar
100 dilution method. Disk diffusion method was used for the other antimicrobial classes
101 (penicillins, cephalosporins, carbapenems, monobactams, fluoroquinolones, aminoglycosides,
102 macrolides, tetracyclines, phenicols and inhibitors of the folic acid pathway). Results were
103 interpreted using both the Clinical and Laboratory Standards Institute (25) and the European
104 Committee on Antimicrobial Susceptibility Testing (2018) (<http://www.eucast.org/>)
105 guidelines.

106 DNA extraction was performed using XpressDNA Bacteria kit (MagGenome
107 Technologies Pvt Ltd., India). Whole-genome sequencing data were generated using (i) an
108 Illumina NextSeq-500 platform with a 2 x 150 nt paired-end protocol (Nextera XT library,
109 Illumina, San Diego, CA); and (ii) long-read Oxford Nanopore sequencing using MinION
110 device integrated with a FLO-MIN-106 flow cell and libraries prepared using a 1D ligation
111 sequencing kit (SQK-LSK109) following the protocol for 1D genomic DNA (gDNA) long
112 reads without BluePippin (Oxford Nanopore Technologies, New York, NY, USA). *De novo*
113 assemblies of the reads were obtained using SPAdes v3.12.0 (26) for Illumina data and using
114 Unicycler v0.4.4 (27) for hybrid assembly. Assembled sequences were annotated using

115 Prokka v1.12 (28). Reads and assembly were deposited at the European Nucleotide Archive
116 database (under the BioProject PRJEB41234).

117

118 **Global dataset of publicly-available genomic sequences**

119 All publicly available CG147 Kp genomes from NCBI RefSeq repository of genome
120 assemblies (May 2019) were downloaded. From the 245 CG147 genomes available,
121 duplicated (n=5) and poor-quality genomes (n=6; genome size and G+C content not matching
122 with Kp and/or more than >1000 contigs), and those without attached isolation year (n=17)
123 were excluded. The final dataset comprised 218 genomes, including strain DJ. Sample
124 information, accession numbers, and biological characteristics of the genomes are given in
125 **Table S1**.

126

127 **Phylogenetic analyses**

128 For phylogenetic analyses, a core-genome alignment based on the concatenation of
129 4,529 core genes was obtained using Roary v3.12 (29) using a blastP identity cut-off of 90%
130 and core genes defined as those being present in more than 90% of the isolates.
131 Recombination events were removed from the core-genome alignment using Gubbins v2.2.0
132 (30). The final recombination-free alignment comprised 8,450 single-nucleotide variants
133 (SNVs) and was used to construct a maximum-likelihood phylogenetic tree using IQ-TREE
134 v1.6.11 (model GTR+F+ASC+G4). The tree was rooted with a Kp ST258 NJST258_2
135 (accession number: GCF_000597905.1) and a Kp ST37 INF042 (GCF_002752995.1)
136 **(Figure S1)**.

137 To evaluate the strength of the temporal signal of our molecular phylogeny, we first
138 conducted a linear regression analysis of the root-to-tip genetic distances as a function of the
139 sample collection year, using TempEst v1.5.3 (<http://tree.bio.ed.ac.uk/software/tempest/>)

140 **(Figure S2)**. The final recombination-free alignment was then subjected to Bayesian
141 phylogenetic analysis using BEAST v2.6.1 (run with a Markov chain Monte Carlo length of
142 1×10^9 , sampling every 5×10^3 steps) (31). We used model parameters that had the best fit:
143 GTR substitution model, lognormal relaxed clock and constant population size. Parameters
144 estimates were computed using Tracer v1.7.1, and a maximum clade credibility tree was
145 obtained with TreeAnnotator v2.6.0. and visualized in FigTree v1.4.4.

146

147 **MLST and genomic analyses of resistance, virulence and other genetic elements**

148 Multilocus sequence typing (MLST, 7 genes) was performed using the Institut Pasteur
149 *Klebsiella* MLST (32) database (<https://bigsdB.pasteur.fr/klebsiella/>). Kleborate (33) and
150 BIGSdb analytical tools (<https://bigsdB.pasteur.fr/klebsiella/>) (34) were used to define the
151 presence of antimicrobial resistance, heavy metal tolerance, virulence genes and to
152 characterize the capsular gene cluster . Geneious Prime 2019.1.1 software
153 (<https://www.geneious.com>) was used for further manual curation of antibiotic resistance
154 genes, and ISFinder (<https://isfinder.biotoul.fr>) was used to look for the insertion sequences
155 in the resistance genes or in their genetic context. Plasmid replicons were detected using
156 PlasmidFinder (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>) (35), whereas prophages,
157 integrative and conjugative elements (ICEs) and CRISPRs were identified using PHASTER
158 (<https://phaster.ca>) (36), ICEfinder (<https://db-mml.sjtu.edu.cn/ICEfinder/ICEfinder.html>)
159 and CRISPRCasFinder (<https://crisprcas.i2bc.paris-saclay.fr/CrisprCasFinder/Index>) (37),
160 respectively. To depict co-resistance genotypes and plasmid networks, we constructed a
161 correlation matrix for binary variables (1, presence; 0, absence) using the ‘corr.test’ function
162 (Pearson method, which for a pair of binary variables compares to the Phi coefficient) from
163 the ‘corrplot’ R package. Significant correlations were visualized with the ‘corrplot’ function
164 from the same package. Statistical analyses to check the association of the different

165 categorical variables within the phylogeny groups were calculated using the χ^2 test (*P* values
166 of <0.05 were considered statistically significant).

167 RESULTS

168 Phenotypic and genomic features of pan-drug resistant strain DJ

169 Strain DJ was resistant to all tested antimicrobial agents, including last-resort
170 antimicrobials such as carbapenems, colistin, tigecycline and fosfomycin (**Table 1**), and is
171 therefore pandrug-resistant (6). To define its genetic mechanisms of resistance, a hybrid
172 complete genome assembly was produced (**Figure S3a**). The 5.7 Mb sequence was 56.9%
173 G+C-rich and made up of one chromosome and 6 circularized plasmids [123kb
174 IncFII(pKPX1); 57 kb IncR; 5.6 kb ColRNAI; 4.7 kb ColRNAI; 2.0 kb ColpVC; and 1.5 kb
175 ColMG828]. In addition, there were two non-circularized contigs: a 84 kb IncFII plasmid and
176 a 57 kb contig corresponding to a N15-like phage-plasmid (P-P) coding for a protelomerase
177 (*telN*) responsible for the maintenance of its linear genome (38). PHASTER identified four
178 other prophages within the chromosome. Phylogenetic analysis of strain DJ showed it
179 belonged to *K. pneumoniae sensu stricto* (*i.e.*, phylogroup Kp1) and to the ST147-KL64
180 lineage previously described as endemic in India (12).

181 The long-read sequencing followed by hybrid assembly of strain DJ allowed the
182 identification of multiple copies of unique antibiotic resistance genes in the different genomic
183 elements depicted (chromosomes and plasmids). Strain DJ harbored four carbapenemase
184 genes, corresponding to two copies of each *bla*_{NDM-5} and *bla*_{OXA-181}. Additionally, three copies
185 of *bla*_{CTX-M-15} were detected (**Table 1**). The above genes were localized as follows (**Figure**
186 **S3, Figure 1**): (i) *bla*_{NDM-5}: one copy in the chromosome, and the second copy in the 84 kb
187 IncFII plasmid; (ii) *bla*_{OXA-181}: two copies in the chromosome; and (iii) *bla*_{CTX-M-15}: two
188 copies in the chromosome and one copy in the 57 kb IncR plasmid. In addition, a 61 kb
189 region from the IncFII plasmid, comprising several antimicrobial resistance genes included in
190 a class 1 integron and the replication machinery of the plasmid, was duplicated in the
191 chromosome (**FigureS3a,c**).

192 Additional molecular determinants of antimicrobial resistance were observed in strain
193 DJ. Most notably, the gene *mgrB* was disrupted by an IS5 transposase (1057 bp), consistent
194 with the observed colistin resistance phenotype (MIC=4 µg/ml). This strain also carried *rmtF*,
195 *rmtB*, *strA*, *strB* and *aadA2* genes, associated with resistance to aminoglycosides including
196 amikacin (**Table 1**). Quinolone resistance determining region (QRDR) mutations were
197 observed, leading to GyrA-S83I and ParC-S80I amino acid alterations. Last, there was a
198 premature stop codon caused by a A580T substitution in RamR, a negative regulator of
199 RamA, itself a transcriptional activator of the *acrAB* genes. Higher production of AcrAB
200 increases the efflux of tigecycline (39), consistent with the resistance phenotype observed for
201 this agent (MIC=16 µg/ml).

202 Regarding virulence genes, strain DJ harbored a complete yersiniabactin gene cluster
203 (*ybt10*) located on an ICEKp4 mobile genetic element (**Figure S3a**). Type 1 (*fimAICDFGH*)
204 and type 3 (*mrkABCD*) fimbriae gene clusters were also observed, but none of the *rmpACD*,
205 aerobactin and salmochelin cluster genes, typically associated with hypervirulence, were
206 present.

207

208 **Table 1.** Antimicrobial susceptibility of *Klebsiella pneumoniae* strain DJ, and genes
 209 potentially conferring resistance.

Class and antimicrobial agent	Diameter (mm) or MIC (µg/ml)	Interpretation ¹	Associated resistance genes (copy number)
Beta-lactams			<i>bla</i> _{NDM-5} (2), <i>bla</i> _{OXA-181} (2), <i>bla</i> _{CTX-M-15} (3), <i>bla</i> _{TEM-1} (2), <i>bla</i> _{SHV-11} (1), disrupted <i>ompK35</i> , mutation in <i>ompK36</i>
Ampicillin	9	R	
Piperacillin	13	R	
Amoxicillin-clavulanic acid	11	R	
Ticarcillin-clavulanic acid	14	R	
Piperacillin-tazobactam	14	R	
Cefuroxime	11	R	
Cefotaxime	12	R	
Ceftazidime	15	R	
Cefepime	15	R	
Aztreonam	14	R	
Ertapenem	12	R	
Imipenem	14	R	
Meropenem	15	R	
Aminoglycosides			<i>rmtB</i> (1), <i>rmtF</i> (2), <i>aac(6')-Ib</i> (2), <i>aadA2</i> (3), <i>strAB</i> (2)
Amikacin	10	R	
Gentamycin	10	R	
Tobramycin	9	R	
Quinolones and Fluoroquinolones			<i>gyrA</i> and <i>parC</i> mutations
Nalidixic acid	11	R	
Ofloxacin	11	R	
Folate pathway inhibitors			<i>sul1</i> (3), <i>sul2</i> (1), <i>dfrA12</i> (3)
Trimethoprim/Sulfamethoxazole	19	R	
Trimethoprim	8	R	
Polymyxins			disrupted <i>mgrB</i>
Colistin	MIC=4	R	
Tetracyclines			<i>ramR</i> mutation ²
Tigecycline	MIC=16	R	
Phenicol			<i>catA2</i> (1), <i>catB</i> (2)
Chloramphenicol	12	R	
Other			
Fosfomycin	MIC=128	R	<i>fosA</i>
Rifampicin	NI	-	<i>arr-2</i> (2)
Macrolides	NI	-	<i>mphA</i> (1), <i>ermB</i> (1)

210 NI, not included in the antimicrobial susceptibility testing panel; R, resistant; ¹Interpretations were based on CLSI and
 211 EUCAST guidelines; ² Nucleotide mutation resulting in a premature stop codon.

212 **Time-scaled phylogenetic structure of the global population of sublineage CG147**

213 We investigated the evolutionary origins of strain DJ within the global diversity of
214 CG147 using 217 publicly available genomes of isolates collected between 2002 and 2018.
215 CG147 genomes were mainly isolated from human samples (90%, 196/218) and hospital
216 environment (6%, 12/218), mostly in European (40%), Southeastern Asia (17%), Southern
217 Asia (11%) and Northern America (10%) countries (**Figure 2**).

218 CG147 was deeply structured into three main branches (**Figure S1**), each
219 corresponding to a single MLST sequence type: ST147, ST273 and ST392; the two latter are
220 *tonB* variants of ST147. The number of genome-wide nucleotide substitutions was associated
221 with isolation dates (root-to-tip regression analysis: $R^2 = 0.1123$, **Figure S2**), enabling to infer
222 a time-scaled phylogeny (**Figure 3**). The evolutionary rate within CG147 was estimated at
223 1.45×10^{-6} substitutions/site/year (95% HPD, $1.12 \times 10^{-6} - 1.78 \times 10^{-6}$), corresponding to 6.2
224 SNPs per genome per year. The last common ancestor of CG147 was estimated around year
225 1896, with a large uncertainty (95% highest posterior density (HPD): 1817-1962). The ST273
226 lineage was the first to diverge, whereas ST147 and ST392 shared a common ancestor,
227 estimated around 1921 (95% HPD, 1868-1970) (**Figure 3**).

228 ST147 was the most represented (79%, 172/218 genomes) and geographically
229 widespread lineage (**Figure 2**), and emerged around 1965 (95% HPD, 1940-1986) (**Figure**
230 **3**). The phylogenetic structure within ST147 revealed five main clades characterized by
231 distinct capsular (KL type) and liposaccharide O antigen loci. Clades KL64-O2 (74%;
232 128/172) and KL10-O3a (20%, 34/172) emerged in recent years: 1994 [95% HPD, 1990-
233 1998] and 2002 (95% HPD, 1995-2008), respectively (**Figure 3**). Whereas KL64-O2
234 genomes were predominantly from Europe (54%), KL10-O3a was mainly sampled from Asia
235 (85%; **Figure 2**).

236 The ST392 and ST273 branches emerged in 2005 (95% HPD, 2001-2009) and 1995
237 (95% HPD, 1987-2001), respectively (**Figure 3**). ST392 (16% of genomes, 34/218) is
238 distributed globally and harbors a KL27 capsular gene cluster and a O4 antigen. In contrast,
239 ST273 (6%, 12/218) was predominantly found in Asia (64%) and carries KL74 and O3b gene
240 clusters (**Figure 2, Figure 3**). Of note, a group of closely related genomes from the
241 Philippines (n=5) lacked a capsular gene cluster, with only *ugd* being detected. This gene had
242 100% identity with the *ugd* gene from the KL74 reference strain, suggesting a recent loss of
243 the capsular gene cluster.

244

245 **Acquired antimicrobial resistance genes and their evolutionary dynamics within CG147**

246 All genomes presented QRDR alterations in GyrA and ParC. The topoisomerase ParC
247 S80I alteration was fully conserved, whereas the GyrA gyrase subunit S83I amino-acid
248 change was observed in all genomes except for clade ST147-KL10, which had S83Y, caused
249 by an ATC to TAC codon change. In addition, this clade had an D87A alteration (**Figure 3**).

250 The number of acquired antimicrobial resistance genes or mutations among CG147
251 genomes ranged from 2 to 23 (mean: 13; **Table S1**). Regarding beta-lactam resistance, in
252 addition to the conserved chromosomal *bla_{SHV-11}* gene, a majority of CG147 isolates carried a
253 *bla_{CTX-M}* (n=181, 83%), 94% of which was the *bla_{CTX-M-15}* variant. In addition, 63% (n=137)
254 genomes harbored at least one carbapenemase gene, with 14% (n=19) of these harboring
255 more than one copy of the same carbapenemase gene, and/or two or more carbapenemase
256 genes from different families (**Table S1**). Noteworthy, carbapenemase genes were
257 significantly more frequent in ST147 (69%) compared to ST392 (35%) and ST273 (58%;
258 $p=0.0005$). The two main ST147 clades were similar to this respect (ST147-KL10: 74%;
259 ST147-KL64: 71%; $p=0.78$). However, their carbapenemase genes were distinct across world
260 regions: there was a predominance of *bla_{NDM}* in Southeastern Asia and Northern America,

261 whereas the combination of *bla*_{NDM} and *bla*_{OXA-48-like} was almost exclusively detected in
262 Southeastern Asia, as observed in strain DJ. In contrast in Europe, *bla*_{OXA-48-like}, *bla*_{KPC-2} and
263 *bla*_{VIM-1/-27} were the most frequent carbapenemases (**Figure S4; Table S1**).

264 Different *ybt*/*ICEKp* subtypes (associated with hypervirulence) and OmpK35/K36
265 mutations (associated with multidrug resistance) were observed within the ST147-KL64
266 clade (**Figure 3; Table S1**). First, a group of 29 genomes (denominated subclade 1; mean
267 number of SNPs among them: 57), emerged around 2007, was characterized by the presence
268 of *ybt*16/*ICEKp*12 and an altered OmpK35 protein, due to a deletion of 2 nt resulting in a
269 premature stop codon. Within this subclade itself, a subgroup of genomes (n=19/29) harbored
270 *bla*_{OXA-48-like} genes and the OmpK36GD mutation observed previously (40). Second, a group
271 of 22 genomes emerged around 2009 (subclade 2; mean of 60 SNPs) was defined by the
272 presence of *ybt*10/*ICEKp*4 and an OmpK35 gene disrupted by *ISEcp1-bla*_{CTX-M-15} (**Figure 1,**
273 **Figure 3, Table S1**). As observed in subclade 1, all subclade 2 isolates carrying *bla*_{OXA-181}
274 shared the same OmpK36TD mutation (40). Subclades 1 and 2 also differed in plasmid
275 replicon content: whereas the former were rich in IncR (90%) and IncHIB/IncFIB(Mar)
276 (41%), in contrast IncFII (pKPX1) (82%), IncFII (59%) and IncR (64%) were frequent in the
277 latter (**Table S1**). Differently, the remaining ST147-KL64 genomes (n=77) often carried
278 IncFIB_K (65%), IncFII_K (49%; a common pKPN-3-derived plasmid found in Kp harboring
279 *pco* and *sil* clusters; (4,41) and IncFIA(HI1) (38%).

280 Strain DJ belonged to subclade 2 and was phylogenetically closely related (<28
281 SNPs) to five other isolates recovered between 2014 and 2015 in different Asian countries
282 (**Figure S3b; Figure3**) and described as extremely drug resistant or pandrug-resistant
283 (11,42,43), with identical plasmids being observed among them but no chromosomal
284 integration of *bla*_{NDM-5} (**Figure S3**).

285

286 **Convergence of antimicrobial resistance and virulence**

287 Whereas the yersiniabactin virulence factor gene cluster was rare amongst genomes of
288 ST392 (6%; 2/34) and ST273 (8%; 1/12), it was observed in 53% of ST147 genomes. There
289 were two predominant variants (*ybt16/ICEKp12* and *ybt10/ICEKp4* associated with subclades
290 1 and 2, respectively), and four minority ones (**Figure 3; Table S1**).

291 Two isolates with hypervirulence genotypes, defined by the presence of *rmpA* and/or
292 *rmpA2* or aerobactin, were observed: KpvST147L (ST147-KL14, 2016, United Kingdom)
293 and B-8658 (ST147-KL10, 2014, Russia). The plasmid from KpvST147L was fully
294 sequenced previously (GenBank accession number NZ_CM007852) and carries *rmpA*,
295 *rmpA2* and aerobactin; it corresponds to a 343 kb IncFIB-IncHIB (pNDM-MAR) plasmid
296 (**Figure S5**) (44). This plasmid was recently described in three ST147 *Kp* isolates recovered
297 in 2018-2019 in the United Kingdom and in other sublineages (45). Here, we found that
298 Russian strain B-8658 (ST147-KL10, 2014) also acquired the pNDM-MAR plasmid, but it
299 lacked the *rmpA* gene (**Figure S5**).

300

301 **Co-occurrence of antimicrobial resistance and heavy metal tolerance genes and** 302 **plasmids**

303 Antimicrobial resistance genes, mutations and plasmids co-occurred in a structured
304 way (**Figure S6; Table S1**). For example, Col(BS512), ColKp3, IncFIA (HI1), Cml, EreA/B
305 and GyrA S83Y/D87A co-occurred frequently within the ST147-KL10 clade. Associations of
306 (i) VIM, QnrA1, AadB and IncHI2; (ii) OXA-48-like and IncL(pOXA-48) or (iii) KPC and
307 IncFIB(pQIL) were also observed, consistent with previous descriptions of genetic elements
308 co-carrying these genes (46–48). In addition, genes conferring tolerance to copper and silver
309 were associated with IncFII_K/IncFIB_K plasmids (*pco* and *sil* operons; $p < 0.00001$), whereas
310 genes for mercuric resistance were linked to IncR plasmids ($p = 0.0082$). Similarly, a positive

311 association between the tellurite cluster and IncHIB/IncFIB (MAR) was observed ($p<0.0001$)
312 (4). Last, a negative association between IncR and IncFII_K/IncFIB_K was observed.

313

314 **CRISPR-Cas systems**

315 We investigated whether resistance gene dynamics could be influenced by CRISPR-
316 Cas systems in CG147. There were either one (83%; 180/218) or two (17%; 37/218)
317 CRISPR-Cas systems amongst CG147 genomes. A conserved subtype IV-E system was
318 found in all genomes, located in the *iap – cysH* region, with a %G+C of 60.6%, and defined
319 arbitrary as CRISPR1. Direct repeat sequences were highly conserved but the number and
320 sequences of spacers varied across CG147 genomes. CRISPR1 variant v0 (43 spacers) was
321 present in 56% of the genomes (122/218) and was used as reference to define CRISPR1
322 variants (**Figure S7; TableS2**). Four of the CRISPR1 v0 spacers (spacers 1, 28, 29 and 42)
323 matched sequences on MDR IncF plasmids disseminated among different
324 *Enterobacteriaceae*, including Kp. Spacer 1 matched a multicopy intergenic region, whereas
325 spacers 28 and 29 matched a DUF3560-domain containing protein and spacer 42 a
326 hypothetical protein located upstream of SAM-methyltransferase. Although these spacers
327 were highly conserved among CRISPR1 variants, they were also found at high frequency (in
328 28% to 53% of the strains) as protospacers located in plasmid contigs. Noteworthy, in 28%
329 (62/218) of CG147 genomes these plasmid protospacers were not detected, and the majority
330 of these strains (65%, 40/62) belonged to subclades 1 and 2, which were also characterized
331 by the absence of IncFII(K) and IncFIB(KpQIL) plasmid replicons. The association of the
332 lack of IncF plasmids with IncF-targeting spacers in CRISPR1, suggests a possible activity of
333 this CRISPR system in subclades 1 and 2. Finally, twelve of the forty-three spacers from
334 CRISPR1 v0 targeted prophages; of these, spacers 6 and 25 were found as protospacers in
335 some of the CG147 genomes (28% and 18%, respectively).

336 Six other CRISPR-Cas systems, distinct from CRISPR1, were observed in 37
337 genomes. CRISPR2 to CRISPR4, of type IV-A3, were strongly associated with IncHIB
338 and/or IncFIB (pNDM-MAR) plasmids ($p < 0.00001$), as previously described (49). Two
339 variants of CRISPR2 (v1; n=12; 17 spacers and v3; n=5, 25 spacers) were prevalent (**Figure**
340 **S7; TableS2**). CRISPR2 to CRISPR4 systems shared identical direct repeat sequences but
341 showed a high diversity in the number and sequences of spacers. Their tendency to target
342 IncFII_K/IncFIB_K plasmids has led to suggest a role in inter-plasmid competition (49,50).
343 However, here the presence of CRISPR2 to CRISPR4 systems was not uniformly associated
344 with an absence of IncFII_K/IncFIB_K plasmids.

345

346 **Prophage elements**

347 CG147 genomes harbored 0 to 7 prophages (mean of 4 prophages per genome;
348 considering only the intact ones). Some prophages were frequent, including (i) ST147-
349 VIM1phi7.1-like (GenBank accession no. NC_049451; *Myoviridae* family), which was
350 present in 90% of the genomes (196/218); (ii) *Salmonella* phage 118970_sal3-like (GenBank
351 accession no. NC_031940; *Myoviridae* family; 70% [152/218]); and (iii) *Enterobacteria*
352 phage mEp237-like (GenBank accession no. NC_019704; *Siphoviridae* family; 56%
353 [121/218]) (**Figure S7**).

354 The N15-like phage-plasmid (*Siphoviridae* family) we uncovered in the genomic
355 assembly of strain DJ was present in 37% of CG147 genomes (81/218). It was strongly
356 associated ($p < 0.00001$) with ST147-KL64 subclades 1 and 2 and present in 92% of these
357 genomes (**Table S1, Figure S7**). These two subclades were also enriched in other prophages
358 (mean number of prophages: 5) compared with the remaining CG147 genomes (mean
359 number of prophages=3).

360

361 **DISCUSSION**

362 This study was triggered by the discovery of a pandrug resistance phenotype in strain
363 DJ from India, which prompted us to analyzed its genomic features and understand their
364 dynamics in the context of a large genome dataset of CG147 from multiple world regions.
365 The results revealed its deep phylogenetic structure and a capacity of CG147 members to
366 acquire a wide range of antimicrobial resistance or virulence elements, plasmids and
367 prophages. Of these, *Klebsiella* capsular locus (KL) switches, which occurred repeatedly,
368 represent prominent phylogenetic markers of CG147 clades. The evolutionary dynamics of
369 these surface structure in CG147 echo those observed in other MDR clonal groups, such as
370 ST258 or ST307 (13,47,51). The multiple clonal expansions of sublineages with distinct KL
371 or O types has clear implications for diagnostic, control or therapeutic strategies such as
372 vaccination and phage therapy. Although its reliance on public genomic sequences may
373 expose this study to a bias towards antibiotic-resistant CG147 isolates, as well as those
374 involved in nosocomial outbreaks, this study provides new insights into the evolutionary
375 history, epidemiology and population dynamics of this important emerging clonal group.

376 The genomic arsenal of antimicrobial resistance features of subclade 2, to which strain
377 DJ belongs, suggest extensively drug and pandrug resistance is not restricted to strain DJ, but
378 rather is a shared characteristic in this particular subclade, already disseminated among Asian
379 countries (11,42,43,52–54). Clonal spread of this subclade between different countries was
380 reported (52). This subclade should be closely monitored and may represent a pioneering
381 situation ushering the worrying prospect of pan-resistance in other CG147 subclades.

382 In the case of strain DJ, we observed the integration of *bla*_{NDM-5} in the chromosome.
383 Previous studies have reported the one or multiple copies of *bla*_{CTX-M-15} and *bla*_{OXA-181}
384 integrated into the chromosome of Kp isolates (55,56), including in the high-risk ST147
385 (52,57). However, the chromosomal integration of *bla*_{NDM} was, to our knowledge, only

386 reported once in Kp, in two NDM-1 producing ST14 clinical isolates from Thailand (58). In
387 that case, the chromosomal integration was mediated by IS5 and the Tn3 transposase. In
388 strain DJ, the integration of *bla*_{NDM-5} may have been mediated by *ISEcp1*/IS26 (**Figure 1a**).
389 The genomic rearrangement due to the replicative transposition of IS26 has previously been
390 shown for IncFII-*bla*_{NDM-5} bearing plasmids (59). The chromosomal incorporation of
391 carbapenemase and ESBL genes is concerning, as it may stabilize these genes by promoting
392 their vertical dissemination (52).

393 The convergence of MDR and hypervirulence genotypes is being increasingly
394 observed in Kp (3). Here, this worrisome association was detected in two phylogenetically
395 distinct CG147 isolates from 2014 (Russia) and 2016 (United Kingdom), which shared an
396 MDR-Hv IncHIB/FIB plasmid (44). The recent observation of this plasmid in ST101 and
397 ST147 isolates from the United Kingdom (45), with no epidemiological link with the isolate
398 from 2016, suggests its continuous circulation and further risk of horizontal spread.

399 The ST147-KL64 lineage is globally disseminated. The evolutionary rate we
400 estimated (1.03×10^{-6} substitutions/site/year) is very similar to other MDR global sublineages,
401 such as ST258 (1.03×10^{-6} substitutions/site/year) and ST307 (1.18×10^{-6}
402 substitutions/site/year) (47,51), and slightly slower than the one estimated for ST101
403 (2.85×10^{-6} substitutions/site/year) (60). It is striking that the emergence of ST147-KL64
404 lineage occurred approximately at the same time as other MDR sublineages ST258 (year
405 1995), ST307 (1994) and ST101 (1989) (47,51,60). In addition, the presence of GyrA and
406 ParC QRDR alterations is a common characteristic to these MDR high-risk sublineages
407 [CG258 and ST307: GyrA-S83I and ParC-S80I; ST101: GyrA-S83I, GyrA-D87G/N/A and
408 ParC-S80I] (47,51,60). This phenotypic and temporal conjunction points to common drivers
409 and suggests a role of the usage of fluoroquinolones, introduced into clinical practice in the
410 end of the 1980s, in the emergence of MDR Kp sublineages. This is reminiscent of the

411 scenarios of emergence of *Escherichia coli* ST131 and methicillin-resistant *Staphylococcus*
412 *aureus* ST22 (61–63).

413 The drivers of genomic diversification of emerging Kp sublineages may include a
414 combination of ecological opportunities to acquire genetic elements, local selective pressure,
415 and molecular mechanisms that enable or restrict genetic flux. Of these, CRISPR-Cas
416 systems may play a role (64–67). In CG258, an association was suggested between the
417 absence of these systems and the ability to acquire IncF plasmids [such as *bla*_{KPC}-
418 IncF(pKpQIL-like plasmids)] (68–70). Here we found a conserved type IV-E CRISPR-Cas
419 system (CRISPR1) within CG147, consistent with early reports (9), with four spacers
420 matching IncF plasmid sequences. The distribution of CRISPR1 in the broader Kp species
421 shows a unique association with CG147, with only two exceptions (ST2746 and ST3700;
422 based on 1001 Kp genomes representing unique STs; selected from a dataset of 4222
423 genomes from NCBI, November 2018; data not shown). Type IV CRISPR-Cas systems
424 primarily target plasmids (50). However, in the majority of CG147 strains, corresponding
425 protospacers were found in plasmid sequences, advocating that the immunity provided by this
426 CRISPR1 system might not be fully functional. In contrast, ST147-KL64 subclades 1 and 2
427 were largely devoid of these protospacers and did not carry IncFIA, IncFII_K, IncFIB_K and
428 IncFIB (pQil) plasmids (**Figure S7**). This observation may suggest a possible activity of the
429 CRISPR1 system in these recently emerged subclades. Future experimental studies are
430 needed to explore this hypothesis. Noteworthy, it was also among these subclades that the
431 N15-like phage-plasmid was prevalent. Among bacterial phyla, this phage-plasmid family
432 was found almost exclusively in Kp (38), and we found that 58.9% of these belonged to
433 CG147. A possible biological role of plasmid-prophages has yet to be established.

434

435 **CONCLUSIONS**

436 The presence of pandrug-resistant and extremely-drug resistant isolates in CG147,
437 together with the high genetic plasticity and rapid emergence dynamics of this clone,
438 represents a clear threat to public health. CG147 is globally disseminated but shows a strong
439 phylogenetic structure, with different clades being associated with specific genomic features
440 and geographical distributions. These observations underline how different variants of
441 CG147 contribute to the major public health threat posed by Kp, and call for specific
442 surveillance and directed control strategies of this clone and its particularly concerning clades
443 (12).

444 A possible link between the absence of IncF plasmids and the activation of CRISPR1
445 defense system is intriguing. This observation calls for more work on mechanistic drivers of
446 the flux of genetic elements across MDR bacterial lineages. Precise phylogenetic mapping
447 and understanding of the dynamics of antimicrobial resistance features are needed to guide
448 the development of control strategies, especially those that target specific subsets of strains
449 within pathogenic bacterial species, such as CRISPR delivery or toxic conjugation systems
450 (71,72).

451 **DECLARATIONS**

452 **Ethics approval and consent to participate:** To conduct the research, we used bacterial
453 strain DJ, which is not considered a human sample. Accordingly, this research was not
454 considered human research and is out of the scope of the decree n° 2016-1537 of November
455 16, 2016 implementing law n° 2012-300 of March 5, 2012 on research involving human
456 subjects. Therefore, no ethics approval was needed and no informed consent was required.

457 **Consent for publication:** No personal identifying data was used; therefore, no consent was
458 necessary.

459 **Availability of data and materials:** All data generated or analysed during this study are
460 included in this published article and its supplementary information files.

461 **Competing interests:** The authors declare that they have no competing interests.

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468 **Author contributions:** S.D. and D.G. coordinated the microbiological cultures of the isolate
469 DJ and its antimicrobial susceptibility testing and biochemical characterization. V.P., C.R.
470 and S.D. performed the genomic sequencing. C.R. designed and coordinated the comparative
471 genomics study, with input from S.D. C.R. wrote the initial version of the manuscript. All
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478 Manuscript version arising from this submission.

479 **FIGURE LEGENDS**

480

481 **Figure 1.** Genetic context of the different copies of *bla*_{NDM-5} (**a**), *bla*_{OXA-181} (**b**) and *bla*_{CTX-M-15}
482 (**c**) genes identified in *K. pneumoniae* strain DJ.

483 Within each panel, the chromosomal and/or plasmid-encoded copies of *bla*_{NDM-5}, *bla*_{OXA-181}
484 and *bla*_{CTX-M-15} detected in DJ are compared between them. Predicted open reading frames
485 (ORFs) are represented on each line by colored arrows, with arrowheads indicating the
486 direction of transcription: antimicrobial resistance genes (red), mobile genetic elements
487 (MGEs) or gene mobilization-related genes (green); RNA-binding proteins (dark yellow);
488 other functions (light gray). Disrupted genes are outlined with dotted lines. Dark gray blocks
489 connecting the distinct gene regions represent homology levels, as indicated in the gradient
490 key. The nucleotide positions of the represented regions are indicated at the left below the
491 copy descriptors. Figures were created using the Easyfig (<https://mjsull.github.io/Easyfig/>).

492

493 **Figure 2.** Geographic (**main**) and temporal (**inset**) distribution of genomes included in this
494 study.

495 The pie charts represent the frequency of each CG147 clade in each country (see size and
496 color keys). **Inset:** Bars represent the number of isolates per year for which genome
497 assemblies were available (NCBI RefSeq) as of May 2019, coloured by clade. Red lines
498 represent (solid line) the number of PubMed-indexed records as of March 2020 (identified
499 using the search criteria '*Klebsiella pneumoniae*' and 'ST147' or 'ST392' or 'ST273',
500 resulting in a total of 99 distinct entries); and (dotted line) the cumulative number of
501 genomes. The scale on the left Y-axis refers to total number whereas the one on the right
502 refers to cumulative numbers.

503

504 **Figure 3.** Time-scaled phylogeny of 218 CG147 genomes and their epidemiological and
505 molecular characteristics.

506 The phylogeny was obtained using the BEAST tool. The three main branches correspond to
507 the three main sequence types (ST). Tree tips are colored by world region of isolation (see
508 key). Black dots on main nodes indicate $\geq 95\%$ posterior probability. The grey boxes
509 delineate subclades 1 and 2, as indicated. Capsular (KL) and O-antigen (O) locus types and
510 the yersiniabactin-carrying ICEKp elements are colored according to their variants as shown
511 in the legend. Antimicrobial resistance determinants are indicated by colored rectangles when
512 present. In the antimicrobial resistance determinants columns:¹ dark pink indicates *bla*_{KPC-2}
513 and light pink indicates *bla*_{KPC-3}; ² dark pink indicates *bla*_{CTX-M-15} and dark pink indicates other
514 *bla*_{CTX-M} variants; ³ dark pink indicates *mgrB* mutations and dark pink indicates *pmrB*
515 mutations. AMG: aminoglycosides; FPI: folate pathway inhibitors.

516 **Supplementary Data:**

517

518 **Figure S1. Phylogenetic structure of CG147.**

519 The tree was obtained by maximum likelihood analysis (IQ-TREE) based on the final
520 recombination-free alignment of concatenated nucleotide sequence alignments of 4,517 core
521 genes. The tree was rooted with a Kp ST258 NJST258_2 (GCF_000597905.1) and a Kp
522 ST37 INF042 (GCF_002752995.1). Branch lengths represent the number of nucleotide
523 substitutions per site (scale, 0.001 substitution per site). The three main branches are colored
524 by sequence type (ST) and branch tips are colored by world region of isolation (see key).
525 Capsular (KL) and O-antigen (O) locus types and the yersiniabactin-carrying
526 ICEKp elements are colored according to their variants (see key). Subclades 1 and 2 are
527 denoted by rectangle outlines.

528

529 **Figure S2. Temporal signal in genomic sequences.**

530 The linear correlation between year of isolation and root-to-tip distance from the maximum
531 likelihood phylogeny for CG147 genomes collection was calculated using TempEst.

532

533 **Figure S3. Replicons of strain DJ.**

534 (a) Visual representation of the hybrid Unicycler assembly graph, obtained using Bandage
535 (Wick et al., 2015). Nine replicons were obtained, including the circularized chromosome, 6
536 circularized plasmids, 1 linear phage-plasmid and 1 non-circularized plasmid contig. The size
537 of replicons is shown.

538 (b) Sequence alignments of the chromosomes of strain DJ and the four closest genomes
539 belonging to subclade 2 (<28 SNPs): MS6671 (GenBank Accession Number:
540 GCA_001455995), DA48896 (GCA_003006175.1); CRKP-1215 (GCA_002786755;

541 =CRKP-2297) and SKGH01 (GCA_001644765.1). Strain DJ was used as reference. The
542 outermost circle is an annotation of the reference strain showing the location of antimicrobial
543 drug resistance genes, ytb/ICEKp, KL64 capsular locus and the IncFII chromosomal
544 integration region. The figure was created using BRIG (Alikhan et al., 2011).

545 (c) Sequence alignments of the non-circular IncFII replicon of strain DJ with the closest
546 plasmids of subclade 2 strains: pCRKP-2297_2 (CP024836.1); pCRKP-1215_2
547 (CP024840.1), MS6671_plasmidE (LN824138.1), SKGH01_p3 (CP015503.1), p48896_1
548 (CP024430.1) and the region integrated in the chromosome of strain DJ. The IncFII non-
549 circularized replicon was used as reference. The outermost circle is an annotation of the
550 reference strain showing the location of antimicrobial drug resistance genes in red. In the
551 table the coverage, identity, size and antimicrobial resistance genes of the plasmids that were
552 used for the comparison. In bold the resistance genes also present in DJ replicon.

553 (d) Sequence alignments of the p48896_1 (CP024430.1) plasmid with the plasmids of the
554 closest strains: pCRKP-2297_2 (CP024836.1); pCRKP-1215_2 (CP024840.1),
555 MS6671_plasmidE (LN824138.1), SKGH01_p3 (CP015503.1), DJ-IncFII non-circularized
556 replicon and DJ-IncR plasmid. p48896_1 was used as reference plasmid. The outermost
557 circle is an annotation of the reference strain showing the location of antimicrobial drug
558 resistance genes in red.

559 (e) Sequence alignments of the 123 kb IncFII(pKPX1) plasmid of strain DJ with the closest
560 plasmids of subclade 2 strains: pCRKP-2297_1 (CP024835.1), pCRKP-1215_1
561 (CP024839.1), MS6671_plasmidB (LN824135.1), SKGH01_p2 (CP015502.1), p48896_2
562 (CP024431.1). The IncFII(pKPX1) plasmid of strain DJ was used as reference. The
563 outermost circle is an annotation of the reference strain showing the location of antimicrobial
564 drug resistance genes in red. In the table the coverage, identity, size and antimicrobial

565 resistance genes of the plasmids that were used for the comparison. In bold the resistance
566 genes also present in DJ replicon.

567

568 **Figure S4. Geographic origins of the genomes included in this study and their associated**
569 **carbapenemase profile.**

570 The pie charts represent the frequency of each carbapenemase profile in each country (see
571 key). CPKP-: no carbapenemase.

572

573 **Figure S5. Sequence alignments of MDR-Hv plasmids identified in CG147 genomes.**

574 Comparison of MDR-Hv plasmids identified in CG147 genomes (KpvST147L_NDM and B-
575 8658) with the reference virulence plasmid pK2044 (AP006726.1) and a similar plasmid
576 recovered in 2019 (pKpvST147B, CP040726.1), which was used as reference. The outermost
577 circle shows annotations of the reference strain.

578

579 **Figure S6. Correlation between genotypes (Pearson method).**

580 Blank squares represent correlations without statistical significance ($p > 0.05$). The plot was
581 created with the corrplot R package.

582

583 **Figure S7. Time-scaled phylogeny of CG147 genomes and their heavy metal tolerance**
584 **genes, plasmid replicons, CRISPR systems, and prophages profile.**

585 The phylogeny was obtained using the BEAST tool. The three main branches correspond to
586 the three main sequence types (ST). Tree tips are colored by world region of isolation (see
587 key). Black dots on main nodes indicate $\geq 95\%$ posterior probability. The grey boxes
588 delineate subclades 1 and 2, as indicated. Capsular (KL) and O-antigen (O) locus types and
589 the yersiniabactin-carrying ICEKp elements are colored according to their variants as shown

590 in the legend. The presence of heavy metal tolerance genes, plasmid replicons, CRISPR-Cas
591 systems, protospacers and prophages is indicated. In heavy metal tolerance genes columns,
592 lighter yellow indicates an incomplete operon. In prophages lighter orange indicates an
593 questionable prophage. HMTG, heavy metal tolerance genes.

594

595 **Table S1.** Epidemiological and genomic characteristics of the 218 CG147 *K. pneumoniae*
596 genomes analysed in this study.

597

598 **Table S2.** CRISPR/Cas systems and variants identified within CG147 *K. pneumoniae*
599 genomes.

600

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