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1 *Research Note*

2 **Bloodstream infection caused by KPC-producing *Klebsiella pneumoniae* resistant to**
3 **ceftazidime/avibactam: Epidemiology and Genomic characterization**

4 Paolo GAIBANI^{a*}, Maria Carla RE^{a,c}, Caterina CAMPOLI^b, PierLuigi VIALE^b, and Simone
5 AMBRETTI^a

6

7 ^aOperative Unit of Clinical Microbiology, S. Orsola-Malpighi University Hospital, Bologna, Italy; ^b
8 Operative Unit of Infectious Diseases. S. Orsola-Malpighi University Hospital; ^c University of
9 Bologna

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11

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13 mutation, porins-deficiency, *bla_{KPC}*/copy-number

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19 ***Corresponding Author:**

20 Paolo Gaibani, PhD

21 Operative Unit of Microbiology, S.Orsola-Malpighi University Hospital, Regional Reference
22 Centre for Microbiological Emergencies (CRREM), 9 via G. Massarenti – 40138 Bologna,
23 ITALY.

24 Telephone: +39 051 6364316

25 Fax: +39 051 6363076

26 e-mail: paolo.gaibani@unibo.it

27 **Abstract**

28 *Objective*

29 Aim of this study was to evaluate the incidence of ceftazidime/avibactam resistance among KPC-
30 producing *Klebsiella pneumoniae* (KPC-Kp) strains isolated from patients with bloodstream
31 infection.

32 *Methods*

33 We collected 120 carbapenemase producing *Enterobacteriaceae* (CPE) strains from unique patients
34 hospitalized in two Italian hospitals between January 2018 to February 2019. Strains were
35 phenotypically characterized for the type of carbapenemase production and susceptibility to
36 ceftazidime/avibactam. Ceftazidime/avibactam-resistant strains were characterized by whole-
37 genome sequencing.

38 *Results*

39 During the study period, we characterized 105 (87,5%) KPC producers among a total of 120 CPE
40 strains. Ceftazidime-avibactam resistance was found in three KPC-Kp strains isolated from patients
41 with no history of previous ceftazidime/avibactam-based treatment. Of note, two out of three
42 ceftazidime-avibactam-resistant KPC-Kp were also resistant to meropenem/vaborbactam. Genomic
43 characterization showed that a ceftazidime/avibactam-resistant KPC-Kp harbored a mixed
44 population with D179Y mutated KPC-2, while the other two ceftazidime-avibactam-resistant KPC-
45 Kp possessed non-functional ompK35-ompK37 and mutated ompK36 porins associated with higher
46 copy number of *bla_{KPC}* gene.

47 *Conclusion*

48 Our results showed that incidence of ceftazidime/avibactam resistance emerged in KCP-Kp strains
49 independently from previous antimicrobial exposure. Resistance to ceftazidime/avibactam was
50 associated to mutations within *bla_{KPC}* gene or porins deficiency associated to higher *bla_{KPC}* copy
51 number that is also related to the meropenem/vaborbactam resistance.

53 **Introduction**

54 Carbapenemase-producing *Enterobacteriaceae* (CPE) represent a serious threat for human health
55 and a serious challenge for clinicians. CPE are often resistant to several antimicrobial agents
56 commonly used for the treatment of Gram-negative infections. As a consequence, severe infections
57 due to CPE are associated to high of mortality and morbidity rates [1]. Recently, a novel β -lactam
58 combination, ceftazidime/avibactam (CAZ/AVI), was approved for treatment of infections due to
59 Gram-negative bacteria producing class A, class C and class D β -lactamase. Clinical studies
60 demonstrated that CAZ-AVI therapy exhibited higher rate of clinical success than other
61 antimicrobial regimens for bloodstream infections (BSIs), especially against KPC-producing
62 *Klebsiella pneumoniae* (KPC-Kp) bacteremia [2]. Although CAZ/AVI-based treatments exhibited
63 initial promising clinical efficacy for treatment of severe KPC-Kp infections, resistance
64 development has been recently reported [3-4]. Previous studies showed that development of
65 resistance often emerged during prolonged CAZ/AVI-based treatment [4-5] and pneumonia and
66 renal replacement therapy are independent risk factors for microbiological treatment failure and the
67 emergence of resistance [6]. CAZ/AVI resistance was associated to either different mutations of
68 KPC enzyme or hyperexpression of *bla_{KPC}* gene associated with nonfunctional porins [5-8].
69 Aim of this study was to evaluated the incidence of ceftazidime/avibactam resistance in KPC-Kp
70 isolates recovered from patients with bloodstream infections and characterize the mechanisms of
71 resistance.

72

73 **Methods**

74 Between January 2018 and February 2019, we collected all carbapenemase-producing
75 *Enterobacteriaceae* (CPE) strains isolated from BSIs of patients recovered into two large tertiary
76 Hospitals in Bologna metropolitan area. The study was conducted in accordance with the
77 Declaration of Helsinki. Samples were coded and analysis was performed with anonymized
78 database. Antimicrobial susceptibility testing was performed by Microscan, confirmed by MIC test

79 strip (Liofilchem, Italy), and MIC results were interpreted following EUCAST clinical breakpoints
80 v9.0. [9]. The types of carbapenemase were determined with NG-Test CARBA 5 (NG Biotech,
81 France) and confirmed with molecular assay (Xpert Carba-R, Cepheid) in doubtful cases.
82 Ceftazidime/avibactam activity was tested only against confirmed KPC-Kp and OXA-48 strains,
83 excluding KPC and OXA-48 or MBL co-producing strains. Whole-genome sequencing of KPC-Kp
84 resistant strains was performed to identify the genetic determinants responsible of
85 ceftazidime/avibactam-resistance. Briefly, genomic DNA was sequenced using the Illumina MiSeq
86 platform (Illumina, San Diego, USA) with a 2x250 paired-end run and assembled as previously
87 described [5]. Bacteria genomes were automatically annotated on the RAST server and
88 antimicrobial resistance genes were investigated against bacterial isolate genome sequence database
89 (BIGSdb) (<http://bigsdb.web.pasteur.fr>). Sequence type and allele frequencies was investigated as
90 previously described [5]. The core genome single nucleotide polymorphism (SNP) phylogeny was
91 performed as previously described [10]. Relative quantification of *bla*_{KPC} gene in comparison to
92 16S *rDNA* gene was performed in triplicate by quantitative real-time PCR (qPCR) from log-phase
93 cultures.

94

95 **Results**

96 During the study period, we isolated 120 CPE strains harboring different types of carbapenemase.
97 Phenotypic characterization revealed that vast majority (87.5%) were KPC producers, while 10%
98 were MBL and 2.5% were OXA-48 producers. Among different CPE, *K. pneumoniae* was the most
99 common species identified. The KPC-Kp distribution revealed that 53.4% of bacteremic patients
100 were hospitalized in Intensive Care Units (ICUs), 33.3% in medicine, 5.8% in haemato-oncology,
101 5% in surgical and 2.5% in transplantation wards.

102 Antimicrobial susceptibility rates of 105 KPC-Kp strains are shown in Figure S1 in the
103 Supplementary material. *In vitro* activity of ceftazidime-avibactam demonstrated that 102 (97.1%)
104 out of 105 KPC-Kp strains were susceptible, while 3 (2.9%) isolates were resistant. In detail,

105 median MIC of ceftazidime/avibactam-susceptible isolates was 2 µg/ml (interquartile range, IQR,
106 1-4), while median MIC for resistant strains was 32 µg/ml (IQR, 16-256) (Figure 1). The
107 ceftazidime/avibactam-resistant KPC-Kp strains were also resistant to carbapenems.

108 Interestingly, two out of ceftazidime/avibactam-resistant KPC-Kp strains were also resistant to
109 meropenem/vaborbactam (Table 1). Retrospective clinical data showed that 43 out of 105 patients
110 with BSI due KPC-Kp isolates were treated with CAZ/AVI-based treatment. At the same time, none
111 of the patients with BSI due to CAZ/AVI-resistant KPC-Kp strains received ceftazidime/avibactam
112 during antimicrobial therapy. CPE rectal screening demonstrated that patient with BSI due to
113 KpBO5 was colonized 19 days before, while the other two patients were not or colonized few days
114 before BSIs (Table 1).

115 A summary of the genetic characteristics of ceftazidime/avibactam-resistant KPC-producing *K.*
116 *pneumoniae* strains are shown in Table 1. Genetic analysis demonstrated that all KPC-Kp strains
117 belonged to the Clonal Complex (CC) 258, harbored the same genes coding for antimicrobial
118 resistance to fluoroquinolone and sulfonamide, while differed for genes responsible for
119 aminoglycoside resistance. Analysis of genetic determinants responsible for β-lactam resistance
120 showed that KPC-Kp strains harbored different β-lactamase gene patterns. Of note, two out of three
121 ceftazidime/avibactam-resistant KPC-Kp strains harbored a wild-type *bla_{KPC-3}* gene, while KpBO5
122 carried a SNP (*i.e.* 537 G>T) within *bla_{KPC-2}* genes, which determined an amino acid substitution at
123 position 179 (D179Y). In detail, reads alignment against *bla_{KPC-2}* gene revealed that KpBO5
124 genome had a mutant allele frequency of 45.7%. Genetic environment of *bla_{KPC}* gene demonstrated
125 that all three KPC-Kp strains harbored Tn4401 isoform a.

126 Analysis of outer membrane porin genes showed that ceftazidime/avibactam-resistant KPC-Kp
127 strains with wild-type *bla_{KPC}* had truncated *ompK35* and *ompK37*, and GD insertion at 134-135
128 aminoacid within *ompK36* and were also resistant to meropenem/vaborbactam (Table 1). At the
129 same time, ceftazidime/avibactam-resistant KPC-Kp strain with mutated KPC (*i.e.* KpBO5) had
130 wild type *ompK36* gene (Table 1).

131 Examination of plasmid content showed that ceftazidime/avibactam-resistant KPC-Kp strains
132 carried similar plasmid replicon types (*i.e.* IncFIB (K), IncFIB, IncX3, ColRNAI), while only
133 KpBO7 possessed additional plasmid replicon types [IncFII(K), Col(BS512) IncFIB (pQIL)].

134 Relative quantification analysis of *bla_{KPC}* gene demonstrated that copy number of porin-deficient
135 KpBO3 and KpBO7 strains were 2.5- and 1.5-fold higher rather than KPC-mutated KpBO5
136 isolates.

137 The phylogenetic tree of the KPC-Kp genomes showed KpBO3 and KpBO7 clustered closely and
138 formed a monophyletic group with KPC-Kp 27318 strain (Figure S1 in the Supplementary
139 Material). At the same time, KPC-Kp strain (e.g. KpBO5) harbouring D179Y mutation within
140 *bla_{KPC-2}* gene belongs to a different clade, thus suggesting no clonal relationship.

141

142 **Discussion**

143 Here we described the emergence of resistant KPC-Kp strains isolated from patients with BSI and
144 no history of previous antimicrobial exposure to ceftazidime/avibactam treatment. Our results
145 showed that resistance was associated to porins ompK35-ompK37 deficiency associated with an
146 ompK36 variant in two out of three ceftazidime/avibactam-resistant KPC-Kp strains. Interestingly,
147 porins deficiency was also related to meropenem/vaborbactam resistance while no others
148 mechanisms or mutations were found (*i.e.* IS5 insertion in the *ompK36* gene promoter region) [11].

149 At the same time, only one KPC-Kp strain resistant to ceftazidime/avibactam exhibited a specific
150 mutation within *bla_{KPC}* gene (e.g. D179Y). Similar finding was observed in a carbapenem-
151 susceptible ST307 KPC-Kp strain harboring mutated *bla_{KPC-2}* gene that emerged during
152 ceftazidime/avibactam treatment [12]. Although we observed a similar mutation, our original
153 assembly gave wild type *bla_{KPC-2}* and only successive reads alignment demonstrated the presence of
154 a mixed subpopulations harboring wild-type and polymorphic gene. These results are in accordance
155 with our previously findings [5] that demonstrated as two distinct subpopulations within a single

156 isolate determine a hybrid phenotype which result resistant both to carbapenems and
157 ceftazidime/avibactam.

158 Phylogenetic analysis demonstrated that porins-deficient KPC-Kp strains were closely related and
159 segregated into a monophyletic group with a *bla_{KPC-36}*-carrying KPC-Kp strain recently described
160 [13].

161 We observed that none of three patients with BSI due to resistant KPC-Kp strains emerged
162 following ceftazidime/avibactam-based treatment. Although these findings seem to be reliable for
163 porins-deficient strains it was doubtful for mutated-KPC strain. Therefore, considering the high
164 incidence of KPC-Kp strains in our country, it was plausible that patient acquired mutated-*bla_{KPC-2}*
165 during hospitalization and that mixed subpopulations emerged during colonization. Previous studies
166 demonstrated that mechanisms responsible to resistance includes specific mutations within *bla_{KPC}*
167 gene and that emerged after ceftazidime/avibactam treatment or hyperexpression of *bla_{KPC}* gene
168 associated with porins loss with no previously exposure to ceftazidime-avibactam [3-8,12].

169 A limitation of this study is that the expression of *bla_{KPC}* gene was not determined in CAZ/AVI-
170 resistant strains. However, our results demonstrated that an increased KPC copy number was
171 associated to CAZ/AVI resistance in porin-deficient KPC-Kp strains.

172 In conclusion, our data described the emergence of ceftazidime/avibactam-resistance in a country
173 endemic for KPC-Kp, reinforce the role of rectal CPE screening to prevent spreading of
174 ceftazidime/avibactam resistant strains and highlight the importance of susceptibility testing of
175 these new molecules.

176

177 **Transparency declarations**

178

179 **Conflict of interest**

180 We declare no conflict of interest

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186 **Author contributions**

187 PG developed the study, performed genotypic and data analysis, write the manuscript; CC and PLV
188 collected clinical data; SA and MCR supervised the study design and reviewed the manuscript.

189

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240

241

242 **Figure 1.** Distribution of ceftazidime/avibactam MICs of KPC-producing *K. pneumoniae* strains
243 isolated from bacteremic patients included in this study. Dotted line represents the trend line
244

Table 1. Phenotypic and genotypic characteristics of ceftazidime/avibactam-resistant KPC-producing *Klebsiella pneumoniae* strains

Isolate	Previous Rectal Swab (days before BSI)	Previous exposure to ceftazidime/avibactam	MIC ($\mu\text{g/ml}$)									ST	Genetic determinants				Porins			Plasmid replicons (Inc)	
			ETP	IPM	MEM	CRO	CAZ/AVI	MER/VAB	GEN	AMK	TGC		Beta-lactam	Aminoglycoside	Fluoroquinolone	Sulfonamide	<i>OmpK35</i>	<i>OmpK36</i>	<i>OmpK37</i>		
KpBO3	Negative	No	≥ 32	≥ 32	≥ 32		32	256	2	128	2	512	<i>bla_{KPC-3}</i>	<i>bla_{SHV-11}</i>	<i>aac(6')-Ib</i>	<i>oqxA, oqxB, aac(6')Ib-cr</i>	<i>sulI</i>	truncated at aa 38	GD insertion at aa 134-135	truncated	IncFIB (K), IncFIB(pKPHS1), IncX3, ColRNAI
KpBO5	19	No	≥ 32	≥ 32	≥ 32	≥ 32	16	0.032	2	64	2	258	<i>bla_{KPC-3}</i> †	<i>bla_{SHV-12}</i>	<i>aadA2, aph(3')-Ia, aac(6')Ib-cr</i>	<i>oqxA, oqxB, aac(6')Ib-cr</i>	<i>sulI</i>	truncated at aa 38	WT	truncated	IncFIIK, IncFIB(K), IncX3, ColRNAI
KpBO7	7	No	≥ 32	≥ 32	≥ 32	≥ 32	≥ 256	256	4	64	2	1519	<i>bla_{KPC-3}</i>	<i>bla_{TEM-1A}</i> <i>bla_{OXA-11}</i>	<i>aadA2, aph(3')-Ia, aac(6')Ib-cr</i>	<i>oqxA, oqxB, aac(6')Ib-cr</i>	<i>sulI</i>	truncated at aa 38	GD insertion at aa 134-135	truncated	IncFIB (pQIL), IncFIB (pKPSH1), IncFIB(K), IncFII(K), IncX3, ColRNAI, Col(BS512)

Abbreviations: ETP, Ertapenem; IPM, imipenem; MEM, meropenem; CRO, ceftriaxone; CAZ/AVI, ceftazidime/avibactam; MER/VAB, meropenem/vaborbactam GEN, gentamicin; AMK, amikacin; TGC, tigecycline; ST, sequence type; WT, wild type; BSI, bloodstream infection.

† heteroresistant subpopulation harboring D179Y mutation in *bla_{KPC-2}* gene

