Research Note

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

Paolo GAIBANI[a*], Maria Carla RE[a,c], Caterina CAMPOLI[b], PierLuigi VIALE[b], and Simone AMBRETTI[a]

[a] Operative Unit of Clinical Microbiology, S. Orsola-Malpighi University Hospital, Bologna, Italy; [b] Operative Unit of Infectious Diseases. S. Orsola-Malpighi University Hospital; [c] University of Bologna

Keywords: ceftazidime/avibactam-resistance, meropenem/vaborbactam-resistance, D179Y-mutation, porins-deficiency, bla_{KPC}/copy-number

Word count on the text: 1348

Number of Table: 1

Number of Figure: 1

[*]**Corresponding Author:**

Paolo Gaibani, PhD

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

Telephone: +39 051 6364316

Fax: +39 051 6363076

e-mail: paolo.gaibani@unibo.it

Abstract

Objective

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

Methods

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

Results

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

Conclusion

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

Introduction

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

Aim of this study was to evaluated the incidence of ceftazidime/avibactam resistance in KPC-Kp isolates recovered from patients with bloodstream infections and characterize the mechanisms of resistance.

Methods

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

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

Results

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

Antimicrobial susceptibility rates of 105 KPC-Kp strains are shown in Figure S1 in the Supplementary material. *In vitro* activity of ceftazidime-avibactam demonstrated that 102 (97.1%) 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 ß-lactam resistance
120 showed that KPC-Kp strains harbored different ß-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 Tn*4401* 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 KCP-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

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

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

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

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

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

Transparency declarations

Conflict of interest

We declare no conflict of interest

Funding

This work was supported by Italian Ministry of Health (Ricerca Finalizzata, Giovani Ricercatori, GR-2018-12367572).

Acknowledgements

We would like to thank bacteriologists involved in isolates collection.

Author contributions

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

References

1. Rodríguez-Baño J, Gutiérrez-Gutiérrez B, Machuca I, Pascual A. Treatment of Infections Caused by Extended-Spectrum-Beta-Lactamase-, AmpC-, and Carbapenemase-Producing Enterobacteriaceae. *Clin Microbiol Rev*. 2018; **31**: pii:e00079-17
2. Tumbarello M, Trecarichi EM, Corona A, De Rosa FG, Bassetti M, Mussini C, *et al*. Efficacy of Ceftazidime-Avibactam Salvage Therapy in Patients With Infections Caused by *Klebsiella pneumoniae* Carbapenemase-producing *K. pneumoniae*. *Clin Infect Dis*. 2019; **68**: 355-64.
3. Galani I, Antoniadou A, Karaiskos I, Kontopoulou K, Giamarellou H, Souli M. Genomic characterization of a KPC-23-producing Klebsiella pneumoniae ST258 clinical isolate resistant to ceftazidime-avibactam. *Clin Microbiol Infect*. 2019; **25**: 763.e5-763.e8.
4. Shields RK, Chen L, Cheng S, Chavda KD, Press EG, Snyder A, et al. Emergence of Ceftazidime-Avibactam Resistance Due to Plasmid-Borne bla(KPC-3) Mutations during Treatment of Carbapenem-Resistant *Klebsiella pneumoniae* Infections. *Antimicrob Agents Chemother*. 2017; **61**: pii: e02097-16.
5. Gaibani P, Campoli C, Lewis RE, Volpe SL, Scaltriti E, Giannella M, et al. *In vivo* evolution of resistant subpopulations of KPC-producing *Klebsiella pneumoniae* during ceftazidime/avibactam treatment. *J Antimicrob Chemother*. 2018; **73**: 1525-9.
6. Shields RK, Nguyen MH, Chen L, Press EG, Kreiswirth BN, Clancy CJ. Pneumonia and Renal Replacement Therapy Are Risk Factors for Ceftazidime-Avibactam Treatment Failures and Resistance among Patients with Carbapenem-Resistant *Enterobacteriaceae* Infections. *Antimicrob Agents Chemother*. 2018; **62**: pii: e02497-17
7. Nelson K, Hemarajata P, Sun D, Rubio-Aparicio D, Tsivkovski R, Yang S, et al. Resistance to Ceftazidime-Avibactam Is Due to Transposition of KPC in a Porin-Deficient Strain of *Klebsiella pneumoniae* with Increased Efflux Activity. *Antimicrob Agents Chemother* 2017; **61**: pii:e00989-17

8. EUCAST. Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0, 2019. http://www.eucast.org/clinical_breakpoints/
9. Errico G, Gagliotti C, Monaco M, Masiero L, Gaibani P, Ambretti S, *et al*. Colonization and infection due to carbapenemase-producing *Enterobacteriaceae* in liver and lung transplant recipients and donor-derived transmission: a prospective cohort study conducted in Italy. *Clin Microbiol Infect.* 2019; **25**: 203-9.
10. Humphries RM, Hemarajata P. Resistance to Ceftazidime-Avibactam in *Klebsiella pneumoniae* Due to Porin Mutations and the Increased Expression of KPC-3. *Antimicrob Agents Chemother.* 2017; **61**: pii: e00537-17
11. Sun D, Rubio-Aparicio D, Nelson K, Dudley MN, Lomovskaya O. Meropenem-Vaborbactam Resistance Selection, Resistance Prevention, and Molecular Mechanisms in Mutants of KPC-Producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2017; **61**: pii:e01694-17.
12. Giddins MJ, Macesic N, Annavajhala MK, Stump S, Khan S, McConville TH, et al. Successive Emergence of Ceftazidime-Avibactam Resistance through Distinct Genomic Adaptations in bla(KPC-2)-Harboring *Klebsiella pneumoniae* Sequence Type 307 Isolates. *Antimicrob Agents Chemother* 2018; **62**: pii:e02101-17.
13. Gaibani P, Ambretti S, Campoli C, Viale PL, Re MC. Genomic characterization of a *Klebsiella pneumoniae* ST1519 carrying a novel KPC variant (KPC-36) resistant to ceftazidime/avibactam. *J Antimicrob Chemother* 2019; In Press (Submitted).
14. Shields RK, Potoski BA, Haidar G, Hao B, Doi Y, Chen L, Press EG, Kreiswirth BN, Clancy CJ, Nguyen MH. Clinical Outcomes, Drug Toxicity, and Emergence of Ceftazidime-Avibactam Resistance Among Patients Treated for Carbapenem-Resistant *Enterobacteriaceae* Infections. *Clin Infect Dis*. 2016; **63**: 1615-8.

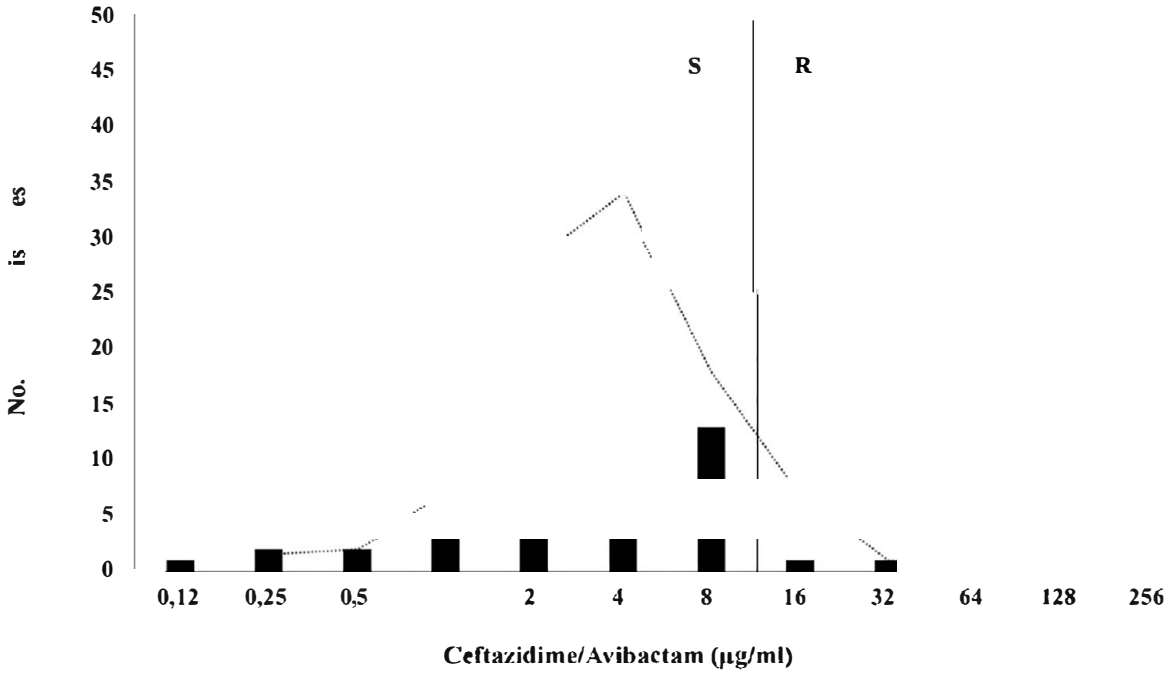
242 **Figure 1.** Distribution of ceftazidime/avibactam MICs of KPC-producing *K. pneumoniae* strains
243 isolated from bacteremic patients included in this study. Dotted line represents the trend line
244

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 (µg/ml)									ST	*bla*$_{KPC}$ allele	Genetic determinants				Porins			Plasmid_replicons (IncC)
			ETP	IPM	MEM	CRO	CAZ/AVI	MER/VAB	GEN	AMK	TGC			Beta-lactam	Aminoglycoside	Fluoroquinolone	Sulfonamide	*OmpK35*	*OmpK36*	*OmpK37*	
KpBO3	Negative	No	≥32	≥32	≥32		32	256	2	128	2	512	*bla*$_{KPC-3}$	*bla*$_{SHV-11}$	*aac(6')-Ib*	*oqxA, oqxB, aac(6')Ib-cr*	*sul1*	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	*bla*$_{KPC-3}$†	*bla*$_{SHV-12}$	*aadA2, aph(3')-Ia, aac(6')Ib-cr*	*oqxA, oqxB, aac(6')Ib-cr*	*sul1*	truncated at aa 38	WT	truncated	IncFIIK, IncFIB(K), IncX3, ColRNAI,
KpBO7	7	No	≥32	≥32	≥32	≥32	≥256	256	4	64	2	1519	*bla*$_{KPC-3}$	*bla*$_{TEM-1A}$, *bla*$_{OXA-}$ *bla*$_{SHV-11}$	*aadA2, aph(3')-Ia, aac(6')Ib-cr*	*oqxA, oqxB, aac(6')Ib-cr*	*sul1*	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


50
45
40
35
30
25
20
15
10
5
0
No. is es
S
R
0,12
0,25
0,5
2
4
8
16
32
64
128
256
Ceftazidime/Avibactam (µg/ml)