AKADÉMIAI KIADÓ

Acta Microbiologica et Immunologica Hungarica

68 (2021) 4, 235–239

DOI:
10.1556/030.2021.01626

RESEARCH ARTICLE

Ceftazidime/avibactam resistance is associated with different mechanisms in KPC-producing *Klebsiella pneumoniae* strains

SARA CAVALLINI, ILARIA UNALI, ANNA BERTONCELLI, RICCARDO CECCHETTO and ANNARITA MAZZARIOL*

Department of Diagnostics and Public Health, University of Verona, Verona, Italy

Received: October 10, 2021 • Accepted: October 22, 2021
Published online: November 5, 2021

ABSTRACT

This study focused on *Klebsiella pneumoniae* isolates that were resistant or had low susceptibility to a combination of ceftazidime/avibactam. We aimed to investigate the mechanisms underlying this resistance. A total of 24 multi-drug resistant isolates of *K. pneumoniae* were included in the study. The phenotypic determination of carbapenemase presence was based on the CARBA NP test. NG-Test CARBA 5 was also performed, and it showed KPC production in 22 out 24 strains. The molecular characterisation of bla_{KPC} carbapenemase gene, ESBL genes (bla_{CTX-M}, bla_{TEM}, and bla_{SHV}) and porin genes *ompK35/36* was performed using the PCR. Finally, ILLUMINA sequencing was performed to determine the presence of genetic mutations.

Various types of mutations in the KPC sequence, leading to ceftazidime/avibactam resistance, were detected in the analysed resistant strains. We observed that KPC-31 harboured the D179Y mutation, the deletion of the amino acids 167–168, and the mutation of T243M associated with ceftazidime/avibactam resistance. The isolates that did not present carbapenemase alterations were found to have other mechanisms such as mutations in the porins. The mutations both on the KPC-3 enzyme and in the porins confirmed, that diverse mechanisms confer resistance to ceftazidime/avibactam in *K. pneumoniae*.

KEYWORDS

ceftazidime-avibactam resistance, KPC mutations, OmpK35-Ompk36 mutations

INTRODUCTION

During the past decade, carbapenemase-producing *Enterobacteriaceae* (CPE) have spread worldwide. The initial clinical studies demonstrated that ceftazidime/avibactam is effective for the treatment of infections caused by carbapenem-resistant *Enterobacteriaceae* (CRE). However, the emergence of ceftazidime/avibactam resistance in CPE has recently been reported [1]. Mortality rates among patients with serious CRE infections are as high as 70% [2]. The carbapenem-resistant *Klebsiella pneumoniae* strain is associated with high morbidity and mortality and is one of the most serious clinical threats to human health [3]. The most common determinants of carbapenem resistance are the KPC-2 and KPC-3 enzyme variants [2]. Recent studies identified the presence of mutations such as D179Y or plasmid-borne bla_{KPC-3}, resulting in an impact on ceftazidime/avibactam resistance and causing comparable reductions in meropenem MICs [2]. An additional mutation reported in the bla_{KPC3} gene is the deletion of six nucleotides in the position 498–503, resulting in a mutant variant with the deletion of glutamic acid and leucine at position 167 and 168 [4]. This study aimed to investigate the resistance mechanisms underlying ceftazidime/avibactam resistant strains isolated in same hospital setting.

*Corresponding author.
E-mail: annarita.mazzariol@univr.it

AKJournals

MATERIALS AND METHODS

Strains

Twenty-four *K. pneumoniae* strains that were resistant to or showed low susceptibility to ceftazidime/avibactam and showed resistance to carbapenems were included in the study. The strains were isolated from various infection sites during routine clinical analyses from 2018 to 2020.

Antimicrobial susceptibility

Antimicrobial susceptibility testing was performed using the broth microdiluition method. The antibiotics tested in this study were cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, ertapenem, colistin, and the new drug combination ceftazidime/avibactam. An ETEST was performed to confirm the MIC values of ceftazidime/avibactam resistant strains. The results were interpreted following the last EUCAST clinical breakpoint guideline (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_10.0_Breakpoint_Tables.pdf).

Detection of beta-lactamases

The CARBA NP test [5] is used for the rapid detection of carbapenemase production in Enterobacterales. The NG-Test CARBA 5 (NG Biotech, Guipry, France) has been used for the rapid detection of the five most widespread carbapenemase families: KPC, NDM, VIM, IMP, and OXA-48-like enzymes.

The molecular characterisation of the carbapenemase gene bla_{KPC} [6] and the ESBL genes ($bla_{CTX\text{-}M}$, bla_{TEM}, and bla_{SHV}) [7] was performed via PCR.

The Porin *ompK35* and *ompK36* genes were detected using the following primers: ompK35 fw: CGCAATATTCTGGCAGTGGT, ompK35rv: GAACTGGTAAACGATACCCACG, ompK36fw: AGTTAAAGTACTGTCCCTCCTGG ompK36rv: TAGAACTGGTAAACCAGGCCC, that we designed for this study. The PCR conditions that we have set up were: 95 °C × 60 s, 54 °C × 60 s, 72 °C × 90 s for 30 cycles for *ompK35*, while the annealing temperature was 55 °C in the case of *ompK36* gene detection.

The PCR products were purified using a QIAquick PCR purification Kit (Qiagen, Germany) and then sequenced by Eurofins Genomics (Ebersberg, Germany). The derived sequences were analysed using the Basic Local Alignment Search Tool (BLAST) on the NCBI website to investigate the presence of mutations in the ESBL, carbapenemase, and porin genes that could explain the ceftazidime/avibactam resistance.

The molecular function, the active and substrate-binding sites, and the interactions were analysed by searching for the identified protein in UniProt. The Chimera 1.12 programme was used for the interactive visualisation and analysis of the molecular structures and associated data as well as the characterisation of the mutations.

RESULTS AND DISCUSSION

The MIC values for the 24 strains are reported in Table 1. All strains were observed to be resistant to cephalosporins and aztreonam. All 24 strains were resistant to at least one of three tested carbapenem antibiotics, i.e. imipenem, meropenem, and ertapenem. This resistance may be explained by the fact that all strains harboured the bla_{KPC} gene. A total of 19 of the 24 analysed strains were positive in the CARBA NP test. Moreover, the NG-Test CARBA 5 showed the production of KPC enzymes in 22 of the 24 strains.

Table 2 presents the molecular and phenotypic characteristics of the strains under study. All 24 of the analysed strains harboured the bla_{SHV} gene, 21 strains harboured the bla_{TEM} gene and 18 strains harboured the $bla_{CTX\text{-}M}$ gene (9 of which harboured the $bla_{CTX\text{-}M25}$ gene and 9 harboured the $ba_{CTX\text{-}M1}$ gene). None of the strains harboured carbapenemases except for KPC.

For all the strains, the bla_{KPC} gene was sequenced through ILLUMINA analysis. All KPC variants resulted in KPC-3 except the AMP 989 and AMP 2543 strains which presented the KPC-31 variant and the AMP 1572 strain which presented the KPC-2 variant.

The strains harbouring the KPC31 variant, as reported by Giani et al. [8], showed a negative result in the NG-Test CARBA 5, which was not capable of detecting this variant. KPC-31 is a D179Y variant of KPC-3. Moreover, it significantly reduces ceftazidime/avibactam susceptibility and typically behaves like ESBL. The mutation (D179Y) is relevant because the amino acids 163–179 include the Ω-loop that surrounds the active site of KPC [9, 10]. This mutation explains the inability of the KPC enzyme to hydrolyse the antibiotic imipenem in the CARBA NP test and confirms the hypothesis that the KPC enzyme is faulty. Furthermore, the mutation may explain the resistance of the strain to ceftazidime/avibactam because avibactam is unable to bind the KPC enzyme due to the mutation.

Unlike the previous case, the NG-Test CARBA 5 was positive for the AMP 1209 isolate, which indicates that the protein is expressed. However, the CARBA NP test was negative, thus, the protein does not function correctly. Through ILLUMINA sequencing, the presence of KPC-3 was highlighted and, in particular, was detected with the T243M mutation.

The T243M mutation observed in the AMP 1209 strain is relevant because the amino acids 240–243 are close to the hinge-loop that surrounds the active site of KPC [9]. Therefore, a similar situation to the previous case was present. This aminoacidic substitution may explain the negative result obtained in the CARBA NP test and may justify the resistance of the strain to ceftazidime/avibactam.

The analysis of AMP 1009 and AMP 1425 showed the presence of the KPC-3 variant. A mutation in the bla_{KPC} gene was confirmed in both strains and, in particular, a deletion of two amino acids (six nucleotides) at the position 167–168 in the KPC sequence was observed. This mutation

Table 1. Antimicrobial susceptibility of the strains under study selected to be resistant or with low level of susceptibility to ceftazidime/avibactam

strains	*CTX*	*CAZ*	*FEP*	*ATM*	*IPM*	*MEM*	*ETP*	*COL*	*CAZ-AVI*
920	64	>128	64	>128	32	64	>128	0.125	2
939	128	>128	128	>128	128	>128	<128	0.125	4
968	>128	>128	64	>128	64	128	>128	0.125	4
989	32	>128	128	32	0.5	2	32	0.125	>128
1009	4	>128	16	4	0.25	2	8	0.125	32
1209	16	>128	128	>128	16	16	32	0.125	64
1425	4	>128	16	4	0.25	4	16	8	64
1765	>128	>128	>128	>128	64	>128	>128	0.125	32
1764	>128	>128	>128	>128	32	128	>128	16	16
1767	>128	>128	>128	>128	128	>128	>128	0.125	16
1818	>128	>128	>128	>128	16	128	>128	0.125	16
1827	>128	>128	>128	>128	16	32	16	0.25	4
1546	>128	>128	>128	>128	16	16	32	0.125	8
1572	128	128	64	>128	4	8	32	0.125	2
1658	32	>128	128	>128	64	64	128	0.125	4
1714	>128	>128	>128	>128	8	16	16	0.25	8
1740	>128	>128	>128	>128	16	128	>128	0.25	16
1741	>128	>128	128	>128	8	8	16	0.125	8
1782	128	>128	>128	>128	128	128	>128	0.25	8
1795	64	<128	128	>128	16	64	>128	0.25	4
1822	32	>128	32	>128	4	8	8	0.25	2
1872	>128	>128	128	>128	8	8	16	0.25	8
1896	128	>128	>128	>128	4	>128	>128	0.25	32
2543	16	>128	128	32	0.5	2	16	0.125	64

CTX: cefotaxime, CAZ: ceftazidime, FEP: cefepime, ATM: aztreonam, IPM: imipenem, MEM: meropenem, ETP: ertapenem, COL: colistin, CAZ-AVI: ceftazidime/avibactam.

affects the proton acceptor active site located at position 167, which corresponds to one of the two amino acids deleted. The UniProt analysis of the carbapenem-hydrolysing β-lactamase KPC showed that the proton acceptor active site, composed of a glutamic acid (LDRWELELNS), is located at position 167, precisely at the site of the deletion [6].

All the strains showed a KPC enzyme mutation at the Ω-loop level. Substitutions in the KPC Ω-loop (amino acid positions 165–179) enhance the affinity for ceftazidime, which is postulated to prevent the subsequent binding of avibactam [9].

Omp35 and Omp36 allow the diffusion of avibactam across the outer membrane [11]. Porin research was performed on the isolates that were considered to be resistant according to the EUCAST clinical breakpoint, as well as the isolates that showed an MIC value of 8 μg ml^{-1} and did not have mutations in the bla_{KPC} gene.

The ILLUMINA sequencing analysis showed that at least one of the two porin genes for each strain was mutated or deleted. The mutation or deletion of the *ompK35/36* genes resulted in a significant increase in the MIC value of CAZ-AVI in *K. pneumoniae* [12]. The AMP 1896 strain did not present mutations in the *ompK36* gene, and PCR analysis showed negative results for the *ompK35* gene. The remaining strains showed mutations in at least one of the two porins (OmpK35 and OmpK36). AMP 1546 and AMP 1714 carried the non-functional OmpK35 porin and AMP 1741 carried a non-functional OmpK35 porin in addition to a mutated *ompK36* gene provided by a guanine insertion at position 448.

AMP 1782 showed a mutated OmpK35 porin from the amino acids 302 to 327. Moreover, the AMP 1872 PCR analysis result was negative for the *ompK35* and *ompK36* genes.

The strains that showed a ceftazidime/avibactam MIC value between 2 μg ml^{-1} and 8 μg ml^{-1} were considered for ESBL analysis. In particular, the bla_{SHV} genes were sequenced to detect mutation, as reported by Marisa et al. [12]; however, no mutations were detected. The strains with ceftazidime/avibactam MIC values between 2 μg ml^{-1} and 4 μg ml^{-1} did not show the onset of resistance mechanisms. It should be noted that the ceftazidime/avibactam EUCAST clinical breakpoint was 8 μg ml^{-1}.

CONCLUSIONS

The ceftazidime/avibactam resistance in this group of strains could not be attributed to a single mechanism; therefore, it does not seem to be clonal. With the increased use of ceftazidime/avibactam, it is expected that resistance will continue to emerge and plasmids carrying mutant genes may be disseminated via horizontal gene transfer [13]. Ceftazidime/avibactam resistance is an increasing phenomenon that must be monitored, and the strains that show resistance must be constantly examined to identify the mechanism underlying the resistance.

Table 2. Genetic and phenotypic characteristics related to carbapenemases, ESBL and porins for the strains under study

	Carbapenemase				ESBL		Porins			MIC (μg ml^{-1})
strain	*CarbaNP*	*Carba5*	*Gene*	*Mutation*	*Gene*	*Mutation*	*ompK35*	*ompK36*	*Mutation*	Ceftazidime/ avibactam
920	+	KPC	KPC3	no	SHV, TEM, CTX-M25	no	+	+	no	2
939	+	KPC	KPC3	no	SHV11,TEM, CTX-M25	no	+	+	no	4
968	+	KPC	KPC3	no	SHV11,TEM	no	+	+	no	4
989	−	−	KPC31	D179Y	SHV, TEM	no	nd	nd	\	>128
1009	−	KPC	KPC3	Deletion of 167–168 aa	SHV, TEM, CTX-M25	no	nd	nd	\	32
1209	−	KPC	KPC3	T243M	SHV	no	nd	nd	\	64
1425	−	KPC	KPC3	Deletion of 167–168 aa	SHV, TEM, CTX-M25	no	nd	nd	\	64
1765	+	KPC	KPC3	no	SHV, TEM, CTX-M25	no	+	+	Non functional *ompK35*	32
1764	+	KPC	KPC3	no	SHV, TEM, CTX-M1	no	+	+	Non functional *ompK35* and mutated *ompK36* (S147A, T154S, N189S, N190D, N272D, V291L)	16
1767	+	KPC	KPC3	no	SHV, TEM,	no	+	+	Non functional *ompK35*	16
1818	+	KPC	KPC3	no	SHV, TEM, CTX-M1	no	+	+	Non functional *ompK35* and *ompK36* mutated from 349 to 359 aa	16
1827	+	KPC	KPC3	no	SHV1, CTX-M1	no	+	+	no	4
1546	+	KPC	KPC3	no	SHV1, TEM, CTXM-1	no	+	−	Non functional *ompK35*	8
1572	+	KPC	KPC2	no	SHV1, TEM, CTX-M1	no	+	+	no	2
1658	+	KPC	KPC3	no	SHV11, TEM, CTX-M25	no	+	+	no	4
1714	+	KPC	KPC3	no	SHV1, TEM, CTX-M1	no	+	−	Non functional *ompK35*	8
1740	+	KPC	KPC3	no	SHV, TEM, CTX-M1	no	+	−	Non functional *ompK35*	16
1741	+	KPC	KPC3	no	SHV1, TEM, CTX-M1	no	+	+	Non functional *ompK35* and mutated *ompK36* (guanine insertion in position 448)	8
1782	+	KPC	KPC3	no	SHV11, TEM, CTX-M25	no	+	+	*ompK35* mutated from 302 to 327 aa	8
1795	+	KPC	KPC3	no	SHV11, CTX-M25	no	+	+	no	4
1822	+	KPC	KPC3	no	SHV33, TEM	no	+	+	no	2
1872	+	KPC	KPC3	no	SHV, TEM, CTX-M1	no	−	−	\	8
1896	+	KPC	KPC3	no	SHV, TEM, CTX-M25	no	−	+	no	32
2543	−	−	KPC31	D179Y	SHV, TEM	no	nd	nd	\	64

Nd = Not determined.
\ = Not examined.

Conflict of interests: None

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCES

1. 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.
2. Shields RK, Chen L, Cheng S, Chavda KD, Press EG, Snyder A, et al. Emergence of ceftazidime-avibactam resistance due to plasmid-borne *blaKPC-3* mutations during treatment of carbapenem-resistant *Klebsiella pneumoniae* infections. Antimicrob Agents Chemother 2017 Feb 23; 61(3): e02097–16.
3. Li D, Liao W, Huang H, Du F, Wei D, Mei Y, et al. Emergence of Hypervirulent Ceftazidime/Avibactam-resistant Klebsiella pneumoniae isolates in a Chinese tertiary hospital. Infect Drug Res 2020; 13: 2673–80.
4. Antinori E, Unali I, Bertoncelli A, Mazzariol A. *Klebsiella pneumoniae* KPC producer resistant to ceftazidime-avibactam due to a deletion in the bla_{KPC3} gene. Clin Microbioland Infect 2020; 66: 946e1–e3.
5. Nordmann P, Poirel L, Dortet L. Rapid detection of carbapenemase-producing *Enterobacteriaceae*. Emerg Infect Dis 2012; 18: 1503–7.
6. Mazzariol A, Lo Cascio G, Ballarini P, Ligozzi M, Soldani F, Fontana R, et al. Rapid molecular technique analysis of a KPC-3-producing *Klebsiella pneumoniae* outbreak in an Italian surgery unit. J Chemother 2012 Apr; 24(2): 93–6.
7. Dallenne C, Da Costa A, Decre D, Favier C, Arlet G. Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in *Enterobacteriaceae*. J Antimicrob Chemother 2010; 65: 490–5.
8. Antonelli A, Giani T, Di Pilato V, Riccobono E, Perriello G, Mencacci A, et al. KPC-31 expressed in a ceftazidime/avibactam-resistant *Klebsiella pneumoniae* is associated with relevant detection issues. J Antimicrob Chemother 2019; 74: 2464–6.
9. Winkler ML, Papp-Wallace KM, Bonomo RA. Activity of ceftazidime/avibactam against isogenic strains of *Escherichia coli* containing KPC and SHV beta-lactamases with single amino acid substitutions in the Omega-loop. J Antimicrob Chemother 2015; 70: 2279.
10. Haidar G, Clancy CJ, Shields RK, Hao B, Cheng S, Nguyena MH. Mutations in bla_{KPC-3} that confer ceftazidime-avibactam resistance encode novel KPC-3 variants that function as extended-spectrum beta-lactamases. Antimicrob Agents Chemother 2017; 61:1–6.
11. Wang Y, Wang J, Wang W, Cai Y. Resistance to ceftazidime-avibactam and underlying mechanisms. J Glob Antimicrob Res 2019; 1–35.
12. Winkler ML, Papp-Wallace KM, Taracila M, Bonomo RA. Avibactam and inhibitor-resistant SHV β-lactamases. Antimicrob Agents Chemother 2015; 59: 3700–9.
13. Rozwandowicz M, Brouer MSM, Fischer J, Wagenaar JA, Gonzalez-Zorn B, Guerra B, et al. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. J Antimicrob Chemother 2018; 73: 1121–37.