

## Original Article

**Ceftazidime-avibactam resistance determination in carbapenem-resistant *Klebsiella pneumoniae* infections before its use in practice**

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**Abstract**

**Introduction:** To ensure the appropriate usage of ceftazidime-avibactam (CAZ-AVI), recently introduced in our hospital, we aimed to determine susceptibility rates, enzyme analysis, and clonal relationship among strains, together with clinical data.

**Methodology:** Between June 1 and September 30, 2021, demographic and microbiological data of the patients were recorded. In the obtained samples, meropenem and colistin minimal inhibitory concentration (MIC) levels, carbapenem resistance genes, and the clonal relationship were studied by molecular methods. CAZ-AVI was not used in any of the patients.

**Results:** 140 carbapenem-resistant *Klebsiella pneumoniae* were isolated from 57 patients. Resistance to CAZ-AVI was found in 76 (54.3%) strains. Out of 57 patients, 31 (54.4%) isolates could be reached. Meropenem MIC level was  $\geq 32$   $\mu\text{g}/\text{mL}$  in 26 (83.9%), and colistin MIC level was  $\geq 4$   $\mu\text{g}/\text{mL}$  in 17 (54.8%) isolates. Enzyme analysis revealed NDM in 20 (64.5%), OXA-48 in 17 (54.8%), and KPC in seven (22.6%). NDM + OXA-48 was determined in 10 (32.2%) strains. NDM was determined in all CAZ-AVI resistant strains, OXA-48 in 16.1% (2/5) strains. Seven genotypes were detected. The largest cluster was genotype 3 clusters (11 isolates). Of 31 patients, 22 (71.0%) died. CAZ-AVI was susceptible in one of the patients who survived and four who died.

**Conclusion:** Before using a new antibiotic, each center should determine the basal data and phenotypic/genotypic resistance ratios specific to that antibiotic. While a high NDM rate and low CAZ-AVI sensitivity limit the use of the drug in our center, it is clear that CAZ-AVI use in sensitive strains will decrease mortality.

**Key words:** Ceftazidime-avibactam; carbapenem resistance; *Klebsiella pneumoniae*; NDM; AP-PCR.

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**Introduction**

Treatment of resistant Gram-negative bacterial (GNB) infections is difficult, and new antibiotics aim to contribute to the success of therapy in practice. Ceftazidime-avibactam (CAZ-AVI) is the first beta-lactam/beta-lactamase inhibitor combination recommended for treating carbapenemase-producing resistant GNB infections, which was introduced in Turkey in April 2021 [1]. The use of the drug in the treatment of resistant GNB infections, which were aminoglycoside-sensitive, was limited according to government procedures [2]. Currently, the CAZ-AVI therapy can only be started if the patient is in the intensive care unit (ICU) and can continue when the patient leaves the ICU. Still, it cannot be used in the wards for treatment. For this reason, to ensure the appropriate use of CAZ-AVI, which has just begun to

be used in our hospital, in the treatment of infections caused by carbapenem-resistant *Klebsiella pneumoniae* (CR-Kp), we aimed to determine the antibiotic susceptibility rates of strains, enzyme analysis, and clonal relationship between strains together with clinical data.

**Methodology***Study samples and data collection*

The study center is the second largest hospital in the province, with 1051 beds (105 intensive care beds). It is a university hospital where immunosuppressed patient groups (such as organ and bone marrow transplant recipients and oncology patients) are treated.

It is a retrospective cross-sectional study covering June 1 to September 30, 2021. Demographic data (age, gender, underlying diseases) of patients with CR-Kp

**Table 1.** Primers and amplification conditions used to search for carbapenemase genes.

Resistance Genes	Primers	Amplicon (bp)	PCR Heat Cycles
<i>blaOXA-48</i> [5]	A: 5'-TTG GTG GCA TCG ATT ATC GG-3'	438 bp	94 °C → 5 minutes, 30 cycles (94 °C → 1 minutes, 55 °C → 1 minutes, 72 °C → 1,5 minutes), 72 °C → 10 minutes
	B: 5'-GAG CAC TTC TTT TGT GAT GGC-3'		
	F: 5'-CAA TAT TAT GCA CCC GGT CG-3'		
<i>blaNDM</i> [6]	R: 5'-ATC ATG CTG GCC TTG GGG AA-3'	826 bp	94 °C → 5 minutes, 30 cycles (94 °C → 1 minutes, 55 °C → 1 minutes, 72 °C → 1,5 minutes), 72 °C → 10 minutes
	F: 5'-TGTCACTGTATCGCCGTC-3'		
	R: 5'-TATTTTCCGAGATGGGTGAC-3'		
<i>blaKPC</i> [7]	A: 5'-GAA GGY GTT TAT GTT CAT AC-3'	331 bp	94 °C → 5 minutes, 30 cycles (94 °C → 1 minutes, 54 °C → 1 minutes, 72 °C → 1 minutes), 72 °C → 10 minutes
	B: 5'-GTA MGT TTC AAG AGT GAT GC-3'		
	A: 5'-GTT TGG TCG CAT ATC GCA AC-3'		
<i>blaIMP</i> [8]	B: 5'-GTA MGT TTC AAG AGT GAT GC-3'	586 bp	94 °C → 5 minutes, 30 cycles (94 °C → 1 minutes, 53 °C → 1 minutes, 72 °C → 1 minutes), 72 °C → 10 minutes
	A: 5'-GTT TGG TCG CAT ATC GCA AC-3'		
	B: 5'-TCG GTC GAA TGC GCA GCA CC-3'		
<i>blaVIM</i> [8]	A: 5'-GTT TGG TCG CAT ATC GCA AC-3'	388 bp	94 °C → 5 minutes, 30 cycles (94 °C → 1 minutes, 57 °C → 1 minutes, 72 °C → 1 minutes), 72 °C → 10 minutes
	B: 5'-TCG GTC GAA TGC GCA GCA CC-3'		

isolated from cultures, length of hospital and ICU stay, culture samples (blood, urine, tracheal secret, CSF, bronchoalveolar lavage, tissue, abscess, catheter/catheter type) were recorded. One isolate was selected to represent each patient. The minimal inhibitory concentration (MIC) of meropenem and colistin was studied in accessible cultures. The molecular method investigated carbapenem resistance genes and the clonal relationship among strains in isolates whose MIC values were determined. There was no intervention in the patient's follow-up and treatment. CAZ-AVI was not used in any of the patients.

#### Identification of Isolates and Antibiotic Susceptibility Tests

Growing strains were identified by conventional methods and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Germany). The disk diffusion method determined the antimicrobial susceptibility of isolates according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Colistin sensitivity was studied with the colistin liquid microdilution kit (Sensititre™ FRCOL, Thermo Fisher Scientific, West. Sussex, UK) by the manufacturer's recommendations. Meropenem MIC values were determined by the broth microdilution method [3]. All susceptibility results were interpreted according to EUCAST criteria [4].

#### Identification of Carbapenem Resistance Genes

Carbapenemase genes of the isolates (*blaOXA-48*-like, *blaNDM*-like, *blaKPC*, *blaVIM*, and *blaIMP*) were investigated by polymerase chain reaction (PCR) method with the specific primers used in the literature and specified in Table 1 [5-8]. Isolates stored in Tryptic Soy Broth (TSB) containing 10% glycerol at -40 °C

were inoculated into a blood agar medium and incubated overnight at 37 °C. For the isolation of template DNA, several pure colonies were diluted in 300 µL sterile distilled water and centrifuged at 100 RPM for 5 minutes after 10 minutes of boiling at 100 °C. The supernatant formed after centrifugation was used as PCR template. Reaction mixtures for PCR were prepared in 50 µL, containing 25 pmol of *blaOXA-48* primer, 20 pmol of *blaNDM*, *blaKPC*, *blaVIM*, *blaIMP* primers, and 5 µL of template DNA. PCR conditions were as indicated in Table 1. PCR products were analyzed by agarose gel electrophoresis, and product sizes were evaluated.

#### AP-PCR

The arbitrarily primed-polymerase chain reaction (AP-PCR) method was used to determine the clonal relationship among isolates. This method was performed as described by Kuzucu *et al* [9]. DNA isolation was performed using the QIAamp DNA mini-Kit (Qiagen, Germany) and the QIASymphony SP Automated Nucleic Acid Purification System (Qiagen, Germany). DNA samples were kept at -80 °C until use. DNA amplification was performed using the Gene Amp™ PCR System 9700 thermal cycler (Applied Biosystems, USA) under the following conditions: 2 cycles at 94 °C for 5 minutes, 40 °C for 5 minutes, and 72 °C for 5 minutes; 40 cycles were performed at 94 °C for 1 minute, at 40 °C for 1 minute, and at 72 °C for 2 minutes. The amplified products were resolved on a 2% agarose gel at 120 V for 220 minutes. The gel was imaged and recorded using the Kodak Gel Logic 200 Imaging System (Eastman Kodak Company, Rochester, NY, USA). Band profiles were analyzed using the GelCompar II software system (version 6.5; Applied Maths, Sint-Martens-Latem, Belgium). The Dice correlation coefficient was used to calculate similarity

**Table 2.** Caz-avi susceptibility, meropenem and colistin MIC levels, resistance genes, and genotypes of the obtained isolates

	Sample	CAZ-AVI	Mer-MIC	Col-MIC	NDM	OXA 48	KPC	VIM	genotype	Prior Mer (+)	Prior Col (+)	Prior Quinolones (+)	STATUS
1	Blood	R	32	16,0	+	-	-	-	g3b	+	-	+	Alive
2	TAsp	R	16	1,0	-	+	-	-	g7	-	-	-	Ex
3	Blood	Sensitive	32	0,5	-	-	+	-	g6	-	-	-	Ex
4	Tissue	R	32	4,0	+	-	-	-	g3b	+	-	-	Alive
5	Urine	R	16	0,5	-	-	+	-	g1	-	-	+	Alive
6	Blood/catheter	R	32	8,0	+	-	-	-	3b	-	-	-	Alive
7	TAsp	R	8	1,0	-	+	-	-	g1	-	-	-	Alive
8	Blood	Sensitive	16	8,0	-	+	-	-	g4	-	-	+	Ex
9	Blood	R	32	0,5	+	+	-	-	g5	+	-	+	Ex
10	Blood	R	32	32,0	+	-	-	-	g5a	+	-	-	Ex
11	Urine	R	32	128,0	+	+	-	-	3b	+	-	+	Alive
12	Blood/catheter	R	32	2,0	+	+	-	-	g5	-	-	-	Ex
13	TAsp	R	32	0,5	+	+	-	-	g5	-	-	-	Ex
14	TAsp	Sensitive	4	8,0	-	+	-	-	g4	+	-	+	Ex
15	Blood	R	32	128,0	+	-	-	-	g3b	+	-	-	Ex
16	Blood	R	32	1,0	+	+	-	-	g5	-	-	+	Ex
17	Urine	R	32	4,0	+	+	-	-	g3	-	-	-	Alive
18	TAsp	R	32	1,0	+	-	-	-	g3	-	-	-	Ex
19	Blood/catheter	R	32	0,5	-	-	+	+	g2	+	-	+	Alive
20	TAsp	R	32	16,0	+	+	-	-	g3	-	-	+	Ex
21	Catheter	R	32	0,5	-	+	+	-	g4b	+	-	-	Ex
22	Blood	R	32	32,0	+	-	-	-	g3	-	-	-	Ex
23	Catheter	R	32	16,0	+	-	-	-	g3	+	-	-	Ex
24	Blood	R	32	8,0	+	-	-	-	g3	-	-	+	Ex
25	TAsp	R	32	32,0	+	+	-	-	g5	+	-	+	Ex
26	Blood	R	32	16,0	+	-	-	-	g1	+	-	-	Ex
27	Blood	R	32	16,0	+	+	-	-	g1	+	+	-	Ex
28	Blood	Sensitive	32	32,0	-	+	+	-	g4a	+	+	-	Ex
29	Blood	R	32	0,5	-	-	+	-	1b	-	-	-	Ex
30	Blood	R	32	0,5	+	+	-	-	g1	+	-	-	Ex
31	Blood	Sensitive	32	0,5	-	+	+	-	g6	-	-	+	Alive

TAsp: Tracheal aspirate, Mer: meropenem, Col: colistin.

for band analysis. UPGMA ("Unweighted Pairwise Grouping Mathematical Averaging" method of grouping unweighted pairs with mathematical mean) was used for cluster analysis [10].

*Statistical analysis*

Statistical analysis of the data was done with SPSS 15.0 program. CR-Kp growth in culture was defined as a dependent variable where age, gender, underlying disease, carbapenem, quinolone and colistin use, hospitalization, and intensive care admission were evaluated as independent variables.

*Ethical Approval*

The Ethics Committee of Dokuz Eylul University, Faculty of Medicine (Approval Date: 13/04/2022, No: 2022/14-04) approved the study.

**Results**

One hundred and forty CR-Kp were isolated from 57 patients and sent to the bacteriology laboratory between June and September 2021. Thirty-nine (68.4%) of the patients were male, and the median age was 71.00

± 16.59 (22-96). A total of 47 (82.5%) patients had comorbidities; cardiac disease in 31 (54.4%) patients, diabetes mellitus (DM) in 13 (22.8%), immunosuppressive disease in 19 (33.3%), chronic renal failure (CKD) in eight (14%), seven (12.5%) had an acute cerebrovascular disease, and six (10.5%) had a chronic neurological disorder. Fifty-four (94.7%) of the culture-positive patients were hospitalized. Of these patients, 47 (82.5%) were hospitalized in the intensive care unit (ICU). The median length of stay in the hospital was 31 days, while the median length in the ICU was 25 days. Culture growth was detected on the median ninth intensive care unit hospitalization day.

Of the cultures, 92 (65.7%) blood, 24 (17.1%) deep tracheal aspirate, 11 (7.9%) urine, six (4.3%) catheter tips, four (2.9%) cerebrospinal fluid (CSF), two (1.4%) were bronchoalveolar lavage (BAL), and one (0.7%) tissue sample. Growth was detected in both blood and non-blood samples in nine (15.7%) patients. And in at least one and at most eight blood and/or non-blood samples from one patient.

By disc diffusion method, resistance to gentamicin in 72 (52.9%), amikacin in 82 (58.6%), ciprofloxacin in

139 (99.3%), cefepime in 130 (92.9%), and trimethoprim-sulfamethoxazole in 134 (95.7%) and CAZ-AVI in 76 (54.3%) isolates were detected. One sample was selected to represent each patient from 31 (54.4%) patients whose isolates could be reached. In 22.1% (31/140) of all strains, meropenem and colistin MIC levels, carbapenem resistance genes, and the clonal relationship between strains by the molecular method were studied and presented in Table 2.

From the available 31 patients' cultures, meropenem MIC level was  $\geq 32$   $\mu\text{g/mL}$  in 26 (83.9%) of 31 strains, and colistin MIC level was  $\geq 4$   $\mu\text{g/mL}$  in 17 (54.8%) strains. While 20 (64.5%) NDM, 17 (54.8%) OXA-48, seven (22.6%) KPC, and one (3.2%) VIM were detected in carbapenem-resistant isolates. IMP type resistance gene was not detected in any of the strains. However, NDM + OXA-48 was detected in 10 (32.2%) isolates, and KPC + OXA-48 was detected in three (9.6%) isolates. In CAZ-AVI susceptible five strains, OXA-48 was determined in four (80.0%) strains, while OXA-48 + KPC in two and KPC in one.

Among the 31 CR-Kp isolates in which clonal association was investigated, seven different genotypes were identified, and the isolates were collected in five clusters (tolerance 1, optimization 1, cut off 90%). Thirty of the 31 CR-Kp isolates are in any cluster, with a clustering rate of 93%. The largest cluster is the genotype 3 cluster, with 11 isolates. Afterward, there are Genotype 1 and Genotype 5 with six isolates, genotype 4 with four isolates, genotype 6 with two isolates, and Genotype 2 and Genotype 7 clusters with one isolate each (Table 2).

In the previous period in which no CR-Kp growth could be detected, all 31 patients were treated with third and/or fourth-generation cephalosporin and/or piperacillin-tazobactam. Also, 15 (48.4%) patients were treated with carbapenem, three (9.7%) with colistin, and 12 (38.7%) with quinolone. Fungal infection was not detected in any of the patients. Out of 31, 22 (71.0%) patients died. Colistin MIC values were  $4 \leq \mu\text{g/mL}$  in six out of nine (29.0%) surviving patients, while meropenem MIC values were  $\geq 8$   $\mu\text{g/mL}$  (seven  $\geq 32$   $\mu\text{g/mL}$ ). CAZ-AVI was susceptible in one of the patients who survived and four who died.

## Discussion

This study emphasized the importance of knowing the host, microorganism, characteristics, and regional resistance data before CAZ-AVI came into use. CAZ-AVI susceptibility was 45.7% in the study population, and NDM was found in 64.5% of the isolates, OXA-48 in 54.8%, and NDM + OXA-48 resistance gene in 32.2%

of the isolates. In numerous centers, including ours, detecting the gene encoding the enzyme that causes resistance for all factors and/or studying genotypic analyses in routine is difficult. The procedures to be applied are evaluated within the possibilities. When necessary, considering the patient's characteristics with the cooperation of the clinician and laboratory team, non-routine procedures (such as susceptibility tests of new antibiotics and enzyme analysis) will contribute positively to patient mortality and morbidity. While the high NDM rate and low CAZ-AVI sensitivity limit the use of the drug, the fact that the strains were found to be sensitive to CAZ-AVI in four of the patients who died also indicates that mortality can be reduced if the drug is used in appropriate patients. In the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and IDSA multi-drug resistant GNB infections guidelines, CAZ-AVI is recommended in treating CR-Cp if it is sensitive in vitro [11,12].

The patient population referred to our center is elderly (median 71 years), with high comorbidity (82.5%). Age and comorbidity are risk factors for resistant GNB infections, even before encountering the microorganism. Studies have reported that the frequency of multi-drug resistance increases with age and comorbidities [13,14]. In addition, extensive and intensive antibiotic use in all patients, hospitalization for approximately one month, and ICU length of stay of twenty days contribute to developing infection with CR-Kp. When intensive antibiotic use, mechanical ventilation, and long hospital and ICU stays are added to unchangeable host characteristics, the risk of resistant *K. pneumoniae* infections increases, and their treatment becomes more difficult [15]. It is tried to be resolved with antibiotic combination treatments. However, the fact that all the strains in our study were resistant to carbapenems, and the lowest rate of resistance to gentamicin (52.9 %) and the highest rate of resistance to quinolone (99.3%) among other antibiotics further reduced the treatment alternatives.

In a study conducted in 18 centers, including ours, in 2014, during which microorganism-specific resistance properties were investigated, it was reported that OXA-48 was found in 83.1%, NDM in 6.5%, and OXA-48 + NDM in 2.4% of CR-Kp strains [16]. Subsequently, in 2017, 26 hospitals from Turkey participated in the European Antimicrobial Resistance Genes Surveillance Network (EURGen-Net) project conducted by the European Center for Disease Prevention and Control (ECDC). And 74.3% of the strains were *K. pneumoniae*, and the resistance to any carbapenem was 58.7%. At least one carbapenemase

resistance gene was found in all resistant strains; 52.2% of the isolates were OXA-48, 16.4% KPC, 15% NDM, 0.5% VIM, 12.6% OXA-48 + NDM-1, 2.8% KPC+NDM-1 and 0.5% OXA-48 + VIM [17]. Although these data show that OXA-48 is the predominant carbapenemase enzyme in our country, we found NDM in 64.5%, OXA-48 in 54.8%, and NDM+OXA-48 in 32.2% of the strains which carbapenemase gene was examined in our study. These results indicate that there may be regional or interhospital differences and an increase in NDM metallo-beta-lactamase type. Identifying the molecular characteristics of resistance in isolates will reduce treatment failure and mortality. For instance, while CAZ-AVI is used effectively in treating KPC and OXA-48 positive resistant isolates, it is not an alternative treatment in class B beta-lactamases positive strains such as VIM and NDM because it is ineffective, and MIC levels are also high. The literature has reported that carbapenem genes are associated with an increase in meropenem MIC level and vary according to the enzyme type. The mean MIC value associated with each of KPC and NDM has been reported to be  $> 8 \mu\text{g/mL}$  for resistance [18]. In our study, all were  $\geq 32 \mu\text{g/mL}$ . In addition, 83.3% (5/6) of KPC-positive strains were susceptible to colistin and 50.0% (3/6) to CAZ-AVI, indicating that these antibiotics may be an alternative treatment. However, 30% (6/20) of NDM-positive strains were susceptible to colistin, and none were sensitive to CAZ-AVI, limiting the treatment options (Table 2).

The study's clonal relationship in the strains was investigated using the AP-PCR (PCR-based DNA fingerprinting method). The detection of seven different genotypes, the collection of isolates in five clusters, the differences in susceptibility patterns, and enzyme analysis show that *K. pneumoniae* strains are heterogeneous and there is no clonal relationship. However, the detection of genotype-3 clustering in 35.4% (11/31) of the patients in whom the clonal relationship was investigated highlights the necessity of monitoring for healthcare-associated infections. Although Pulsed Field Gel Electrophoresis (PFGE) is still the gold standard genotyping method for many microorganisms, AP-PCR stands out as a fast and reliable method for investigating the spread of healthcare-associated infections and taking precautions [19,20].

The study's limitations are its retrospective design, the inaccessibility of all resistant strains, and the ability to perform enzyme analysis and genotyping for only one strain from each patient. However, this study contributes to determining the appropriate use of the drug and the resistance rate before CAZ-AVI enters into

practice. Because if the isolate is susceptible to CAZ-AVI, morbidity and mortality will decrease.

## Conclusions

As a result, before a new antibiotic such as CAZ-AVI is used, each center should determine the basal data and phenotypic/genotypic resistance ratios specific to that antibiotic. While high NDM rate and low CAZ-AVI sensitivity limit the use of the drug in our center, it is clear that mortality will decrease if the strains are found sensitive to CAZ-AVI and the drug is used. Using the medicine in centers where the OXA-48 resistance gene is dominant will further reduce mortality. These data highlight the need to monitor resistance and the epidemiology of resistance genes. Continuing epidemiological monitoring of resistance and resistance genes after CAZ-AVI is used will ensure the correct drug positioning.

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OÖEK and VAO proposed the concept, designed the study, wrote the protocol, and managed the study. OÖEK, HCC, ANSK, EST, and VAO performed the statistics, interpreted the data, and wrote the manuscript. ANSK, EST, ÖAÖ, BO, and İMAÖ were involved in microbiological testing. OÖEK, ÖAÖ, BO, İMAÖ, and VAO critically reviewed the manuscript. All authors provided input for the revision of the manuscript. OÖEK communicated with the journal and addressed comments from reviewers. All authors contributed to data acquisition, analysis, and interpretation and reviewed and approved the final version.

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