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In Vitro Efficacy of Ceftazidime-avibactam Against *bla*$_{OXA-48}$-producing *Klebsiella pneumoniae* Isolates

bla$_{OXA-48}$ Üreten *Klebsiella pneumoniae* İzolatlarına Karşı Ceftazidime-avibaktamın *İn Vitro* Etkinliği

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Abstract

Introduction: The healthcare burden of carbapenem-resistant *Klebsiella pneumoniae* (*K. pneumoniae*) infections is growing. The newly developed beta-lactam/beta-lactamase inhibitor combination, ceftazidime-avibactam, shows promise in the treatment of such infections. We aimed to explore the *in vitro* efficacy of ceftazidime-avibactam against carbapenem-resistant *K. pneumoniae* isolates carrying the *bla*$_{OXA-48}$ gene.

Materials and Methods: The isolates were identified using MALDI-TOF MS (Brucker, USA). The isolates that were non-susceptible to imipenem, meropenem, or ertapenem by the disk diffusion method using the European Committee of Antimicrobial Susceptibility Testing (EUCAST) breakpoints were screenes. Minimum inhibitory concentration (MIC) values were determined via broth microdilution according to the EUCAST criteria. A time-kill study was performed according to Clinical and Laboratory Standards Institute guidelines. Beta-lactamase genes were screened for using polymerase chain reaction with previously published primers.

Results: A total of 129 *K. pneumoniae* isolated between April 2011 and February 2021 were studied. Of these, 98, 23, and eight isolates carried the *bla*$_{OXA-48}$, *bla*$_{NDM}$, and *bla*$_{OXA-48}$ with *bla*$_{NDM}$ genes, respectively. All isolates carrying the *bla*$_{NDM}$ gene were resistant to ceftazidime-avibactam. Approximately 79.6% of the *bla*$_{OXA-48}$-positive isolates were susceptible to ceftazidime-avibactam. The time-kill study for ceftazidime-avibactam was performed with one *bla*$_{OXA-48}$-positive isolate (MIC, 4 mg/l). Ceftazidime-avibactam time-kill kinetics were evaluated in multiples of MIC. There was a decrease of ≥3-log10 in CFU/ml count at a concentration of 8, 16, and 32 MIC at 6 hours. The minimum bactericidal concentration was 8 mg/l.

Conclusion: Ceftazidime-avibactam is an important treatment alternative alternative for *bla*$_{OXA-48}$ positive carbapenem-resistant *K. pneumoniae* infections. The most rational approach to the treatment of carbapenem-resistant *K. pneumoniae* infections appears to be the initiatiion of targeted therapy according to culture antibiogram results or revision of the empirically initiated combination or monotherapy as early as possible according to culture antibiogram results.

Keywords: *bla*$_{OXA-48}$, *bla*$_{NDM}$, ceftazidime-avibactam, antimicrobial susceptibility, *Klebsiella pneumoniae*

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Öz

Giriş: Karbapenem dirençli *Klebsiella pneumoniae*'nin (*K. pneumoniae*) sağlık hizmeti yükü artmaktadır. Yeni geliştirilen beta laktam/beta-laktamaz inhibitörü kombinasyonu olan seftazidim-avibaktam tedavide umut vadetmektedir. Bu çalışma, seftazidime-avibaktamın bla_{OXA-48} genini taşıyan karbapenem dirençli *K. pneumoniae* izolatlarına karşı *in vitro* etkinliğini araştırmayı amaçlamıştır.

Gereç ve Yöntem: İzolatların tanımlanması MALDI-TOF MS (Brucker, ABD) ile yapılmıştır. İzolatlar European Committee of Antimicrobial Susceptibility Testing (EUCAST) kriterlerine göre disk difüzyon yöntemi ile imipenem, meropenem veya ertapeneme duyarlı değildi. Minimum inhibe edici konsantrasyon (MİK) değerleri, EUCAST'a göre sıvı mikrodilüsyon yoluyla belirlendi. Time-kill çalışması Clinical and Laboratory Standards Institute'ye göre yapılmıştır. Beta-laktamaz genleri, önceden yayınlanmış primerlerle polimeraz zincir reaksiyonu kullanılarak tarandı.

Bulgular: Nisan 2011 ile Şubat 2021 arasında izole edilen 129 *K. pneumoniae* çalışıldı. Bunlardan 98'i, 23'ü ve sekizi sırasıyla bla_{OXA-48}, bla_{NDM} ve bla_{OXA-48} artı bla_{NDM} taşıyordu. bla_{NDM} genini taşıyan izolatlar seftazidim-avibaktama dirençliydi. Genel olarak, bla_{OXA-48}-pozitif izolatların %79,6'sı seftazidim-avibaktama duyarlıydı. Seftazidim-avibaktam için zaman öldürme çalışması, bir bla_{OXA-48}-pozitif izolat (MİK, 4 mg/l) ile gerçekleştirilmiştir. Seftazidim-avibaktam time-kill kinetiği, MİK'nin katları olarak değerlendirildi. Test edilen izolat, 6 saatte 8, 16 ve 32 mg/l konsantrasyonda CFU/ml sayısında ≥3-log10 düşüş gösterdi. Minimum bakterisidal konsantrasyon 8 mg/l idi.

Sonuç: Seftazidim-avibactam, bla_{OXA-48}-pozitif karbapenem dirençli *K. pneumoniae* enfeksiyonlarını tedavi etmek için önemli bir tedavi alternatifidir. Karbapenem dirençli *K. pneumoniae* enfeksiyonlarının tedavisinde en akılcı yaklaşım, kültür antibiyogram sonuçlarına göre hedefe yönelik tedaviye başlamak veya ampirik olarak başlanan kombine veya monoterapiyi kültür antibiyogram sonuçlarına göre en kısa sürede revize etmek gibi görünmektedir.

Anahtar Kelimeler: bla_{OXA-48}, bla_{NDM}, seftazidim-avibaktam, antimikrobiyal duyarlılık, *Klebsiella pneumoniae*

Introduction

Multidrug resistance in *Klebsiella pneumoniae (K. pneumoniae*) isolates is rapidly becoming a significant concern in Turkey as well as worldwide. This results in a significant increase in mortality, morbidity, and treatment costs[1,2]. Due to the increasing incidence of drug resistance, treatments should be initiated after considering the resistance status of the relevant organism in the unit or region and the appropriate antibiotic. Particular difficulties exist in treating infections caused by carbapenem-resistant isolates with the antibiotics currently being used. Because current treatment options are limited, studies are being conducted to identify new treatment methods. The newly developed beta-lactam/beta-lactamase inhibitor combinations containing ceftazidime-avibactam is a promising candidate for the treatment of these multidrug-resistant infections[3]. Avibactam is effective against class A and C beta-lactamases, partially effective against class D beta-lactamases, and ineffective against metallo-beta-lactamases[4]. The OXA-48 enzyme was first detected in a *K. pneumoniae* isolate in Turkey in 2001[5,6]. Since 2008, *K. pneumoniae* isolates producing OXA-48 carbapenemase have been reported in European countries such as France, Greece, Belgium, the Netherlands, Spain, and Turkey[7,8]. In their multicenter and multinational study, Castanheira et al.[9] determined that the rate of isolates producing OXA-48-like beta-lactamase increased from 0.5% in 2016 to 0.9% in 2018, with OXA-48 being the most common variant.

Most of the previous studies have focused on investigating the susceptibility of *K. pneumoniae* carbapenemase (KPC)-type beta-lactamases to ceftazidime-avibactam[10,11]. In Turkey, OXA-48 (70.5-83.1%) and NDM (6.5-25%) are the most commonly detected beta-lactamases in *K. pneumoniae* isolates, with a coexistence rate of 2.4-4.5%[12,13]. According to a study that evaluated the incidence of transferable carbapenemase genes in carbapenem-resistant *K. pneumoniae* in Turkey, 95 of the 100 isolates carried at least one of the genes (OXA-48: 81.05%, NDM: 38.9%, KPC: 9.47%, VIM: 1.05%). One isolate carried the OXA-48 and KPC genes and two isolates carried the KPC and NDM genes[14]. Only a few studies have assessed the susceptibility of OXA-48 type beta-lactamases, which are mainly responsible for carbapenem resistance in *K. pneumoniae* isolates in Turkey, to ceftazidime-avibactam[15-17]. Herein, we aimed to explore the *in vitro* efficacy of ceftazidime-avibactam against carbapenem-resistant *K. pneumoniae* isolates carrying the bla_{OXA-48} gene.

Materials and Methods

Study Design and Setting

This study was planned as a national, multicenter, *in vitro* experimental study. A total of 132 carbapenem-resistant *K. pneumoniae* isolates were included in the study. Of these 132 isoloates, 100 were clinical isolates obtained from 100 patients between November 2019 and February 2021 at the Başkent University's and İstanbul Medeniyet University, Göztepe Training and Research Hospital's medical microbiology laboratories. The remaining 32 isolates, whose resistance patterns have already been determined molecularly, were obtained from 32 patients between April 2011 and December 2014 at Kartal Dr. Lütfi Kırdar City Hospital's clinical microbiology laboratory[13,18]. Only a single carbapenem-resistant isolate from each patient was included in the study. The isolates were stored at -80 °C until examined.

Three non-viable isolates (one obtained between 2019 and 2021 and two obtained between 2011 and 2014) were excluded from the study. Finally, 129 carbapenem-resistant *K. pneumoniae* isolates were included in the study.

Identification of Isolates and Detection of Carbapenem Resistance and Carbapenemase Genes

The isolates were identified using Bruker Biotyper MALDI-TOF MS (Billerica, MA, USA). The isolates that were not susceptible to imipenem, meropenem, or ertapenem by the disk diffusion method using the European Committee of Antimicrobial Susceptibility Testing (EUCAST)[19] breakpoints were screened for the bla_{OXA-48} and bla_{NDM} genes. The DNA was isolated by boiling the bacterial suspensions at 95 °C for 10 min and immediately cooled on ice. After centrifugation at 14,000 rpm for 10 min, the DNA was recovered from the supernatant[20]. The primer sequences targeting the bla_{OXA-48} and bla_{NDM} genes were 5′-GCGTGGTTAAGGATGAACAC-3′ (OXA-48-F), 5′- CATCAAGTTCAACCCAACCG-3′ (OXA-48-R), 5′-GGTTTGGCGATCTGGTTTTTC-3′ (NDM-F), 5′-CGGAATGGCTCATCACGATC-3′ (NDM-R) (Oligomer, Turkey). These primer sets amplify the 438 and 621 bp fragments. PCR was performed as previously described by Poirel et al.[21]. The PCR amplicon was analyzed using 2% agarose gel electrophoresis at 100 V for 1 h in 1X tris-borate-EDTA containing 0.05 mg/L ethidium bromide. Confirmed positive strains of *K. pneumoniae* CDC529 carrying bla_{NDM-1} or bla_{OXA-48} (n=15) were used as positive controls. *E. coli* ATCC25922 was used as a negative control[18].

Microdilution Tests

To determine the ceftazidime-avibactam minimum inhibitory concentration (MIC) values, the ceftazidime concentrations on microplates were diluted to 0.25-128 mg/l. Avibactam was added to each well with a final concentration of 4 mg/l. A 24-hour culture was used for all isolates to be studied. After preparing the isolate suspensions at 0.5 McFarland standard, the samples were diluted by 1/10 and 5 µl of the suspension was added to each well. The microplates were incubated at 37 °C for 18 to 24 hours. The lowest antibiotic concentration without growth was determined as the MIC value. The susceptibility results of the isolates were interpreted based on the EUCAST standards[19].

Time-kill Study

A time-kill study was performed according to previously published methods, including those described by the Clinical and Laboratory Standards Institute document M26-A[22,23]. Freshly prepared colonies were resuspended in 10 ml of cation-adjusted Mueller-Hinton broth (CAMHB) and incubated in a shaking water bath (at 37 °C and 180 rpm) for 1-2 hours. The cultures were prepared according to the 0.5 McFarland standard (approximately 10^8 CFU/ml) and further diluted to the ratio of 1:20 in CAMHB making the initial inoculum approximately 5×10^6 CFU/ml. Ceftazidime was added to the prepared bacterial suspensions such that the final ceftazidime concentration was 2, 4, or 8 times the MIC of the ceftazidime-avibactam of the isolate selected. Avibactam was added to a final concentration of 4 mg/l. An antibiotic-free growth control was also included. The initial inoculum concentration was determined from the growth control tube immediately after dilution and recorded as the count at time zero. After the addition of antibiotics, the initial inoculum concentration ranged from 1×10^6 to 5×10^6 CFU/ml. The tubes were incubated in a shaking water bath (at 37 °C and 180 rpm) and 100 µl of the culture sample was inoculated onto Tryptic Soy Agar (TSA) at 1, 2, 4, 6, and 24 h. The TSA plates were incubated at 37 °C for at least 18 hours. The colonies were counted and recorded as CFU/ml. A 3-log10 reduction in CFU/ml count was considered bactericidal.

Statistical Analysis

Categorical data are presented as frequencies and percentages. The categorical data were evaluated using the chi-square test, and $p<0.05$ was considered statistically significant.

Results

Of the 129 carbapenem-resistant *K. pneumoniae* isolates included in the study, 98 were bla_{OXA-48} positive, eight were bla_{OXA-48} and bla_{NDM}-positive, and 23 were bla_{NDM}-positive. Of the 129 isolates, 47 were obtained from blood, 36 from urine, 25 from respiratory secretions, 14 from wound and tissue cultures, four from body fluids, and three from catheter cultures.

All the bla_{NDM}-positive isolates were resistant to ceftazidime-avibactam. Approximately 79.6% of the bla_{OXA-48}-positive isolates were susceptible to ceftazidime-avibactam. The *in vitro* activity of ceftazidime-avibactam against carbapenem-resistant *K. pneumoniae* is shown in Table 1.

The *in vitro* activity of ceftazidime-avibactam by the source isolates against carbapenem-resistant *K. pneumoniae* is shown in Table 2. While all the isolates obtained from body fluids and catheters were susceptible to ceftazidime-avibactam, 55.3% of the isolates obtained from blood samples and 50.0% of the isolates obtained from urine samples were susceptible to ceftazidime-avibactam.

The susceptibility rates of bla_{OXA-48}-positive carbapenem-resistant *K. pneumoniae* strains from 2011 to 2014 to ceftazidime-avibactam were higher than those of the bla_{OXA-48}-positive carbapenem-resistant *K. pneumoniae* strains from 2019 to 2021 (85.7% vs. 77.9%); however, this difference was not significant (p=0.4). A yearly comparison of the *in vitro* efficacy of ceftazidime-avibactam against bla_{OXA-48}-positive carbapenem-resistant *K. pneumoniae* is shown in Table 3.

Time-kill Study

The time-kill study performed on a carbapenem-resistant *K. pneumoniae* isolate (OXA 48, MIC=4 mg/l) revealed a 3-log10 reduction in CFU/ml count with 8×, 16×, and 32× the MICs of ceftazidime-avibactam at 6 hours (Figure 1). The minimum bactericidal concentration (MBC) was 8 mg/L.

Discussion

In the present study, a total of 129 carbapenem-resistant *K. pneumoniae* isolates obtained from two different geographical areas and different centers were evaluated. All the isolates that were bla_{OXA-48}- and bla_{NDM}-positive and those that were only bla_{NDM}-positive were resistant to ceftazidime-avibactam. In contrast, 79.6% of the bla_{OXA-48}-positive isolates were susceptible to ceftazidime-avibactam. The time-kill study revealed that treatment with 8×, 16×, and 32× the MICs of ceftazidime-avibactam at 6 hours resulted in at least a 3-log10 reduction in CFU/ml. Furthermore, the MBC was 8 mg/L.

In a study conducted in Spain, which evaluated OXA-48 carbapenemase-producing *K. pneumoniae* isolates from urinary samples, all the isolates were susceptible to ceftazidime-avibactam[15]. In a recent study, Ozger et al. reported a susceptibility rate of 95.2% to ceftazidime-avibactam in 84 OXA-48 and KPC types of carbapenemase-producing *K. pneumoniae* isolates obtained from three different centers from a single city between 2016 and 2018 by broth microdilution[24]. Hoşbul et al.[15] determined that 92.7% of the 150 carbapenem-resistant *K. pneumoniae* isolates evaluated were susceptible to ceftazidime-avibactam. Of the ceftazidime-avibactam-resistant isolates, seven were bla_{NDM}-positive, three were bla_{KPC}-positive, and one was bla_{OXA-48}- and bla_{NDM}-positive. For all isolates without carbapenemase differentiation, the MIC50 and MIC90 values for ceftazidime-avibactam were 1 and 8 µg/ml, respectively. Terzi et al.[25] evaluated the antibiotic susceptibility of 22 carbapenem-resistant *K. pneumoniae* isolates and determined that among the eight isolates producing only OXA-48, all were susceptible to ceftazidime-avibactam. Studies have reported high and low susceptibility rates in carbapenem-resistant *K. pneumoniae* isolates. Karlowsky et al.[16] reported that 51.8% of the 85 carbapenem-resistant *K. pneumoniae* isolates examined

Table 1. *In vitro* activity of ceftazidime-avibactam against carbapenem-resistant *K. pneumoniae*

bla gene(s) (n=129)	Susceptibility* n (%)	MIC50 (mg/l)	MIC90 (mg/l)	MIC range (mg/l)
bla_{OXA-48} (n=98)	78 (79.6)	4	64	0.25 to >128
bla_{OXA-48} and bla_{NDM} (n=8)	0	>128	>128	64 to >128
bla_{NDM} (n=23)	0	>128	>128	64 to >128

*EUCAST cut-off values for susceptibility and resistance to ceftazidime-avibactam are ≤8 mg/l and >8 mg/l, respectively.

EUCAST: European Committee of Antimicrobial Susceptibility Testing, MIC: Minimum inhibitory concentration, MIC50: Minimum inhibitory concentration required to inhibit growth by 50%, MIC90: Minimum inhibitory concentration required to inhibit growth by 90%

Table 2. *In vitro* activity of ceftazidime-avibactam based on isolate source

Sources	Susceptibility* n (%)	MIC50 (mg/l)	MIC90 (mg/l)	MIC range (mg/l)
Blood (n=47)	26 (55.3)	8	>128	0.25 to >128
Urine (n=36)	18 (50.0)	8	>128	0.25 to >128
Respiratory secretions (n=25)	18 (72.0)	2	>128	0.25 to >128
Wound and tissue cultures (n=14)	9 (64.3)	8	64	0.25-64
Body fluids (n=4)	4 (100)	2	4	0.25-4
Catheter (n=3)	3 (100)	4	8	0.25-8

*EUCAST cut-off values for susceptibility and resistance to ceftazidime-avibactam are ≤8 mg/l and >8 mg/l, respectively.

EUCAST: European Committee of Antimicrobial Susceptibility Testing, MIC: Minimum inhibitory concentration, MIC50: Minimum inhibitory concentration required to inhibit growth by 50%, MIC90: Minimum inhibitory concentration required to inhibit growth by 90%

Table 3. *In vitro* ceftazidime-avibactam efficacy on bla_{OXA-48}-positive carbapenem-resistant *Klebsiella pneumoniae* based on the time period when the sample was obtained

Years (n=98)	Susceptibility* n (%)	MIC50 (mg/l)	MIC90 (mg/l)	MIC range (mg/l)
2011-2014 (n=21)	18 (85.7)	4	32	0.25 to >128
2019-2021 (n=77)	60 (77.9)	4	64	0.25 to >128

*EUCAST cut-off values for susceptibility and resistance to ceftazidime-avibactam are ≤8 mg/l and >8 mg/l, respectively.

EUCAST: European Committee of Antimicrobial Susceptibility Testing, MIC: Minimum inhibitory concentration, MIC50: Minimum inhibitory concentration required to inhibit growth by 50%, MIC90: Minimum inhibitory concentration required to inhibit growth by 90%

were susceptible to ceftazidime-avibactam, while 89.8% of the 49 MBL-negative carbapenem-resistant *K. pneumoniae* isolates were susceptible to ceftazidime-avibactam. A single-center study in 2017, reported a ceftazidime-avibactam susceptibility of 52% in 42 OXA-48-producing *K. pneumoniae* isolates, with MIC50 and MIC90 values of 8 and 256 mg/l, respectively[26]. Antibiotic susceptibilities may vary in different geographical locations, centers, and times. This may be due to the presence of undetected resistance genes. In the present study, the ceftazidime-avibactam susceptibility of 98 OXA-48 carbapenemase-producing *K. pneumoniae* isolates obtained from two different geographical regions and different centers was approximately 80%, with MIC50 and MIC90 of 4 and 64 mg/l, respectively.

In our study most of the isolates were obtanined from blood and urine cultures. The resistance rate of isolates obtained from blood and urine samples to ceftazidime-avibactam was higher than that of isolates obtained from respiratory secretions, wound/tissue, body fluids, and catheter cultures. Another study from Turkey reported a higher resistance rate to ceftazidime-avibactam in blood culture isolates than in isolates obtained from other sources[27].

Keepers et al.[22] evaluated the time-kill kinetics of ceftazidime-avibactam for all *Enterobacteriaceae* isolates included in their study (wild-type, ESBL, carbapenemase- and/or AmpC-producing *K. pneumoniae*) and reported a ≥3-log10 reduction in CFU/ml count after 6 hours. In the present study, the time- and concentration-dependent bactericidal effect of ceftazidime-avibactam on OXA-48 carbapenemase-producing *K. pneumoniae* isolates was determined by the time-kill method. Similar to the study findings of Keepers et al.[22], a decrease of at least 3-log10 in CFU/ml count was observed at 6 hours with 8×, 16×, and 32× the MICs of ceftazidime-avibactam.

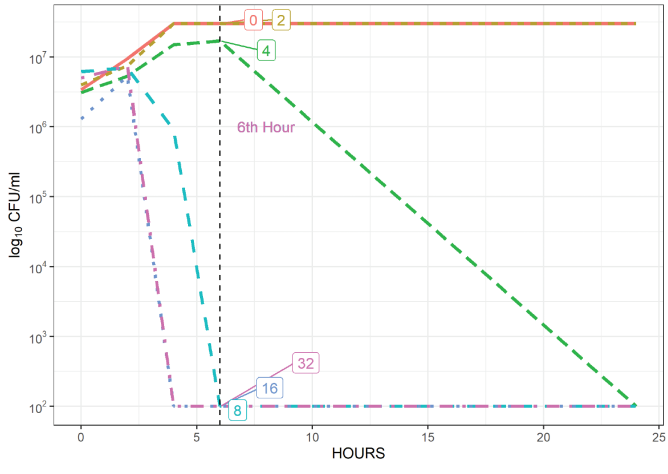

Figure 1. Time-kill graph of ceftazidime-avibactam for bla_{OXA-48}-positive *Klebsiella pneumoniae* isolate (MIC=4 mg/l)

MIC: Minimum inhibitory concentration

When this study was initiated, ceftazidime-avibactam was yet to be covered by insurance for the treatment of carbapenem-resistant *K. pneumoniae* in Turkey. Since then, it has been included in the reimbursement schemes for the treatment of patients with carbapenem-resistant *K. pneumoniae*, who have been hospitalized in the intensive care unit for over a year, with a proven antibiotic susceptibility based on culture antibiogram results. The unnecessary and frequent use of antibiotics may cause antibiotic resistance. Development of resistance during the use of ceftazidime-avibactam has been reported in literature[28,29]. Clinicians should adhere to the principles of rational use of antibiotics and infection control practices to prevent the development of resistance to certain antibiotics which are used as the last line of defense in the treatment of resistant infections.

A study evaluating the efficacy of ceftazidime-avibactam in solid organ transplant recipients with bloodstream infections caused by carbapenemase-producing *K. pneumoniae* reported that patients treated with ceftazidime-avibactam had higher 14-day and 30-day clinical success rates and lower 30-day mortality rates than those receiving the best available therapy. Combination therapy was not associated with better outcomes in the ceftazidime-avibactam group[30]. The optimal treatment of infection caused by the NDM-producing *K. pneumoniae* strain remains uncertain and antibiotic options are limited. The combination of ceftazidime-avibactam and aztreonam or cefiderocol, can be a treatment option for NDM-producing *K. pneumoniae* infections[31].

Study Limitations

This study has some limitations. It was designed as an *in vitro* study and the results are not supported by clinical data. Furthermore, resistance genes other than OXA-48 and NDM beta-lactamases could not be investigated in the isolates.

Conclusion

All the bla_{NDM}-positive carbapenem-resistant *K. pneumoniae* isolates included in the study were resistant to ceftazidime-avibactam. Approximately 80% of the bla_{OXA-48}-producing carbapenem-resistant *K. pneumoniae* isolates were susceptible to ceftazidime-avibactam. Ceftazidime-avibactam appears to be an important treatment alternative for isolates producing OXA-48 beta-lactamase. In the treatment of carbapenem-resistant *K. pneumoniae* infections, the most rational approach appears to be the initiation of targeted therapy according to culture antibiogram results or revision of the empirically initiated combination or mono-therapy as early as possible based on culture antibiogram results.

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Ethics

Ethics Committee Approval and Informed Consent: The study was approved by the Ethical Committee of the İstanbul Medeniyet University, Göztepe Training and Research Hospital, İstanbul, Turkey, on January 02, 2019 with number 2018/0514, and signed informed consent was waived.

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Authorship Contributions

Surgical and Medical Practices: Y.Ç., H.Ç., R.A.Ç., M.E.K., H.V., D.H., H.C.M., A.Ü.G., Concept: Y.Ç., R.A.Ç., M.E.K., H.V., D.H., Design: Y.Ç., M.E.K., Data Collection or Processing: Y.Ç., H.Ç., R.A.Ç., M.E.K., D.H., Analysis or Interpretation: Y.Ç., H.V., Literature Search: H.C.M., A.Ü.G., Y.Ç., H.Ç., R.A.Ç., M.E.K., D.H., Writing: Y.Ç.

Conflict of Interest: The authors declare that they have no conflict of interest.

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