

# Mapping Clonal Landscape and Phenotypic-Genotypic Divergence of *Pseudomonas aeruginosa* Isolates from Three Medical Centers

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## Research Article

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# Abstract

## Background

*Pseudomonas aeruginosa* is responsible for challenging nosocomial infections and is distinguished by high environmental adaptability and survival in different environments. The extensive clonal diversity and complex resistance mechanisms identified among *P. aeruginosa* isolates are highly noteworthy. This study aimed to evaluate the intra- and inter-center clonal relatedness and genotypic-phenotypic divergence of carbapenem-resistant *P. aeruginosa* isolates from three medical centers.

## Methods

A total of 59 carbapenem-resistant *P. aeruginosa* isolates recovered from blood cultures across three different medical centers between 2021 and 2023 were included in this study. The susceptibilities of the isolates to ceftazidime (CAZ), ceftazidime-avibactam (CZA), meropenem (MEM), amikacin, and colistin (CT) were determined by broth microdilution. The presence of *blaKPC*, *blaOXA-10*, *blaOXA-48*, *blaVIM*, *blaIMP*, and *blaNDM* was investigated using conventional PCR. The expression levels of the efflux pump activity gene *mexB* and the outer membrane porin protein OprD were analyzed by quantitative real-time reverse transcription PCR (RT-qPCR) using the delta-delta cycle threshold ( $\Delta\Delta Ct$ ) method. Efflux pump activity was evaluated phenotypically by the ethidium bromide cartwheel agar test. Biofilm formation was quantified by measuring the optical density using a microplate assay. The clonal relatedness of the isolates was analyzed by AP-PCR using the M13 primer. The genotypic dendrogram, figures, and all statistical analyses were performed using R software.

## Results

All isolates were resistant to MEM and susceptible to CT. Significant inter-center differences were observed in CAZ and CZA susceptibility results and in the presence of resistance genes. While *mexB* overexpression was high across all centers ( $p > 0.05$ ), *oprD* downregulation did not reach statistical significance ( $p = 0.06$ ). Phenotypic evaluation of efflux pump activity indicated substantial variation alongside a broad discordance with genotypic expression levels. All isolates were capable of biofilm formation, albeit weakly. Isolates recovered from the same center within a relatively short timeframe tended to be more closely related, whereas those collected from different centers and over more extended periods exhibited greater polyclonal diversity.

## Conclusion

The detected phenotypic-genotypic heterogeneity and high clonal diversity surpass the mere complexity of genomic structure and the diversity of resistance mechanisms. Managing this calls for integrating

antimicrobial stewardship programs, standardized antibiotic restriction policies, and following local guidelines across all centers.

## Introduction

Exhibiting exceptional adaptability to both environmental and hospital settings, alongside the capacity for prolonged survival on moist surfaces, *Pseudomonas aeruginosa* frequently causes highly resistant infections and is a prominent member of the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*), a notorious group of microorganisms responsible for difficult-to-treat nosocomial infections (1). Representing approximately 7% of nosocomial bloodstream infections, *P. aeruginosa* presents remarkable epidemiological diversity, driven not only by its high capacity for adaptation and survival but also by antimicrobial resistance profiles mediated by diverse mechanisms (2).

Antimicrobial resistance mechanisms in *P. aeruginosa* can be generally classified into three groups: chromosomally encoded intrinsic resistance, acquired resistance driven by mutations or horizontal gene transfer, and adaptive resistance induced by environmental stimuli (3–5). The downregulation of OprD (an outer membrane porin that facilitates the cellular entry of carbapenems), the overexpression of the resistance-nodulation-cell division (RND) family efflux pump, mainly MexAB-OprM, and their synergistic interplay are well-established mechanisms associated with the development of carbapenem resistance in *P. aeruginosa* isolates (3). Among horizontally acquired carbapenemases, the class B metallo-beta-lactamase (MBL)-encoding genes *bla<sub>VIM</sub>* and *bla<sub>IMP</sub>* are frequently reported (4). Furthermore, biofilm formation is a phenotypic modification that is upregulated in response to stimuli such as antimicrobial selective pressure, facilitating persistent infections and conferring resistance to antimicrobial therapy due to its structural properties (5).

In recent years, efforts to investigate and prevent nosocomial outbreaks driven by antimicrobial resistance have become increasingly critical (6). A variety of polymerase chain reaction (PCR)-based and non-PCR-based methods are available for molecular profiling and clonal relationship analysis, ranging from pulsed-field gel electrophoresis (PFGE), which has been considered the gold standard for years, to next-generation sequencing (NGS) techniques combined with bioinformatics pipelines that are currently in widespread use driven as a result of technological advancements (7). Although intra-hospital isolates recovered from the same clinical wards and within close temporal proximity are typically expected to exhibit clonal relatedness, the extensive clonal diversity observed among *P. aeruginosa* isolates is highly noteworthy (8).

This study aimed to map intra- and inter-center heterogeneity by comprehensively investigating the phenotypic profiles, genotypic characteristics, and clonal relatedness of carbapenem-resistant *P. aeruginosa* isolates recovered from three distinct medical centers in Türkiye.

## Methods

# Bacterial isolates

A total of 59 *P. aeruginosa* isolates, recovered from blood cultures between 2021 and 2023 across three different centers in Türkiye, namely Gülhane Training and Research Hospital in Ankara (Center G), Erciyes University Hospital in Kayseri (Center E), and Hacettepe University Hospital in Ankara (Center H), were included in the study. All subsequent stages of the study were conducted at a single center (Center G). The isolates were identified using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics, Germany).

## Antimicrobial Susceptibility Testing

To compare results from isolates obtained from three different centers, the minimum inhibitory concentration (MIC) values for ceftazidime (CAZ), ceftazidime/avibactam (CZA), amikacin (AK), meropenem (MEM), and colistin (CT) were determined simultaneously at a single center (Center G) using the standard broth microdilution method. The MIC ranges were established as 0.125-128 (mg/L) for CAZ and CZA, 0.06-64 (mg/L) for AK, 2-128 (mg/L) for MEM, and 0.25-16 (mg/L) for CT. The results were evaluated according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (9).

## Polymerase chain reactions (PCRs)

The presence of *bla*<sub>OXA-48</sub>, *bla*<sub>NDM</sub>, *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>KPC</sub>, and *bla*<sub>OXA-10</sub> resistance genes was investigated by conventional PCR; *mexB* and *oprD* expression levels were assessed by quantitative real-time PCR (RT-qPCR); and the clonal relationship of the isolates was analyzed by arbitrarily primed PCR (AP-PCR) using the M13 primer. All isolates were subjected to the same procedures and run simultaneously in a standard PCR cycle. DNA extraction protocols, master mix contents used in the reactions, gel electrophoresis, and imaging settings were performed as described in a previous study (10). For RT-qPCR, RNA extraction was performed using the TRIzol™ Reagent (Thermo Fisher, USA) kit, and cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad, USA), in accordance with the manufacturers' recommendations. The RT-qPCR reactions were performed in triplicate using the iTaq Universal One-Step Kit (Bio-Rad, USA) on an ABI 7500 Real-Time PCR instrument (Applied Biosystems™, USA) (primer sequences for all genes and PCR reaction conditions are provided in the Supplemental Material). The cycle threshold (CT) values for the RT-qPCR reactions were calculated by normalizing to the housekeeping gene *rpsL* and calibrating to the PAO1 reference strain. The analysis of expression levels was performed using the  $2^{-\Delta\Delta C_t}$  method. A  $\geq 2$ -fold change for *mexB* and a  $< 1$ -fold value for *oprD* were considered significant. (11, 12).

## Phenotypic analysis of efflux pump activity (ethidium bromide cartwheel agar test)

Efflux pump activity was evaluated phenotypically by preparing tryptic soy agar (TSA) containing ethidium bromide (EtBr) at concentrations of 0.5, 1, 1.5, and 2 µg/ml. Suspensions of the isolates prepared at a 0.5 McFarland standard were streaked onto the agars (cartwheel pattern). Fluorescence at 0.5-1 µg/ml concentration was evaluated as mild, fluorescence at 1.5 µg/ml concentration as moderate, and fluorescence at 2 µg/ml concentration as high-level efflux pump activity (13). A sample cartwheel agar image, in which the PAO1 standard strain was used as a negative control, is presented in Fig. 1.

## Biofilm formation

Biofilm formation was determined quantitatively by measuring the optical density (OD) using the microplate method. Brain heart infusion broth supplemented with 2% D-glucose and a bacterial suspension adjusted to a 0.5 McFarland turbidity standard were added to microplates and incubated at 35°C for 24 hours. Following incubation, non-adherent bacteria were removed by washing three times with phosphate-buffered saline. Fixation of the biofilm-forming bacteria was achieved using methanol. The fixed samples were stained with crystal violet. After staining, all wells were washed three times with distilled water, and the microplates were allowed to air-dry. For spectrophotometric measurements, 95% ethanol was added to the wells, and absorbance was measured at 600 nm. The OD cut-off (OD<sub>c</sub>) was defined as the mean OD of the negative control plus 3 standard deviations. The tested isolates were classified based on their mean OD from three replicates (OD<sub>t</sub>) as negative if OD<sub>t</sub> ≤ OD<sub>c</sub>, weak if OD<sub>c</sub> < OD<sub>t</sub> ≤ 2xOD<sub>c</sub>, moderate if 2xOD<sub>c</sub> < OD<sub>t</sub> ≤ 4xOD<sub>c</sub>, and strong biofilm formation if 4xOD<sub>c</sub> < OD<sub>t</sub> (14).

## Data analysis

All statistical analyses and figures were generated using the R software program (v4.6.1, Foundation for Statistical Computing, Vienna, Austria). Categorical variables were expressed as percentages and proportions. Pearson's chi-square test or Fisher's exact test was used for the overall distributional differences among the three centers. In cases where the result was statistically significant, post hoc analyses were adjusted using the Bonferroni correction, and  $p < 0.05$  was considered statistically significant. AP-PCR Banding patterns obtained from molecular typing of the clinical isolates were converted into a binary data matrix, in which the presence or absence of bands was scored as 1 or 0, respectively. The genetic distance and similarity matrices between the isolates were computed using the Sørensen–Dice similarity coefficient. To construct the phylogenetic dendrogram, hierarchical clustering was performed employing the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm. A comprehensive visualization integrating the genotypic dendrogram with phenotypic and genotypic metadata was generated. The distributions of MICs were established by considering the specific MIC ranges for each antibiotic (15).

## Results

The study included 59 *P. aeruginosa* isolates from Center G ( $n = 20$ ), Center E ( $n = 19$ ), and Center H ( $n = 20$ ). In terms of antimicrobial susceptibilities, all isolates were resistant to MEM and susceptible to CT. The susceptibility rates, MIC<sub>50</sub> and MIC<sub>90</sub> values for the tested antibiotics, and the statistical analysis and interpretation of differences across the three centers are presented in Table 1. The distribution of MICs for the five antibiotics tested across the three centers is shown in Fig. 2.

Table 1

Antimicrobial susceptibility rates, MIC<sub>50</sub>/MIC<sub>90</sub> values of the five tested antibiotics, and comparative analysis across the three centers

| Antimicrobials                                                                                                                                                                                                                                                 | Center G | Center E | Center H | p value            | Comments                                                                                                                                      |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------|----------|----------|--------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|
| Ceftazidime susceptibility                                                                                                                                                                                                                                     | 35%      | 79%      | 25%      | *0.001             | A statistically significant difference was observed between Center G <sup>a</sup> and E <sup>a</sup> Center E <sup>b</sup> and H <sup>b</sup> |
| MIC <sub>50</sub>                                                                                                                                                                                                                                              | 32       | 8        | 64       | 0.028 <sup>a</sup> |                                                                                                                                               |
| MIC <sub>90</sub>                                                                                                                                                                                                                                              | 128      | 32       | > 128    | 0.003 <sup>b</sup> |                                                                                                                                               |
|                                                                                                                                                                                                                                                                |          |          |          |                    |                                                                                                                                               |
| Ceftazidime/avibactam susceptibility                                                                                                                                                                                                                           | 55%      | 79%      | 30%      | *0.009             | A statistically significant difference was observed between Center E <sup>a</sup> and H <sup>a</sup>                                          |
| MIC <sub>50</sub>                                                                                                                                                                                                                                              | 8        | 4        | 32       | 0.01 <sup>a</sup>  |                                                                                                                                               |
| MIC <sub>90</sub>                                                                                                                                                                                                                                              | 128      | 32       | 128      |                    |                                                                                                                                               |
|                                                                                                                                                                                                                                                                |          |          |          |                    |                                                                                                                                               |
| Amikacin susceptibility                                                                                                                                                                                                                                        | 65%      | 73.7%    | 50%      | *>0.05             | No significant                                                                                                                                |
| MIC <sub>50</sub>                                                                                                                                                                                                                                              | 16       | 2        | 32       |                    |                                                                                                                                               |
| MIC <sub>90</sub>                                                                                                                                                                                                                                              | 32       | 32       | > 64     |                    |                                                                                                                                               |
|                                                                                                                                                                                                                                                                |          |          |          |                    |                                                                                                                                               |
| Meropenem susceptibility                                                                                                                                                                                                                                       | 0        | 0        | 0        | -                  | Statistical analysis could not be performed                                                                                                   |
| MIC <sub>50</sub>                                                                                                                                                                                                                                              | 32       | 32       | 128      |                    |                                                                                                                                               |
| MIC <sub>90</sub>                                                                                                                                                                                                                                              | 128      | 64       | > 128    |                    |                                                                                                                                               |
|                                                                                                                                                                                                                                                                |          |          |          |                    |                                                                                                                                               |
| Colistin susceptibility                                                                                                                                                                                                                                        | 100%     | 100%     | 100%     | -                  | Statistical analysis could not be performed                                                                                                   |
| MIC <sub>50</sub>                                                                                                                                                                                                                                              | 0.5      | 0.5      | 0.5      |                    |                                                                                                                                               |
| MIC <sub>90</sub>                                                                                                                                                                                                                                              | 1        | 1        | 1        |                    |                                                                                                                                               |
|                                                                                                                                                                                                                                                                |          |          |          |                    |                                                                                                                                               |
| *: Pearson chi-square,ω: Fisher’s exact test, <sup>ab</sup> : Post hoc (Bonferroni-corrected p-values). Center G: Gülhane Training and Research Hospital/Ankara, Center E: Erciyes University Hospital/Kayseri, Center H: Hacettepe University Hospital/Ankara |          |          |          |                    |                                                                                                                                               |

According to conventional PCR results, among the resistance genes, only *bla*<sub>VIM</sub> and *bla*<sub>OXA-10</sub> were detected at varying rates. The *bla*<sub>OXA-10</sub> positivity for Centers G, E, and H is 65%, 26.3%, and 15%, respectively, and the *bla*<sub>VIM</sub> positivity is 5%, 0, and 15% in the same order. When the centers are evaluated for harboring resistance genes, there is a significant difference among the three centers ( $p = 0.008$ ). The

difference between Center G and Center E is statistically significant ( $p = 0.03$ , Bonferroni correction). All isolates are carbapenem-resistant, and the rate of *mexB* overexpression ( $\geq 2$ -fold) is high across all centers ( $p > 0.05$ ). When *oprD* downregulation ( $< 1$ -fold) was evaluated, statistical significance was observed in the initial stage ( $p = 0.026$ ); however, post hoc analyses indicated that the difference between Center E and Center H did not reach statistical significance ( $p = 0.06$ ). The rates of resistance gene harboring across the centers, the results for the other evaluated categories, and their comparative statistical analyses are presented in Table 2.

Table 2

Resistance gene prevalence, phenotypic/genotypic profiles, and comparative analysis across the three centers

| Categories                                                                                                                                                                                                                                                              | Center G         | Center E            | Center H         | p value                             | Comments                                                                                             |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|---------------------|------------------|-------------------------------------|------------------------------------------------------------------------------------------------------|
| <b>Presences of resistance genes</b>                                                                                                                                                                                                                                    | 70%              | 26,3%               | 30%              | *0.008<br>0.03 <sup>a</sup>         | A statistically significant difference was observed between Center G <sup>a</sup> and E <sup>a</sup> |
| <b>mexB overexpression</b>                                                                                                                                                                                                                                              | 90%              | 89.5%               | 100%             | $\omega > 0.05$                     | No significant                                                                                       |
| <b>oprD downregulation</b>                                                                                                                                                                                                                                              | 90%              | 73.7%               | 100%             | $\omega 0.026$<br>0.06 <sup>a</sup> | It did not reach statistical significance after Bonferroni correction <sup>a</sup>                   |
| <b>Efflux pump activity</b>                                                                                                                                                                                                                                             | 75%              | 94,7%               | 95%              | $\omega 0.005$                      | A statistically significant difference was observed between Center G <sup>a</sup> and H <sup>a</sup> |
| <b>none</b>                                                                                                                                                                                                                                                             | 25%              | 5.3% 63.2%<br>21%   | 5%               | 0.01 <sup>a</sup>                   |                                                                                                      |
| <b>mild</b>                                                                                                                                                                                                                                                             | 35%              | 10.5% <sup>ab</sup> | 85%              |                                     |                                                                                                      |
| <b>moderate</b>                                                                                                                                                                                                                                                         | 5%               |                     | 0                |                                     |                                                                                                      |
| <b>high</b>                                                                                                                                                                                                                                                             | 35% <sup>a</sup> |                     | 10% <sup>b</sup> |                                     |                                                                                                      |
| <b>Biofilm formation</b>                                                                                                                                                                                                                                                | 100%             | 100%                | 100%             | $\omega 0.076$                      | Not significant                                                                                      |
| <b>weak</b>                                                                                                                                                                                                                                                             | 65%              | 36.8%<br>57.9%      | 75%              |                                     |                                                                                                      |
| <b>moderate</b>                                                                                                                                                                                                                                                         | 25%              | 5.3% <sup>a</sup>   | 20%              |                                     |                                                                                                      |
| <b>strong</b>                                                                                                                                                                                                                                                           | 10% <sup>a</sup> |                     | 5% <sup>a</sup>  |                                     |                                                                                                      |
| *: Pearson chi-square, $\omega$ : Fisher's exact test, <sup>ab</sup> : Post hoc (Bonferroni-corrected p-values). Center G: Gülhane Training and Research Hospital/Ankara, Center E: Erciyes University Hospital/Kayseri, Center H: Hacettepe University Hospital/Ankara |                  |                     |                  |                                     |                                                                                                      |

The number of isolates negative for all investigated resistance genes was 6 (30.0%), 14 (73.7%), and 14 (70.0%) for Centers G, E, and H, respectively. Among the six isolates from Center G, five exhibited *mexB*

overexpression and/or *oprD* downregulation. In the remaining one isolate, no specific mechanism for carbapenem resistance could be identified; it was identified solely as a weak biofilm producer. For Center E (n = 14), only one isolate demonstrated *oprD* downregulation without concomitant *mexB* overexpression, and this strain was identified as a moderate biofilm producer. Conversely, all 14 isolates from Center H displayed both *mexB* overexpression and *oprD* downregulation.

In our study, efflux pump activity was also evaluated phenotypically. As a result of the phenotypic cartwheel agar test, isolates exhibiting mild efflux pump activity were the majority across the three centers. Statistical analysis showed a significant difference among the centers ( $p = 0.005$ ). Subsequent post hoc analysis indicated that the difference between Center G and Center H was statistically significant ( $p = 0.01$ ) (Table 2). An investigation into the relationship between the four phenotypic categories (none, mild, moderate, and high) and *mexB* expression levels showed a statistically significant difference across the groups ( $p < 0.05$ ). This finding indicates a generalized discordance between the phenotypic profiles and genotypic expression. However, a subsequent trend analysis assessing whether *mexB* expression levels increased in proportion to the intensity of phenotypic efflux activity showed no significant monotonic trend ( $p > 0.05$ ). These findings indicate that the MexAB-OprM system may not be the sole contributor to the observed phenotypic efflux pump activity.

When biofilm-forming capacity was evaluated, it was determined that all isolates were capable of forming biofilms. When biofilm formation levels were evaluated, the majority of Center G and Center H isolates were weak biofilm producers. In contrast, Center E isolates, unlike those from the other two centers, predominantly exhibited moderate biofilm production. The difference in biofilm formation between the three centers was not significant ( $p = 0.076$ ) (Table 2).

Finally, the clonal relationship analysis of the isolates was interpreted using the generated dendrogram. The dendrogram constructed from a binary 0/1 matrix and the associated phenotypic-genotypic characteristics is presented in Fig. 3. The middle-lower section of the tree represents the major cluster. The major cluster, particularly encompassing the G16 and G18-G20 isolates, exhibits a similar phenotypic profile and a 100% compatible banding pattern. While isolates recovered from the same center within a short timeframe exhibited considerable clonal relatedness, extensive polyclonal diversity was found across different centers and among isolates collected throughout extended time intervals.

## Discussion

The remarkable capacity of *P. aeruginosa* to adapt to and survive throughout diverse ecological habitats was first elucidated following the genomic characterization of the PAO1 strain, which was sequenced in 2000. Given their extensive genome size and high number of open reading frames, *Pseudomonas* species display a genetic complexity rivaling that of eukaryotic organisms (16). The wealth of genomic data accumulated since the sequencing of the initial reference strain demonstrates that the *P. aeruginosa* pangenome comprises a conserved core genome and a highly varied array of accessory genes. Although genomic analyses provide important insights into population structure and diversity,

defining the genotype and its corresponding phenotype remains insufficient to explicate the complex mechanisms of pathogenesis (17). In the present study, we aimed to clarify this heterogeneity by evaluating the phenotypic and genotypic characteristics and the clonal relationships of isolates recovered from the three centers. Our data indicate substantial genotypic and phenotypic heterogeneity not only among different centers but also within individual centers and among temporally related isolates.

In addition to their genotypic background, *P. aeruginosa* isolates display marked diversity in their antimicrobial susceptibility profiles (18). A previous bacterial resistome analysis showed that *P. aeruginosa* exhibited the highest rate of phenotypic-genotypic discordance (44.4%) among all evaluated microorganisms (19). In addition, regarding variations in the clinical management of inpatient and outpatient populations within individual hospitals, the absence of uniform antimicrobial stewardship programs across institutions, coupled with disparities in adherence to antibiotic restriction policies and local treatment guidelines, drives substantial intra- and inter-central heterogeneity. Consequently, therapeutic options are becoming severely restricted for isolates resistant to carbapenems, a critical class of antimicrobials that requires stringent surveillance to monitor the emergence of resistance (20). In the present study, we evaluated inter-central differences through analyzing the susceptibility profiles of a collection consisting exclusively of carbapenem-resistant *P. aeruginosa* isolates against five antibiotics, including the last-resort agents CZA and CT. The CZA susceptibility rates for Centers G, E, and H were 55%, 79%, and 30%, respectively. Notably, previous literature includes studies from Centers G and H that evaluated the CZA susceptibility of *P. aeruginosa* isolates before the clinical introduction of this antibiotic in our country. (21, 22). A previous study from Center G by Hosbul et al. (21) reported a 90% CZA susceptibility rate among *P. aeruginosa* isolates recovered between 2016 and 2021; similarly, Hazirolan et al. demonstrated a 91.2% susceptibility rate for isolates collected at Center H between 2019 and 2020 (22). In our current study, which evaluated *P. aeruginosa* isolates recovered after the antibiotic entered clinical use in our country, CZA susceptibility rates were 55% and 30% for Centers G and H, respectively, and 79% for Center E. Following the clinical introduction of this agent, CZA susceptibility among *P. aeruginosa* isolates declined notably at Centers G and H, leading to a statistically significant difference across the three centers.

In the global ERACE-PA surveillance study evaluating isolates collected between 2019 and 2021, CZA susceptibility among carbapenem-susceptible *P. aeruginosa* was reported to be 91%. In contrast, among carbapenem-resistant isolates, in which *bla<sub>VIM</sub>* was the most frequently detected carbapenem resistance gene, this rate varied by continent, with a susceptibility of 79% in Europe (23). The differing findings in the literature and the inter-central discrepancies detected in our study underline the impact of varying antibiotic policies and antimicrobial stewardship strategies on phenotypic resistance profiles, both internationally and among health care facilities within the same country. Notably, all isolates in our study remained susceptible to CT, a critical last-resort antimicrobial. Furthermore, susceptibility to AK, a frequently used agent in combination therapy regimens, was maintained at 50% or higher across all centers. These outcomes have considerable clinical value, as they demonstrate the continued preservation of viable alternative treatment options.

Regarding the resistance determinants evaluated in our study, *bla*<sub>OXA-10</sub> was harbored by 13 (65%), 5 (26.3%), and 3 (15%) isolates from Centers G, E, and H, respectively, while *bla*<sub>VIM</sub> was detected in 1 (5%), 0, and 3 (15%) isolates across these respective centers. OXA-10 is a narrow-spectrum Class D beta-lactamase. Although its production can confer resistance to third-generation cephalosporins, CAZ typically evades hydrolysis by this enzyme. However, under antimicrobial selective pressure, the emergence of OXA-10 variants can broaden the enzyme's hydrolytic spectrum, consequently driving resistance to CAZ, carbapenems, and CZA. (24). In a study evaluating MDR *P. aeruginosa* isolates with WGS, isolates harboring the *bla*<sub>OXA-10</sub> gene were found to be resistant to all tested beta-lactam antibiotics, including CAZ and CZA (25). In our study, CAZ resistance rates among *bla*<sub>OXA-10</sub>-harboring isolates were 69.2% (5/13), 20% (1/5), and 66.7% (2/3) for Centers G, E, and H, respectively. While CZA resistance rates at Centers E and H remained identical to their CAZ resistance rates, Center G exhibited a CZA resistance rate of 53.8% (7/13). This finding alone could point to the emergence of an OXA-10 variant capable of conferring resistance to both CAZ and CZA. However, OXA-10 production was not the sole determinant of resistance. When considering the co-occurrence of VIM-producing isolates and the contribution of transcriptionally regulated mechanisms to carbapenem resistance, the universal carbapenem-resistant phenotype observed across the entire study cannot be attributed to a single mechanism. Furthermore, an evaluation of the clonal relationships among isolates harboring resistance genes and sharing similar phenotypic profiles showed significant intra- and inter-central clonal diversity. Although a few genetically related isolates were identified within individual centers (e.g., G18-G20 and H18-20), there was no evidence of clustering into a single dominant clone.

Downregulation or mutational inactivation of *OprD*, coupled with efflux pump overexpression, represents the most frequently encountered mechanism underlying carbapenem resistance in *P. aeruginosa* isolates. (3). The overexpression of efflux pump systems, including MexAB-OprM, MexXY-OprM, and MexCD-OprJ, mediates resistance to a broad spectrum of antimicrobial agents in *P. aeruginosa* isolates. (13). To delineate the mechanisms underlying carbapenem resistance in our isolates, we performed transcriptomic analyses focusing on *oprD* downregulation and *mexB* overexpression, two of the most frequently encountered resistance drivers. Our transcriptomic data confirm that this resistance is multifactorial and cannot be ascribed to a single mechanism. The rates of *mexB* overexpression in Centers G, E, and H were 90%, 89.5%, and 100%, respectively; concomitantly, *oprD* downregulation was observed in 90%, 73.7%, and 100% of isolates from these centers, respectively. In comparison, a previous meta-analysis reported a pooled frequency of 61% for *oprD* downregulation among MDR *P. aeruginosa* isolates, while the prevalence of *mexB* overexpression ranged between 48% and 77% (26). Another study investigating carbapenem-resistant *P. aeruginosa* isolates reported rates of 74% and 82% for *oprD* downregulation and *mexB* overexpression, respectively (10). Although our findings for Center E are consistent with the current literature, the overall rates across all centers were considerably higher. This discrepancy is likely attributable to the limited number of isolates, which constitutes a limitation of our study.

Furthermore, efflux pump activity was phenotypically evaluated utilizing the EtBr cartwheel agar test. Based on this method, the proportions of isolates demonstrating active efflux were 75%, 94.7%, and 95% for Centers G, E, and H, respectively. We investigated whether *mexB* expression levels correlated with the phenotypic intensity of efflux activity (categorized as none <mild <moderate <high). The absence of a monotonic trend indicates that the MexAB-OprM system does not exclusively mediate the overall efflux activity in these isolates, suggesting the involvement of alternative efflux pumps. EtBr is an intercalating dye that exhibits weak background fluorescence, which is enhanced 20- to 25-fold upon binding to nucleic acids. However, its routine laboratory use has been abandoned due to its toxicity and mutagenicity (27). Although studies evaluating efflux pump activity using EtBr are not novel, they are currently utilized predominantly to assess the efficacy of efflux pump inhibitors rather than to demonstrate the presence of efflux pumps (13). The overexpression of efflux pumps in bacteria is a high-cost process that leads to excessive energy consumption. Upon removal of antibiotic selective pressure, the bacterium may transition to a highly resistant yet biologically inactive (low-cost) state. Under these circumstances, the expression of costly efflux pumps, which impose a burden on the bacterium, may decrease (28). Furthermore, these efflux pumps may not be fully functional under the energy-restricted conditions of the agar-based in vitro assay. Considering all these limitations, the influence of in vitro experimental conditions on the results, and the challenges in standardizing the method, and in light of our study's data, we suggest that the EtBr cartwheel agar test is inadequate for determining the presence or absence of efflux pumps in *P. aeruginosa* isolates.

Finally, when the biofilm-forming characteristics of the isolates in our study were evaluated, it was determined that all isolates from the three centers were capable of biofilm formation, albeit weakly. While the isolates from Center G (65%) and H (75%) predominantly exhibited weak biofilm formation, the majority of isolates from Center E (57.9%) exhibited moderate biofilm formation. Reports in the literature indicating that isolates lacking the MDR phenotype can also show strong biofilm formation support the interpretation that biofilm formation is a virulence determinant independent of the resistance phenotype (29). In our study, the observation that the majority of isolates harboring multiple resistance mechanisms exhibited weak biofilm formation, and that isolates from Center E, which displayed a milder resistance phenotype compared to the other centers, predominantly exhibited moderate biofilm formation, also supports this interpretation.

Our study has several limitations. Although data were collected from three different centers, the relatively low total number of isolates limits the power of statistical comparisons between centers. However, it should be noted that the isolates represent invasive cases collected over a three-year period from three major tertiary healthcare centers in our country, which still provides valuable information on local epidemiology. Furthermore, other efflux systems among the major efflux pumps, such as MexCD-OprJ and MexXY-OprM, were not evaluated. Finally, it should be noted that all tests were conducted in vitro, and the results of the phenotypic assays, particularly those regarding biofilm formation and efflux pump activity, may not reflect the in vivo dynamics where the microorganism is exposed to antibiotics.

## Conclusion

This multicenter study indicates that carbapenem resistance is not a unidimensional process; rather, it is multifactorial, distinguished by complex interactions. The phenotypic-genotypic discordance and high clonal diversity observed at both intra- and inter-center levels cannot be solely attributed to the complex genomic structure of *P. aeruginosa* or the diversity of its resistance mechanisms. To manage this landscape, the integrated implementation of intra- and inter-hospital antimicrobial stewardship programs, along with nationwide standardization of antibiotic restriction policies and following local guidelines, is essential. To better explain the pathogen's genomic dynamics and resistance mechanisms, more comprehensive studies that use clinical data to evaluate behavior under in vivo conditions and apply advanced technologies such as NGS are required.

## Abbreviations

CAZ: Ceftazidime

CZA: Ceftazidime/avibactam

AK: Amikacin

MEM: Meropenem

CT: Colistin

PCR: Polymerase chain reaction

RT-qPCR: Quantitative real-time reverse transcription PCR

AP-PCR: Arbitrarily primed PCR

RND: Resistance-nodulation-cell division

MBL: Metallo-beta-lactamase

NGS: Next-generation sequencing

EtBr: Ethidium bromide

## Declarations

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### Author contributions

TH and IK conceptualized and designed the study, drafted the initial manuscript. Retrospective identification and confirmation of carbapenem-resistant isolates by PS, GH, TKA, and BB. Material preparation was carried out by IK, BB and TH. Data collection and analysis were performed by IK. The first draft of the manuscript was written by IK. TH critically reviewed the manuscript for important intellectual content. All authors reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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No funding was received to conduct this study.

## **Data availability**

No datasets were generated or analyzed during the current study.

## **Ethics approval and consent to participate**

Ethical approval for the study was obtained from the University of Health Sciences, Gülhane Scientific Research Ethics Committee (23.12.2025-2025/583). Consent to participate was waived by the University of Health Sciences, Gülhane Scientific Research Ethics Committee. This study was performed in line with the principles of the Declaration of Helsinki.

## **Consent for publication**

Not applicable.

## **Competing interests**

The authors declare no competing interests.

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## Figures

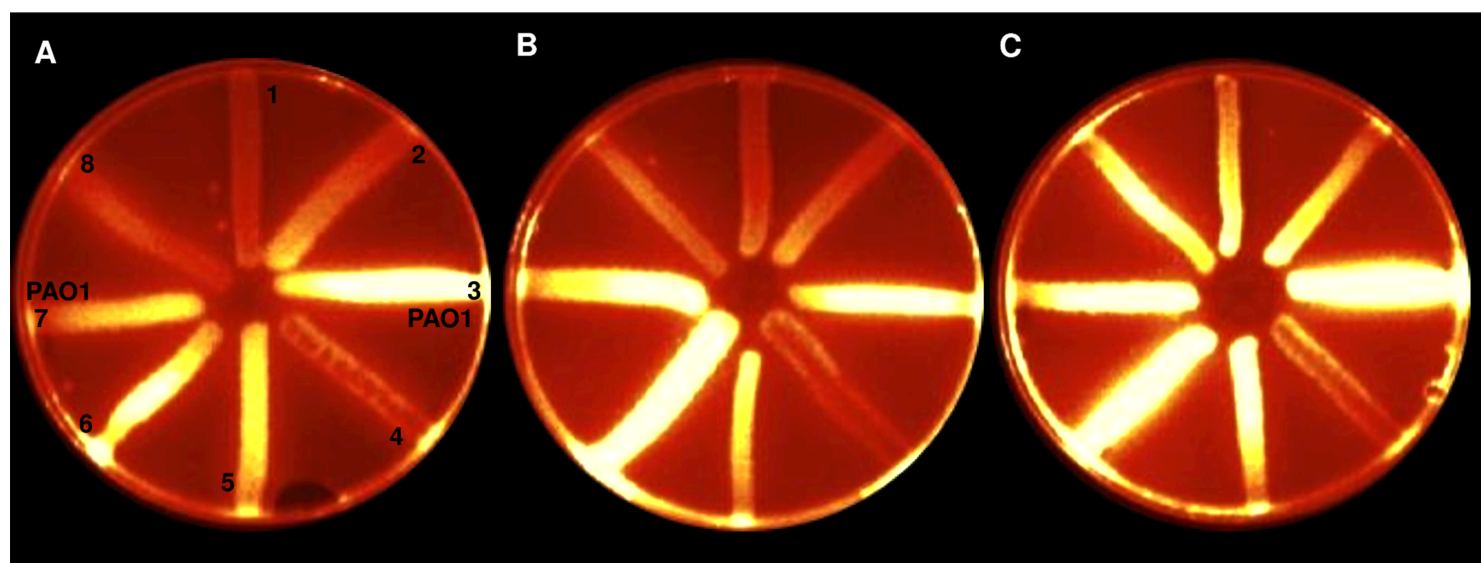


**Figure 1**

**Phenotypic detection of efflux pump activity in *P. aeruginosa* isolates.** Fluorescence visualization of isolates inoculated in a cartwheel pattern on TSA plates supplemented with increasing concentrations of EtBr. Positions 3 and 7 represent the reference strain PAO1, while the other numbers indicate the clinical isolates evaluated. **(A)** TSA containing 0.5-1 µg/mL EtBr. Isolates 5 and 6 exhibit mild efflux pump activity. **(B)** TSA containing 1.5 µg/mL EtBr. Isolates 1, 2, and 8 demonstrate moderate efflux pump activity. **(C)** TSA containing 2 µg/mL EtBr. Isolate 4 displays high efflux pump activity.

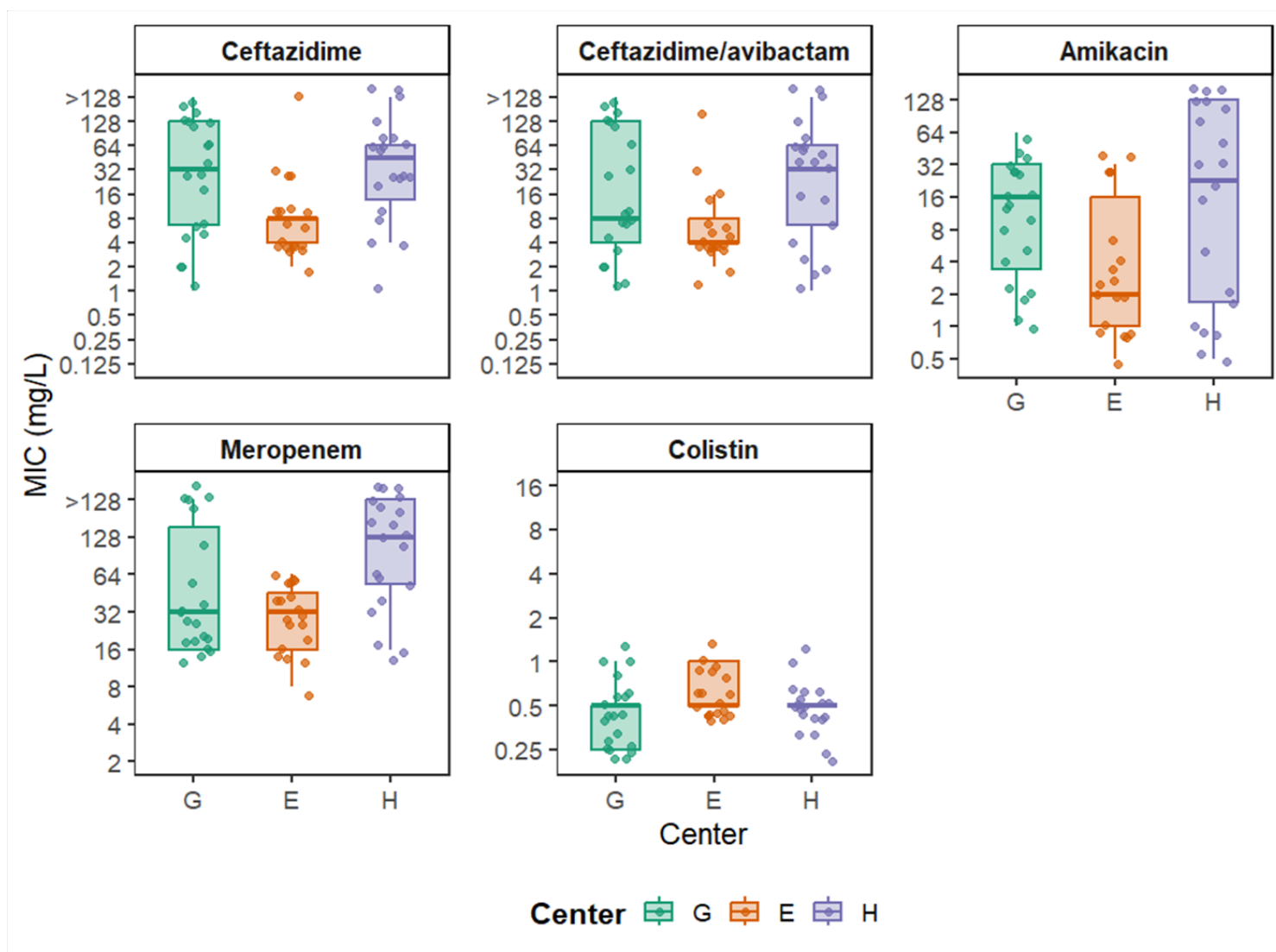


**Figure 2**

**MIC distribution profiles of the five evaluated antibiotics across the three centers, generated using R software.** The box plots represent the interquartile range (IQR), the horizontal lines indicate the median MIC values, and the whiskers show the range of the data. Individual isolate data points are overlaid with a slight jitter to visualize distribution density. (Center G: Gülhane Training and Research Hospital/Ankara, Center E: Erciyes University Hospital/Kayseri, Center H: Hacettepe University Hospital/Ankara)

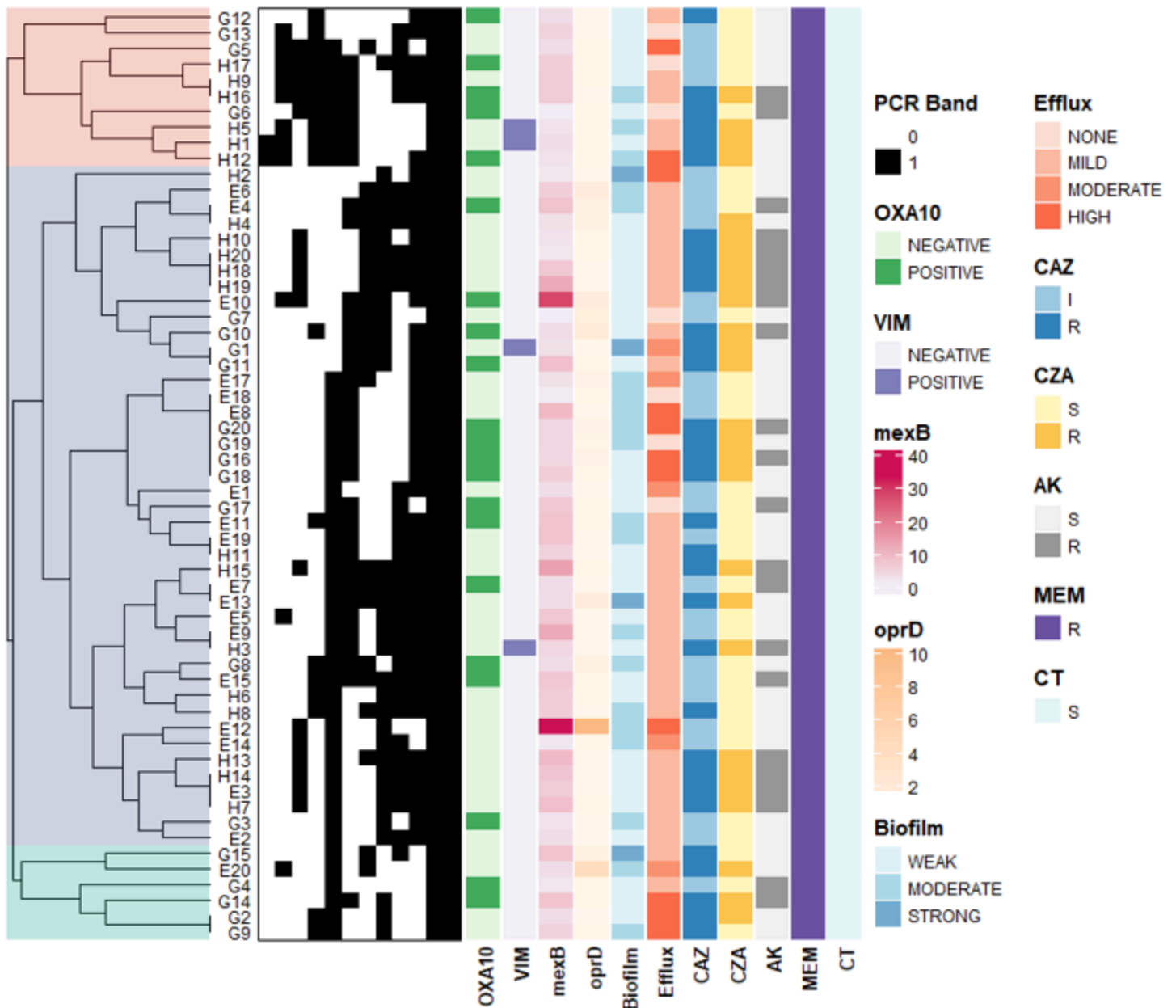


Figure 3

**Integrated hierarchical clustering and comprehensive phenotypic-genotypic profiling of *Pseudomonas aeruginosa* clinical isolates, generated using R.** The dendrogram on the left illustrates the clonal relatedness of the isolates, revealing three major clusters highlighted by distinct background colors (pink, blue, and green). The adjacent binary matrix denotes the presence (black square, 1) or absence (white square, 0) of specific polymerase chain reaction (PCR)-amplified target bands. The clinical source of each isolate (hospital wards and intensive care units) is indicated in the central text column. The right panel presents a multi-variable heatmap, color-coded to indicate both the presence or absence of specific categorical traits, and the relative magnitude (ranging from low to high) of continuous and ordinal variables. The far-right columns display the antimicrobial susceptibility profiles for ceftazidime (CAZ), ceftazidime/avibactam (CZA), amikacin (AK), meropenem (MEM), and colistin (CT), classified as

susceptible-standard dose (S), susceptible-increased exposure (I), or resistant (R) according to standard clinical breakpoints. (G: Gülhane Training and Research Hospital/Ankara, E: Erciyes University Hospital/Kayseri, H: Hacettepe University Hospital/Ankara)

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