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Analysis of Antibiotic Resistance Among *KPC-3* Positive *Pseudomonas aeruginosa* Strains Isolated from Intensive Care Unit Patients

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ABSTRACT

The increase in antibiotic resistance among microorganisms is an issue of utmost importance. Specifically, the resistance to carbapenem and extended-spectrum β-lactam antibiotics in gram-negative bacteria has had a considerable impact on patient morbidity and mortality rates. This research is centered around the examination of severe infections caused by Pseudomonas aeruginosa and *KPC-3* in patients who have been admitted to intensive care units.

P. aeruginosa strains were collected from the intensive care units of Van Training and Research Hospital. In order to conduct a thorough microbiological analysis of these strains, various methods including culture, biochemical tests, antibiogram tests, and polymerase chain reaction (PCR) were employed.

A total of 126 carbapenem-resistant *P. aeruginosa* strains were extracted from patients within intensive care units. Among these isolates, 17 were identified as being both multidrug-resistant and positive for *KPC-3*. Interestingly, all of the identified strains exhibited sensitivity to Amikacin, Piperacillin, Piperacillin/Tazobactam, Gentamicin, Colistin, Fosfomycin and Tigecycline.

The identification of *KPC-3* positive *P. aeruginosa* strains among patients in our hospital highlights a concerning issue. It has been observed that this presence increases the risk of both mortality and morbidity in affected patients. Consequently, it has been deemed crucial to address this matter in terms of hospital surveillance practices and the implementation of strategies to combat antibiotic resistance.

Keywords: *KPC-3*, *P. aeruginosa*, Antibiotics resistant

Introduction

Antimicrobial resistance (AMR) is recognized as a critical global health challenge, with the World Health Organization (WHO) identifying it as a significant threat in 2019 (1). The Centers for Disease Control and Prevention (CDC) estimate that over 2.8 million cases of antibiotic-resistant infections occur annually in the United States (1). Among bacterial species, Pseudomonas aeruginosa has emerged as a particularly concerning pathogen due to its role in severe nosocomial infections and its increasing resistance to carbapenems and other key antimicrobial agents. Surveillance data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) indicate a rising prevalence of antimicrobial resistance in invasive bacterial strains, including P. aeruginosa (2). The issue is especially severe in developing countries, such as Brazil, where Gram-negative bacilli (GNB) are frequent causes of life-threatening hospital-acquired infections (3). The Brazilian Health Surveillance Agency reports that 42.9% of P. aeruginosa isolates causing catheter-related bloodstream infections in intensive care units (ICUs) exhibit carbapenem resistance (3, 4). The high resistance rates in P. aeruginosa are attributed to multiple mechanisms, including overexpression of resistance-nodulation-division (RND) efflux systems, porin (OprD) loss, AmpC β-lactamase absence, and carbapenemase production (4). These adaptations, coupled with horizontal gene transfer through plasmids, significantly enhance its resistance profile and complicate treatment (5). As a major nosocomial pathogen, P. aeruginosa is associated with high morbidity and mortality rates, particularly in ICU settings. Its remarkable adaptability and ability to survive in diverse environments underscore its

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importance as a target for antimicrobial stewardship and novel therapeutic strategies.

The irrational use of antibiotics has led to an increasing proportion of multidrug-resistant (MDR) bacteria (6), with Enterobacteriaceae being the most prevalent. Carbapenems are commonly used to treat infections caused by MDR bacteria. However, the emergence of carbapenem-resistant organisms (CRO) renders these antibiotics ineffective and leads to clinical treatment failure, resulting in high mortality rates, especially in developing countries (7; 8). The primary cause of CRO is the presence of carbapenem-hydrolyzing enzymes, accounting for nearly 90% of CRO cases. A small proportion of CRO occurrence is attributed to the presence of highly efficient AmpC enzymes, which destroy membrane pore proteins or are highly expressed in the excretory system (9). There are several types of carbapenemases, including KPC, NDM, IMP, VIM, OXA-48, and similar enzymes (10). KPC, one of the most significant members of the carbapenemase family, was first identified in a *K. pneumoniae* isolate from a health center in North Carolina in 2001 (11). Since then, various types of KPC genes have been discovered worldwide (12, 13). KPC-3, a common variant of KPC, differs from KPC-2 in structure with His (272)-Tyr (14, 15). KPC-3 was first reported in 2004 at the New York Medical Center and has also been identified in Spain, Brazil, and Africa (16, 17). However, until recently, there have been limited reports of KPC-3 in China (18, 19). The KPC-3 sequence (GenBank AM774409) has been found in Salmonella cubana 4707 (GenBank AF481906) (20) within the same genetic environment as blaKPC-2. This KPC enzyme is now widespread in the USA (21), Israel (22) and the United Kingdom (23).

The objective of this study was to identify *P. aeruginosa* strains that were isolated from the blood of patients in the intensive care unit of our hospital, originating from various provinces within and surrounding Van province. The study aimed to assess their antibiotic susceptibilities and determine the presence of the KPC-3 gene. Additionally, the study aimed to contribute to the surveillance programs for nosocomial infections in this context.

Material and Method

The study focused on identifying carbapenem-resistant *P. aeruginosa* isolates obtained from patients diagnosed with bacteremia in the intensive care units (ICU) of our Van regional training and research hospital between 2021 and 2022. For the analysis of blood culture bottles, the Bactec/Alert 3D device (Biomerieux, USA), was utilized, and the bottles were monitored for a period of five days. Inoculations were carried out on 5% sheep blood agar (Acumedia, USA), as well as McConkey Agar (Oxoid, UK), Eosin Methylene Blue (EMB, Oxoid, UK) agar, and Pseudomonas agar (Millipore, UK). Petri plates were then incubated at 37°C for 48-72 hours, and the colony morphology of the cultures was carefully examined. Subsequently, important biochemical analyses such as oxidase, catalase, lactose test, sucrose test, glucose test, and gram staining were promptly conducted. Bacterial identification and antibiogram testing were performed using the Vitek 2 Compact device (Biomerieux, USA) (24). The following antibiotics, obtained from the antibiotic kit of the Vitek 2 Compact device, were used for testing: Ampicillin (AM), Amoxicillin/Clavulanic Acid (AMC), Amikacin (AK), Piperacillin (PRL), Piperacillin/Tazobactam (TPZ), Cefepime (FEP), Cefazolin (CZ), Cefoxitin (FOX), Cefuroxime (CXM), Ciprofloxacin (SPX), Cefuroxime Axetil (CXA), Colistin (CT), Ceftazidime (CAZ), Ceftriaxone (CRO), Ertapenem (ETP), Fosfomycin (FF), Imipenem (IPM), Gentamicin (CN), Levofloxacin (LEV), Meropenem (MEM), Nitrofurantoin (F), Netilmicin (NET), Aztreonam (ATM), Tobramycin (TOB), Tigecycline (TGC), and Trimethoprim/Sulfamethoxazole (SXT). The European Committee for Microbiological Resistance MIC (mg/L) threshold guideline was applied for the interpretation of the test results (25). Moreover, the presence of carbapenemase was determined using the Modified Hodge test (MHT), with the CLSI 2012 guideline serving as the reference (26, 27).

Bacterial DNA extraction was conducted at the Pharmaceutical Microbiology Laboratory of Van Yüzüncü Yıl University. To obtain bacterial colonies, incubation was carried out at 37°C for 24-48 hours on Tryptone Soy Agar (Accumedia, USA). DNA extraction from the colonies was then performed using the EcoSpin Bacterial Genomic DNA kit (Echotech Biotechnology, Turkey). Subsequently, DNA samples of multidrug-resistant and carbapenem-resistant Pseudomonas aeruginosa strains were obtained. These DNA samples were stored at -20°C for future use.

The DNA amplification of bacteria was carried out using the May Taq™ DNA Polymerase kit (Bioline, Bio-21105). For the Polymerase Chain

Reaction (mPCR), a set of chemical solutions and substances were prepared as follows: 20µL of 5x MyTaq reaction buffer (containing 5 mM dNTPs and 15 mM MgCl2), 10µL of template DNA, 2µL of each primer (20µM), 2µL of MyTaq DNA polymerase, and 16µL of nuclease-free water, resulting in a final solution volume of 50µL. The PCR conditions for amplifying the KPC-3 gene were as follows: an initial denaturation step at 94°C for 10 minutes, followed by denaturation at 94°C for 60 seconds, annealing at 52°C for 62 seconds, elongation at 72°C for 60 seconds, and a total of 35 cycles. A final extension step was performed at 72°C for 5 minutes. To accurately determine the sizes of the amplicons, the HyperLadderTM marker (50 Base Pair, Bioline, USA) was used. The PCR products were visualized by running them on a 2% agarose gel using a Thermo EC300XL2 electrophoresis device at 100 volts for 1.5 hours. The size of the amplicons was determined using the Bio-Print-ST4 system (Vilber Lourmant, France). For the KPC-3 gene amplification of *P. aeruginosa* strains, the following primers were used: Forward Primer: 5'-GCCTGGTCCGAATTCCCTCGTCATCCGCAG ACCAAC-3' (Positions: 727-747) Reverse Primer: 5'-GCCTGGTCCGGATCCCGCGCAGACTCCTA GCCTAAA-3' (Position: 2882-2902) (28).

Statistical Analysis: Descriptive statistics are given as numbers and percentages in terms of the characteristics discussed in the study. MINITAB (ver: 14) statistical package program was used for calculations.

Ethics Committee Approval: Our study received permission from the Van Training and Research Hospital, which was granted based on the recommendation of the clinical research ethics committee. This committee, formed within the hospital's own structure, evaluated the proposed study's application and determined that it adhered to appropriate standards for conducting research on patients. The decision to grant permission was made on 25/01/2018 and assigned the reference number 2018/02.

Results

A comprehensive analysis of 1,680 blood culture bottles collected from intensive care unit (ICU) patients identified 7.5% (126 isolates) as KPC-positive *Pseudomonas aeruginosa*. Among these, 1% (17 isolates) were classified as multidrug-resistant (MDR) strains. Polymerase chain reaction (PCR) analysis confirmed the presence of the bla_{KPC-3} gene in these MDR isolates, which were subsequently included in the study. Antibiotic susceptibility testing revealed that the majority of these isolates exhibited varying degrees of susceptibility to amikacin (AK), piperacillin (PRL), piperacillin-tazobactam (TPZ), gentamicin (CN), ceftolozane-tazobactam (CT), fosfomycin (FF), and tigecycline (TGC). However, resistance was observed against a broad range of antibiotics, with particularly high resistance rates to β-lactam antibiotics, including cefepime (FEP) and ceftazidime (CZ). Additionally, the KPC-3-positive P. aeruginosa strains displayed extensive resistance to carbapenems, such as ertapenem (ETP), imipenem (IPM), and meropenem (MEM). Resistance to quinolone antibiotics was also universal among these isolates. These findings highlight the critical role of *P. aeruginosa* as a multidrug-resistant nosocomial pathogen and underscore the therapeutic challenges posed by KPC-producing strains in ICU settings. Addressing these resistance mechanisms requires robust infection control measures and the development of novel antimicrobial agents.

Discussion

Multi Drug Resistant *P. aeruginosa* is recognized as a significant concern in healthcare settings, leading to challenging treatment scenarios and increased mortality rates, particularly among individuals with weakened immune systems (29; 30). According to a report by ECDC in 2016, *P. aeruginosa* is the predominant microorganism associated with hospital-acquired pneumonia and represents one of the most frequently identified bacteria in bloodstream infections among intensive care patients (31). Furthermore, the WHO has classified *P. aeruginosa* as a priority pathogen for the development and discovery of new antibiotics (32).

Since its first report in 2007 (33), the prevalence of KPC-positive *P. aeruginosa* isolates has been increasing, particularly in Asia and South America. Notably, China, Brazil, Colombia, and Puerto Rico have reported the highest number of cases. The Antimicrobial Testing Leadership and Surveillance program (34) indicates that *P. aeruginosa* clinical strains exhibited the highest resistance to carbapenems in the Middle East, followed by South America, Europe, and North America (35). This suggests that the rise and prevalence of carbapenem resistance mechanisms in *P. aeruginosa*, including KPC and other carbapenemases such as VIM, as well as resistance mechanisms like OprD

porin suppression and efflux pump overexpression, may contribute to these trends (36, 37, 38). Though the KPC gene is more commonly associated with *K. pneumoniae* (39), the findings suggest its potential role in the genomic adaptability of *P. aeruginosa* and its capability to thrive in diverse environments across 14 countries (35). Our study identified *P. aeruginosa* strains that were colonizing both in patients and hospital settings, indicating their significance as a potential source of nosocomial infections. Thus, continuous monitoring of these strains is crucial.

Previous mathematical research has demonstrated that the KPC-2 variant remains the most widespread and successful globally, with the exception of KPC-5, which was reported in Puerto Rico. Recent reports from Zhejiang, China have identified two new variants, KPC-33 and KPC-90, both belonging to the ST463 type (36, 40). While all strains showed resistance to carbapenem antibiotics, certain KPC-90 positive variants exhibited resistance to more modern antibiotic combinations. For instance, carbapenem-resistant *P. aeruginosa* (CRPA) isolates carrying the blaKPC-2 gene were found to display stronger resistance to ceftazidime-avibactam (CZA) treatment (40). Furthermore, a strain that was not included in the initial review has been recently identified (41). This strain represents the first notification of the blaKPC-3 gene in *P. aeruginosa* and is associated with the Tn4401b transposon, commonly found in high-risk, pandemic ST111 clones. The occurrence of the second most common KPC-3 variant (42) within a highly active transposon implicated in KPC dissemination in carbapenem-resistant Enterobacteriaceae (43) warrants attention and suggests a potential new pathway for the spread of this enzyme. Moreover, since the completion of this research, additional reports of KPC-3 variants have emerged, such as KPC-31, which shows concurrent resistance to CZA and is found in a high-risk ST235 clone (44). These findings underscore the importance of ongoing surveillance. Our study revealed that multidrug-resistant *P. aeruginosa* isolates posed a considerable risk of piperacillin-tazobactam resistance.

A recent report from China has unveiled the presence of the blaKPC-3 gene, marking the first occurrence of this gene in the country (45). Across different parts of the world, a total of nine distinct KPC variants have been identified, ranging from KPC-2 to KPC-10. Although KPC-1 and KPC-2 show complete identity, it has been observed that these variants may differ by up to two amino acid substitutions (46, 47). The emergence of carbapenem-hydrolyzing enzymes with KPC properties raises concerns. In a study, *E. coli* transconjugants containing the blaKPC-3 gene, obtained from two clinical strains, exhibited minimum inhibitory concentrations (MICs) of 2 μg/mL for both imipenem and meropenem. Notably, the clinical strains C. freundii HS70 and E. coli HS510 were found to harbor different mechanisms of drug resistance, as their MICs for imipenem and meropenem were higher compared to those of *E. coli* transconjugants. Previous research has indicated a strong connection between the absence of regulated porins, specifically OmpK36 and OmpK35, and the rate at which MICs for carbapenems increase (48). In a study conducted in Turkey by Tuna et al (49) isolated Pseudomonas strains of the hospitalized patients were most commonly found in the chest diseases service (38.6%), urology department (14.3%) and palliative care unit (12.5%). The distribution of samples was followed by sputum (20.4%) cultures, most commonly urine (42.7%). In our study, according to antibiotic resistance rates, the highest drug resistance was observed against cefuroxime, levofloxacin and netilmicin and the lowest resistance was against amikacin. Gentamicin, cefepime, and aztreonam resistance levels significantly decreased with time (P=0.0004, 0.0038, and 0.0321, respectively), but levofloxacin and colistin resistance levels significantly rose (P=0.0407 and, P<0.0001 respectively). Over time, there have been appreciable reductions in resistance to ciprofloxacin, ceftazidime, and cefepime (P=0.0004, 0.0038, and 0.0321, respectively). Only cefepime showed a significant decline in resistance of bacteria isolated from urine culture over time (P=0.0003). Resistance to levofloxacin substantially increased in 2019 for bacteria isolated from cultures of respiratory secretions, urine, and, sputum whereas it increased in 2020 for strains isolated from wound cultures (P=0.0145). In the study conducted by Ağuş et al (50) in Turkey, Ninety-five *P.aeruginosa* strains isolated from various clinical specimens taken from the patients in our Anesthesia Intensive Care Ünite during 2007. Microorganisms were identified by conventional methods and VITEK 2 identification system. Antibiotic resistance were investigated by using E- test method and MIC 50 and MIC 90 values were calculated. Resistance rates were found 39% to S-Sui, 41% FlP/TZ, 43% to IMP. MIC 50 and MIC 90 values were found 16-192, 32-192, 3-32 respectively. This situation once again reveals that reasonable antibiotic usage mandatory. The local antibiotic susceptibility

profiles of *Pseudomonas* spp. Should be surveyed continuously to avoid the spread of Intensive Care Unite isolates carrying high level antibiotic resistance. In a study conducted by Uyar et al (51) Total of 529 *P. aeruginosa* strains isolated from blood culture samples sent from ICUs to microbiology laboratory in a ten years (January 2013–April 2023). Highest resistance percentage among *P. aeruginosa* strains was to imipenem with 50.2% rate. Followed by Meropenem (37.6%), ciprofloxacin (35.7%), ceftazidime (33.4%), piperacillin-tazobactam (31.6%), cefepime (31.6%), gentamicin (19.3%), and amikacin (19.1%). Among 433 isolates, 44 (10, 2%) were defined as colistin resistant. Ceftazidime-avibactam resistance was detected in 42 of 217 (19, 4%) tested strains. In our study, *P. aeruginosa* strains that were positive for KPC-3 displayed multidrug resistance, with high levels of resistance observed against carbapenems such as imipenem, meropenem, and ertapenem.

In a study focusing on conjugation analysis, plasmid restriction enzyme digestion analysis, and PCR of the β- lactamase gene, it was revealed that the blaKPC-3 gene found in two distinct clinical strains was located on the same conjugating plasmid (45). Moreover, sequence analysis demonstrated that the 3.8 Kb DNA sequence encompassing the blaKPC-3 gene in both strains was entirely identical and structurally matched the strains AM774409.1 from Enterobacter cloacae and EU176014.1 from Klebsiella pneumoniae (45). This study provides further clarification and confirmation of a potential transposable element present in both *K. pneumoniae* and *P. aeruginosa* plasmids, which could be accountable for the rapid global dissemination of this gene. Interestingly, the region between the blaKPC-3 promoter and the blaKPC-3 coding region shows the presence of a 671 bp fragment insertion. This finding supports Naas' suggestion that this region of the element exhibits an unstable structure (52), and it suggests the existence of other variants of Tn4401. Naas et al. previously identified a 100 bp deletion upstream of blaKPC in two clinical strains of *K. pneumoniae* (52).

In conclusion, our study conducted at a regional hospital identified *P. aeruginosa* strains in blood samples that exhibited multidrug resistance. Of particular significance is the fact that these strains also displayed resistance to carbapenems. Further analysis revealed the presence of the KPC-3 gene among the carbapenem-resistant isolates. These findings emphasize the importance of implementing surveillance measures to monitor the prevalence of KPC-3 carriage in *P. aeruginosa* strains isolated from blood samples within the hospital.

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