Molecular Detection of MBL Encoding Genes Among Carbapenemase-Producing *Pseudomonas Aeruginosa* Isolated from Sulaimaniyah Hospitals

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ABSTRACT

Background: *Pseudomonas aeruginosa* can quickly develop antibiotic resistance mainly through chromosomal mutations, and its carbapenem-resistant resulting from metallo-β-lactamases (MBL) has been reported to be a significant cause of nosocomial infection. Objectives: To characterize the antibiotic resistance profiles, detect carbapenemase production, and identify MBL encoding genes in *P. aeruginosa* isolates.

Materials and Methods: *P. aeruginosa* was isolated (n=59) from patients at Hiwa Hematology/Oncology (n=31) Hospital and High-Quality Hospital (n=28) from June 2022 to January 2023. An antimicrobial susceptibility test, a CarbaNp test, and a combination detection test (CDT) were used to identify MBL producer isolates. Additionally, PCR targeting MBL-encoding genes for blaVIM, blaVIM, blaIMP, blaSPM, blaSIM and blaGIM was performed to provide additional confirmation.

Results: Approximately 37% of the isolates exhibited multi-drug resistance (MDR), while 22% showed extensive drug resistance (XDR). About 30% of the isolates were resistant to carbapenem. Among carbapenem-resistant *P. aeruginosa* (CRPA), 61% and 56% had positive results for imp/EDTA combined disc and RAPIDEC® CARBA NPs, respectively, while 67% harbored MBL-encoding genes. blaVIM-2 was identified as the most prevalent gene (66.6%), followed by blaIMP-1 (16.6%), and then blaSIM-1 (11.1%).

Conclusions: Multiplex PCR is a rapid and accurate technique to detect MBL-encoding genes. The blaVIM gene has been identified as the primary genetic determinant associated with carbapenemase production.

Keywords: *P. aeruginosa,* antibiotic resistance, metallo-β-lactamase genes, carbapenemase production.

INTRODUCTION

P. aeruginosa is an opportunistic bacterium that causes a wide range of illnesses and is commonly responsible for nosocomial infections[1] and other diseases including and causes various diseases including urinary tract infections, pneumonia, soft-tissue infection and sepsis[2]. *P. aeruginosa* uses a combination of inherent, acquired, and adaptive resistance mechanisms to fight against antibiotics; thus, most anti-pseudomonal antibiotics exhibited efficacy against <80% of isolates[3].

Enzyme synthesis, especially B-lactamase production, is the primary mechanism of acquired resistance in *P. aeruginosa*. Carbapenems are the medicine of last choice for treating infections caused by *P. aeruginosa* because of the rise in strains expressing extended-spectrum B-lactamase (ESBL). However, the alarming rise in carbapenem resistance is a significant cause for concern[4]. The World Health Organization (WHO) has named 12 bacteria as severe threats to the health of humans, and carbapenem-resistant *P. aeruginosa* (CRPA) are included in this category[5].

The Metallo-β-lactamase (MBL) genes have been detected in mobile genetic elements such as plasmids, transposons, and integrons. Furthermore, these genetic elements have been discovered to be linked with insertion sequences and have exhibited a tendency to spread both within and between different species[5]. The emergence of *P. aeruginosa*-acquired MBL is problematic and poses a severe threat during treatment and infection control[2].

Molecular/biochemical techniques have been employed to investigate MBL producer *P. aeruginosa*. Multiple MBL genes, such as Verona integron-mediated metallo-β-lactamase (VIM), imipenemase (IMP), Seul imipenemase (SIM), new Delhi Metallo β-lactamase (NDM), German imipenemase (GIM), Australian imipenemase (AIM), and Sao Paulo MBL (SPM) have been found in *P. aeruginosa*. The majority of acquired MBLs are VIM and IMP genes. Japan was the first country to report MBL resistance in 1991; since then, it has been reported in other countries in Asia, the Middle East, Europe, South America, and North America[6, 7].

According to the research, the mortality rate from MBL-producing *P. aeruginosa* is very high, ranging from 70-90%[8]. Therefore, the current study aimed to determine antibiotic susceptibility and phenotypic and genotypic identification of MBL-producing *P. aeruginosa* isolated from patients of various diseases in Sulaimaniyah City, Iraq.

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MATERIALS AND METHODS

Study Setting: Over seven months spanning from June 2022 to January 2023, the Microbiology Laboratory at a High-Quality Hospital in Sulaimaniyah, Iraq, received a total of 185 clinical samples (blood, urine, wound swabs, sputum, and endotracheal discharges) from patients at Hiwa Hematology/Oncology Hospital and High-quality Hospital.

Bacterial Isolates: Following conventional microbiological procedures, collected samples were cultured on Blood agar (HiMedia, India) and MacConkey agar (HiMedia, India), then incubated at 37°C for 18–24 hours in an aerobic atmosphere. Then, *P. aeruginosa* was isolated and identified based on its Gram-negative staining, colony properties, odor, production of pigment, and the oxidase test. Furthermore, the verification of *P. aeruginosa* isolates was validated by BD Phoenix™ automated identification/susceptibility testing system (BD, M50, USA).

DNA Extraction: Bacterial DNA was extracted using a kit (Geanaid, Taiwan) according to the manufacturer's instructions. Afterwards, DNA's purity and quantity were evaluated using a Nanodrop spectrophotometer (Thermo Scientific, USA).

Primer design and PCR condition: The primer sets of PA-SS-F and PA-SS-R were developed to selectively amplify *P. aeruginosa* at the species level[9]. The total volume of the polymerase chain reaction (PCR) was 20 µl that, consisted of 5.0 µl master-mix (GeneDirex, Taiwan), 2.0 µl DNA template, 0.5 µl 10× of each primer (Sinaclone, Iran), and 12 µl nuclease-free H_2O.

Antibiotic Susceptibility Test (AST): The susceptibility of all *P. aeruginosa* isolates to 14 antimicrobial agents was tested using the BD Phoenix system model (BD, M50, USA) and AST-GN30, following the manufacturer's instructions. The obtained minimum inhibitory concentration (MIC) values were used to categorize samples as susceptible, intermediate, or resistant based on the CLSI MIC breakpoints[10].

Phenotypic detection of carbapenemase

Combination disc test (EDTA-CDT): EDTA solution (0.5 M) was prepared by dissolving 18.6 g EDTA (Sigma-Aldrich, Germany) in 100 mL distilled water with thorough mixing, and the pH was adjusted to 8.0. Bacterial isolates were diluted to 0.5 MacFarland, streaked onto Mueller-Hinton Agar (MHA) plates, and incubated overnight at 37 ºC. Then, imipenem (IMP) discs were put on the plate, and EDTA solution was poured onto them. Positive results were regarded when the inhibition zone of the IMP-EDTA disc was 7 mm larger than that of the IMP disc alone[11].

Rapidec Carba NP Test: Following the manufacturer's instructions, carbapenemase-producing *P. aeruginosa* isolates were identified using the Rapidec Carba NP Test (bioMe'rieux SA, France). This assay is based on the hydrolysis of the β-lactam ring of carbapenem. Following overnight growth on MHA plates, the isolates were inoculated into the wells of the test strip and incubated again at 37 °C for 30 min. Positive results were reported when a colour changed from red to yellow or orange within 30 min. In cases where such a change did not occur, the incubation time was prolonged to 2 hrs[12].

Molecular detection of carbapenemase : Carbapenemase-resistant P. aeruginosa isolates were screened for blaVIM, blaIMP, blaSPM, blaGIM, and blaSIM using multiplex PCR assay[13]. Primer sequences, PCR conditions, and amplicon sizes are detailed in Table 1. The efficacy of these primers has been previously evaluated using 11 reference strains that are known, through DNA sequencing, to express IMP-1, -2, -4, and -7, -12, VIM-1, -2 and -7, SIM-1, GIM-1, and SPM-1 enzymes[13].

Gel electrophoresis and imaging: After amplification, the separation of PCR products was achieved using agarose gel electrophoresis (Cleaver, Scientific Ltd., UK) in 10× TBE buffer at 80 V for 60 min. Then, the gel was stained with ethidium bromide (EtBr) (10 g/ml), and images were captured using Gel Doc XR+ (Bio-Rad, USA)[14].

RESULTS

Antibiotic susceptibility test: A significant proportion of the isolates exhibited resistance to various antibiotics. Most of the isolates were resistant to aminoglycosides (71.1%), fluoroquinolones and monobactam (52.5%), third-generation cephalosporins (50.8%), penicillin (40.6%), carbapenems, and piperacillin/tazobactam (30.5%). Interestingly, colistin showed the best activity against isolates, and only 3.3% of isolates were resistant (Figure 1).
Furthermore, the isolates were classified as multidrug-resistant (MDR), indicating resistance to at least one agent in ≥3 antimicrobial classes, or extensively drug-resistant (XDR), indicating resistance to at least one agent in all but two antimicrobial classes, based on their phenotypic resistance patterns.

Twenty-two (36.67%) isolates demonstrated MDR and displayed 17 distinct patterns, while 13 isolates (22%) were identified as XDR and exhibited 3 different patterns. Among them, pattern 1 was the most commonly observed (9/13) and resisted all tested antibiotics except colistin (Table 2 and Table 3).

Phenotypic detection of carbapenemase-producing *P. aeruginosa*: The study employed BD Phoenix™ automated susceptibility testing (MIC values), Imipenem EDTA Combined Disc Test (CDT), and Rapidec Carba NP test to determine the resistance of isolates to carbapenem. Out of the total isolates, 18 (30.5%) were found to be carbapenem-resistant using BD Phoenix™ (MIC values), 11 (18.6%) using Imipenem EDTA CDT, and 10 (16.9%) using Rapidec Carba NP test. The remaining 41 (69.4%) isolates were determined to be non-carbapenem resistant (Figure 2 and Table 4).

Among *P. aeruginosa* carbapenemase producer (n=18), 6 (33.3%) were MDR, 11 (61.1%) were XDR, and 1.0 (6%) was non-MDR. Notably, 2 (11.1%) MDR-CRPA strains resisted colistin (Figure 3).

Molecular detection of MBL encoding genes: Multiple-PCR on 18 CRPA isolates was conducted to identify the existence of metallo-beta-lactamase (MBL)-encoding genes. Then, it was confirmed that 12 (67%) of them contained genes encoding carbapenemase. The results of the PCR indicated that 12 (66.6%) CRPA isolates harbour the blaVIM gene, 3 (16.6%) contain the blaIMP gene, while blaSIM-1 was found in 2 (11.1%) isolates (Figure 4).

Interestingly, in one isolate, three distinct genes (blaVIM, bla IMP, and blaSIM-1) were detected; another isolate harboured two different genes (blaVIM and blaIMP), and in a single isolate, the co-existence of blaVIM and blaSIM-1 genes was recorded (Table 5).

DISCUSSION

aeruginosa infections are effectively treated by carbapenem; however, resistance toward these antibiotics spread across hospitals due to the extensive use of antibiotics[2]. Thus, we focused on this topic in our

local hospitals in order to screen the rate of CRPA and study the genes responsible for carbapenem resistance among our isolates.

In this study, we indicated that a notable percentage of *P. aeruginosa* isolates had MDR (36.67%) and XDR (22%) resistance phenotypes. In this regard, local very recent studies have reported an incidence rate of MDR and XDR-PA to be 76% and 20% in Erbil[15], 72.6% and 91.3% in Basrah[16], 34.7% (only MDR) in Sulaimaniyah[17], and 30.8% with 33.3% in Diyala, respectively[18]. According to data from neighboring countries, the rates of MDR *P. aeruginosa* were reported as 54% in Syria in 2015[19], 52.5% in Jordan in 2018[20], 13.8% in Kuwait in 2020[21], 7.3% in Kingdom of Saudi Arabia (KSA) in 2020[22], and 58% in Iran in 2019[23]. The mentioned local and regional data highlight the continuing rise in drug resistance rates among *P. aeruginosa* isolates.

Furthermore, the present Investigation has revealed a remarkably high prevalence of CRPA isolates (31%). There has been a notable shift in the resistance pattern of *P. aeruginosa* clinical isolates towards carbapenem antibiotics in many hospitals across Iraq, including the Kurdistan Region. These outcomes are consistent with findings reported in other Iraqi cities, including Wasit in 2018 (34.95%)[24], Al-Diwaniyah in 2019 (24%)[25], Duhok in 2021 (33.3%)[26] and Erbil in 2023 (23%)[15].

Earlier research, however, found a significantly lower incidence of CRPA at the surveyed hospitals in Baghdad (8%)[27] and Duhok (12.7%)[28] during 2011 and 2014, respectively. A study from KSA found that *P. aeruginosa* susceptibility to carbapenem dramatically decreased between 2004 to 2009 from 66 to 26%[29]. However, the prevalence of CRPA has experienced a significant increase in the Middle East, particularly in KSA, UAE, Oman, Kuwait, Qatar, and Bahrain. This increase has been observed over the past decade. A recent study reported that CRPA ranged from 30% to 71% in these countries[29].

Researchers from Iran recorded that 70% of *P. aeruginosa* conferred resistance to carbapenem in burn patients[30]. At the same time, another investigation in Turkey recorded 99% resistance toward imipenem and 74% resistance toward meropenem[10].

Moreover, it has been determined that 66.6% of the CRPA isolates were positive for the blaVIM gene, while 16.6% and 11.1% of the isolates were positive for the blaIMP and blaSIM-1 genes, respectively. The predominance of the blaVIM gene among CRPA isolates was reported locally earlier in Sulaimaniyah[31], Erbil[15], Duhok[26], Najaf[32] and Al-Diwaniyah[33], and even in other countries such as Iran[30], Bahrain[34], and Egypt[35].

The blaIMP gene followed the blaVIM gene in this study, which agreed with another local study in Sulaimaniyah[31] and Najaf cities[32] and a regional study in Egypt[35]. Regarding blaSIM-1 gene detection in *P. aeruginosa*, several studies have been conducted in Iraq, including Baghdad[36] and Basrah[16]. However, the existence of this gene in 33.3% of carbapenemase-producing *P. aeruginosa* in Al-Diwaniyah was reported[33].

Furthermore, in this study, 6 (33.3%) isolates did not show amplification of any of the genes being studied. Additional resistance mechanisms, genes, and gene variants not investigated in this work may be present in these specific isolates. This research shows that multiplex PCR may detect carbapenemase genes rapidly and accurately within a few hours. However, it is essential to consider the geographical regions where specific resistant genes exhibit a higher prevalence.

The remarkable resistance exhibited by *P. aeruginosa* is attributed to its possession of diverse mechanisms of antibiotic resistance. These mechanisms include intrinsic factors such as low outer membrane permeability; efflux pumps overexpression, and the production of enzymes that inactivate drugs. Additionally, it can be attributed to chromosomal mutations and the acquisition of resistance genes through horizontal gene transfer. Furthermore, in adaptive scenarios, *P. aeruginosa* forms biofilms that act as barriers, restricting antibiotic access.

In light of the excessive utilization of carbapenem within our medical facilities and the existence of resistant genes on mobile genetic elements may have played a significant role in the dissemination of resistance among the isolates[2].

CONCLUSION

The blaVIM gene was found to be the most common genetic determinant responsible for carbapenemase production. It is crucial to emphasize the significance of employing phenotypic and genotypic methods in detecting MBL production during routine antibiotic testing. This is a critical step for rapidly detecting carbapenem resistance and preventing the dissemination of this microorganism.

Authorship Contribution: All authors share equal effort contribution towards (1) substantial contributions to conception and design, acquisition, analysis and interpretation of data; (2) drafting the article and revising it critically for important intellectual content; and (3) final approval of the manuscript version to be published. Yes.

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