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Molecular Detection of Carbapenem's Genes Among (*Pseudomonas Areuginosa*) Isolates

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Abstract: *Researching carbapenem-resistant isolates enables the identification of carbapenem's-producing bacteria and prevents their spread. The antibiotic susceptibility of 125 P. aeruginosa isolated from 215 clinical samples (blood, urine, wound swab samples, sputum, and cerebrospinal fluid) was determined using the disk diffusion method on the Muller-Hinton agar medium. The results showed that the isolates had a high resistance rate to carbenicillin (83.2%) and piperacillin (76%). Resistance to Imipenem and meropenem were 7.4% and 14.8%, respectively, while the highest resistance was found against gentamicin (77.9%) and amikacin (75.2%). The modified Hodge test was used to identify carbapenem-resistant isolates, which detects the probability of isolates being able to produce carbapenem's enzyme, and out of the isolates, 11 (33.3%) were Hodge positive. The imipenem-meropenem-EDTA disk synergy test showed that only 17 (51.5%) isolates were positive. The carbapenems' genes were detected using PCR technology, and the most prevalent genes were metallo-beta-lactamase (MBL) genes, which were carried by 17/33 (51%) of the isolates. The bla_{IMP} gene (578 bp) was the most frequently detected MBL gene present in 15 (45.5%) of the carbapenem-resistant isolates. Among the isolates, 11 (33.3%) carried the bla_{IMP}-type gene alone, while 2 (6.06%) carried it with the bla_{VIM}-type gene, and another 2 (6.06%) carried it with the bla_{NDM}-type gene. Moreover, 2 (6.06%) isolates carried the bla_{VIM}-type gene. However, results revealed that bla_{IMP}-type genes were the most common MBL gene combination among the isolates. Moreover, 4 (12.1%) isolates carried a bla_{IMP} -type gene.*

Keywords*: *Antibiotic susceptibility, Bacterial infections, Carbapenem's genes, Molecular detection,

1.Introduction

Pseudomonas aeruginosa is a multi-drug resistance (MDR) opportunistic pathogen, causes acute or chronic infection in immune - compromised individuals with chronic obstructive pulmonary disease (COPD), cystic fibrosis, cancer, traumas, burns, sepsis, and ventilator-associated pneumonia (VAP) including those caused by COVID-19 [1].

Carbapenems are a group of β-lactam antibiotics with a broad spectrum of antibacterial activity. Their structure makes them highly resistant for most β-lactamases [2]. They include meropenem and Imipenem, among the few therapeutic options still available for treating infections caused by *P. aeruginosa* [3]. Although the relative contribution of different carbapenems resistance mechanisms is not well established, Carbapenems resistance in *P. aeruginosa* was reported to increase steadily over the years across the world [4].

Most prevalent β-lactam resistance mechanisms in *P. aeruginosa* PAO1 clinical isolates include β-lactamase derepression (AmpC; penicillins and cephalosporins affected to different degrees), which causes a reduction of the effective concentration of labile drugs in the periplasm and activation or derepression of active efflux systems (MexAB-OprM; penicillins, cephalosporins, monobactams, and meropenem), decreasing the periplasmic concentration of the affected drugs and decreasing outer membrane (OM) expression of the specific porin OprD (imipenem and other carbapenems to a lesser degree) [5].

This study aims to investigate the phenotypic and genotypic characteristics of carbapenemase-producing *P. aeruginosa* isolates from patients in Najaf hospitals.

2. Methodology

Samples Collection and Culturing

One hundred twenty-five (125) clinical *P. aeruginosa* isolates were recovered from different units, and samples (blood, urine, wound swab sample, sputum, cerebrospinal fluid (CSF)) were isolated from Al-sadder medical City/Najaf-Iraq during the period from August 2022 to February 2023. The isolates were identified by conventional methods and the automated Vitek 2 Compact (BioMerieux, France) system. All samples were cultured on blood agar, MacConkey agar, and brain heart infusion broth. bacterial diagnosis depended on morphological features of the colonies and microscopically examination with gram stain as reported by [6].

Antibiotic Susceptibility Assays

Disk Diffusion Method

Susceptibility of all *P. aeruginosa* isolates were evaluated against the commonly used antipseudomonal agents, including Carbenicillin; Piperacillin; Piperacillin-tazoba - ctam; Ceftazidime; Cefotaxime; Ceftriaxone; Cefepime; Imipenem; Meropenem; Amikacin; Gentamicin; Ciprofloxacin. The disk diffusion method on the Muller-Hinton agar medium was used based on the way of (35), and bacterial growth inhibition zones around the disks was measured in (mm) and interpreted as recommended by the National Committee for Clinical Laboratories Standards guidelines (30).

Detection of carbapenem's Production

-Double Disk Synergy Method

MBLs were detected by the double disk synergy method according to [7].

-Modified Hodge Test (MHT)

Detection of MHT was performed according to [8]. The MHT was considered positive if *E. coli* ATCC 25922 growth was observed within the inhibition zone of the imipenem disk. The MHT negative test has no E. coli ATCC 25922 growths along the test organism.

Molecular Determination of Antibiotic Resistance Mechanisms

DNA Extraction and PCR assay

Genomic DNA was extracted from bacteria by using DNA extraction kits (Promega/USA). The protocol used depends on the manufacturer's instructions. All PCR components were assembled in a PCR tube,

which includes 12.5 µl Master mix 2X, 2.5 µl Primer forward (10µM), 2.5 µl Primer reverse (10µM), 5 µl DNA template, and 2.5 µl Nuclease free water. The final volume 25µl. Primers for Detection of Selected Genes in *P. aeruginosa* as in table 1.

Table (1). Primers for detection of selected genes in *P. aeruginosa*

Primer	Gene Name	Sequence (5'-3')	Amplicon Size (bp)	Ref.
KPC	***bla*$_{KPC}$**	**F**: ATGTCACTGTATCGCCGTCT **R**: TTTTCAGAGCCTTACTGCCC	**882**	31
IMP	***bla*$_{IMP}$**	**F**: CATGGTTTGGTGGTTCTTGT **R**: ATAATTTGGCGGACTTTGGC	**488**	32
VIM	***bla*$_{VIM}$**	**F**: ATTGGTCTATTTGACCGCGTC **R**: TGCTACTCAACGACTGAGCG	**780**	32
NDM	***blaNDM***	**F:** ACC GCC TGG ACC GAT GAC CA **R:** GCC AAA GTT GGG CGC GGT TG	**264**	33
OXA-23	***bla*$_{OXA-23}$**	**F**: AAGCATGATGAGCGCAAAG **R:** AAAAGGCCCATTTATCTCAAA	**1066**	34

PCR Thermocycling Conditions

The PCR tubes were placed on the PCR machine, and the right PCR cycling program parameters conditions were installed as shown in Table (2).

Table (2): Programs of PCR Thermocycling conditions for Carbapenemase genes detection.

Gene Name	Temperature (C°)/Time					Cycle No.
	Initial Denaturation	Cycling Condition			Final Extension	
		Denaturation	Annealing	Extension		
bla$_{IMP}$	94/3 min	94/45 sec	55/1 min	70/3min	70/5 min	34
bla$_{NDM}$	95C/2 min	95/30 sec	63.4/ 30sec	72/ 30se	72/5 min	35
bla$_{VIM}$	93/3 min	93/1 min	55/1 min	72/1 min	72/7 min	40
bla$_{OXA-23}$	95/5min	95/1 min	62/ 1min	72/1 min	72/10 min	33
bla$_{KPC}$	95/15 min	94/1 min	62/1 min	72/1 min	72/10 min	40

Agarose Gel and DNA Loading

The gel preparation performed by [9]. Afterwards, five microliters of the amplified PCR product were loaded into the wells of the agarose gel, and a 5 µl DNA ladder was used as a molecular standard for size comparison. The gel tray was then secured in the electrophoresis chamber, and IX TBE buffer was added until it covered the surface of the gel. Finally, the electric current was set to 70 volts and allowed to run for 1 hour.

Statistical Analysis

Statistical analysis was conducted using the SPSS computing program to analyze the results. A P-value of less than 0.05 was deemed statistically significant [10].

4. Results and Discussion

P. aeruginosa characterization

In this study, 215 samples were subjected to bacteriological examination to detect and isolate *P. aeruginosa*. All models were firstly inoculated onto the primary isolation media represented by blood agar, MacConkey agar, and *Pseudomonas* Medium A. Colorless and non-lactose ferment colonies, characteristic of *Pseudomonas* spp., were subjected to biochemical tests for identification. 110 out of 125 isolates of *P.aeruginosa* were able to produce **blue-green (pyocyanin)** pigment—**the diagnosis of all *P. aeruginosa*** confirmed by the VITEK2-automated system.

Antibiotic Susceptibility of *P. aeruginosa*

When the 125 *P. aeruginosa* isolates were subjected to antibiotic susceptibility testing against 12 antibacterial agents, Figure (1) showed that the isolates varied in antibiotic resistance and sensitivity. It was found that 83.2% and 76 % of the isolates were resistant to carbenicillin and piperacillin, respectively. For β-lactam/β-lactamase inhibitor

combination agents, 56% of isolates appeared resistant to piperacillin-tazobactam. The rates of resistance to the third-generation cephalosporins were as follows: cefotaxime (79.2%), ceftriaxone (73.6%), and ceftazidime (71.2%). Additionally, 64.8% of the isolates resisted the fourth-generation cephalosporin, cefepime.

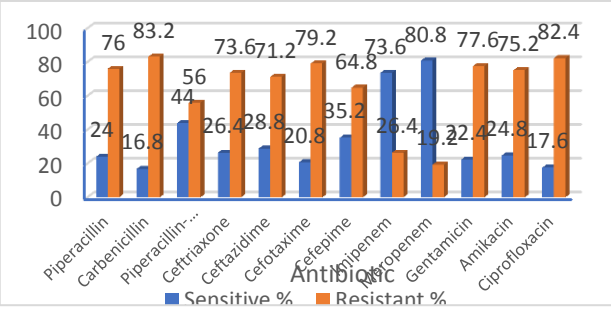

Figure (1): Antiprogram susceptibility of *P. aeruginosa* to isolates toward antibacterial agents (No. 125).

Resistance to Imipenem and meropenem were 7.4% and 14.8%, respectively. The overall resistance rate to aminoglycoside antibiotics with a maximum resistance against gentamicin (77.9%) and amikacin (75.2%). According to the fluoroquinolones susceptibility testing results, 82.4% of the isolates were resistant to ciprofloxacin.

Resistance to three more classes of antibiotics, including aminoglycosides, antipseudomonal penicillin, carbapenems, cephalosporins, β-lactam/β-lactamase inhibitor combination, quinolones and monobactam, was considered either multi-drug resistance (MDR), extensive drug resistance (XDR) or pan-drug resistance (PDR). In this study, antibiotic susceptibility testing of the *P. aeruginosa* isolates by the Kirby-Bauer disk diffusion method showed that 47 (22.8) isolates were MDR.

During the study period, 76% of the isolates resisted piperacillin. Interestingly, present results showed that the resistance to this antibiotic increased compared to previous reports published from Najaf [12, 13]. Descriptions of the susceptibility of *P. aeruginosa* to piperacillin have ranged from as low as 33.7% and 60.5. Similarly, [14] reported a moderate resistance rate against piperacillin among *P. aeruginosa* strains isolated.

Carbenicillin (carboxypenicillins) has a wider spectrum. It has activity against *P. aeruginosa*, strains that are resistant to ampicillin. The carboxypenicillins differ from other penicillins in that they contain a carboxylate moiety in the side chain of the molecule. These molecules are more effective against Gram-negative bacteria, presumably because they penetrate the outer cell wall more extensively than other penicillins. Carbenicillin was tested in Al-Najaf in a previous study [13].

Concerning the β-lactam-β-lactamase inhibitor combinations, piperacillin–tazobactam is preferable to ticarcillin-clavulanate for the treatment of *P. aeruginosa* infections because of the more favourable pharmacokinetics of tazobactam. In the present study, the susceptibility of clinical isolates of *P. aeruginosa* showed that piperacillin-tazobactam was moderately active against isolates (56.0% resistant). Many studies reported piperacillin-tazobactam as the most active antimicrobial agent against *P. aeruginosa* [15, 16].

Cefepime is the most ordinary fourth-generation antibiotic in hospital protocols. This study had a relatively moderate resistance rate to cefepime (64.8%), which was high compared to another local research, 55.5% [13]. Extended-spectrum fourth generation (cefepime) displays an increased stability against enzymatic degradation by β-lactamases (AmpC β-lactamases in particular), and an enhanced ability to penetrate the porins in the outer membrane of Gram-negative bacteria [17].

Carbapenems are a drug that is not readily available in Najaf province and its cost is prohibitive. However, this group of antibiotics has been reported to be very active against *P. aeruginosa* in several recent studies. Moreover, Imipenem has been said to be very

active against *P. aeruginosa* in a number of previous studies [18, 19].

Activity of Carbapenems (Imipenem and meropenem) to *P. aeruginosa* shown in this study (26.4% and 19.2% resistant, respectively) correlated with others which found meropenem had better activity than impenem [20]. Which agree to that reported by [21], who found that meropenem was better than Imipenem and disagree with [13].

Fluoroquinolone susceptibility among *P. aeruginosa* isolates appears to decrease in the Najaf hospitals because of increasing or cumulative fluoroquinolone use. Compared with previous study in Najaf, susceptibility to fluoroquinolones appears to be decreasing at a higher rate (17.6%) than susceptibility to other antimicrobial classes.

All the *P. aeruginosa* isolates from the present study were multiple antibiotics resistant. As expected, however, the difference in MDR rates between the present and other studies could not be compared due to varying definitions of MDR.

Carbapenem-Resistant *P. aeruginosa*

Using an initial screening test according to the guidelines of the Clinical and Laboratory Standards Institute (30) for initially screened for carbapenems susceptibility, all *P. aeruginosa* isolates (125) were tested for carbapenems susceptibility. By disk diffusion method using Imipenem, and meropenem (10µg each) antibiotic disks. They were designated for Imipenem and meropenem as susceptible if the inhibition zone diameter was ≥19 mm, intermediate if the inhibition zone diameter was 16-18 mm, and resistant if the inhibition zone diameter was ≤15 mm, as recommended by (30).

Based on the susceptibility testing results, the isolates' susceptibilities to Imipenem and meropenem are listed in Figure (1). Meropenem showed better activity (80.8%) than Imipenem (73.6 %) in the study period. Resistance for carbapenems by disk diffusion originated in 33 (26.4%) isolates for both meropenem and Imipenem and 10 (8%) isolates for Imipenem alone. However, cross-resistance between meropenem and Imipenem was found in 23 of 33 isolates resistant to both meropenem and Imipenem.

Carbapenemase Producing Isolates

The 33 isolates of *P.aeruginosa* carbapenem-resistant were tested for their ability to produce enzyme carbapenemase. Two methods were used for this purpose; phenotypic and molecular methods. Carbapenem-resistant *P. aeruginosa* isolates were further tested for class B (metallo-β-lactamase, MBL) and class A (serine-β-lactamases) carbapenemase production by modified Hodge test. In this method, the test was considered positive if *E. coli* ATCC 25922 growths were observed within the inhibition zone of the imipenem disk, giving a distorted area and interpreted as carbapenemase production Figure (2). of the 33 carbapenem-resistant isolates 11 (33.3%) were positive, indicating their ability to produce carbapenemases (Figure 3). Unfortunately, the test cannot give any information on the type of carbapenemase enzyme produced by the isolates tested.

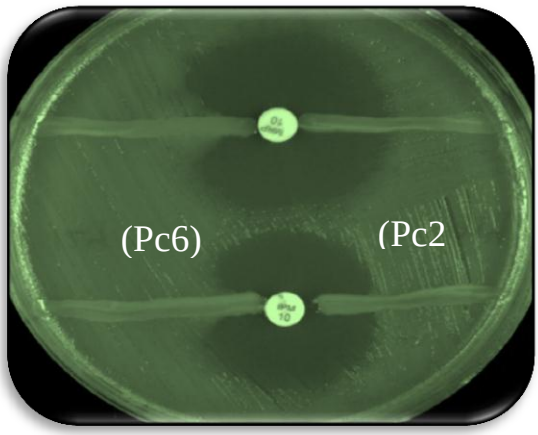

Figure (2): The modified Hodge test of Carbapenemase production. Right-plat; positive test (Pc6) had a clover leaf- like indentation of the *E. coli* 25922. Left-plat; negative test had no growth of the *E. coli* along

the isolate. Pc2 growth streak within the disk diffusion zone.

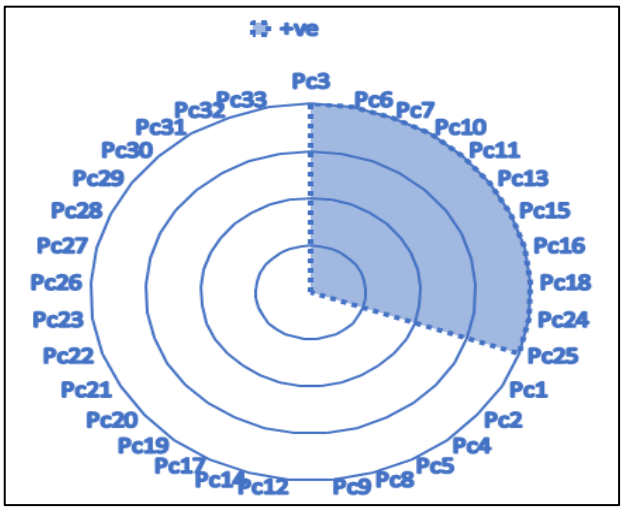

Figure (3): Modified Hodge test for detection of Frequency of production of Carbapenemase in 33 Carbapenemase-resistant *Pseudomonas aeruginosa* isolates

All *P. auruginosa* isolates, positive for Carbapenem resistance by the Kirby-Bauer disk diffusion method, were also tested for metallo-β-lactamase (MBL) production by imipenem-meropenem-EDTA disk synergy test. If the in inhibition zones with the carbapenems (Imipenem and meropenem) and EDTA disk was ≥7 mm than the imipenem and meropenem disks alone, it was considered as MBL positive, as can be seen in Figure (4). However, only 17 isolates (51.5 %) were positive according to this test, suggesting the presence of MBL (the zones around the imipenem and meropenem disks was extended on the side nearest the EDTA disk). Carbapenems disks and an EDTA disk produced large synergistic inhibition zones for the isolate, while no remarkable distinct change was noticed in the remaining 16 (48.5 %) isolates, as shown in Figure (5).

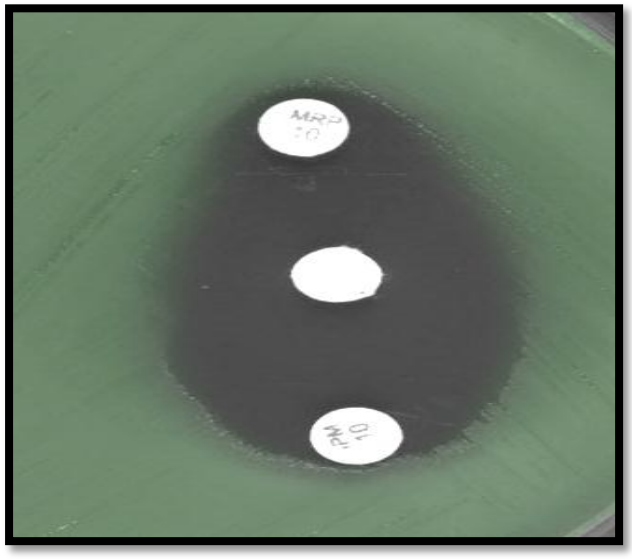

Figure (4): Performances of imipenem-meropenem-EDTA disk synergy test using disks of Carbapenemase (Imipenem, IMP and meropenem, MRP) and disk of EDTA (1.900 µg).

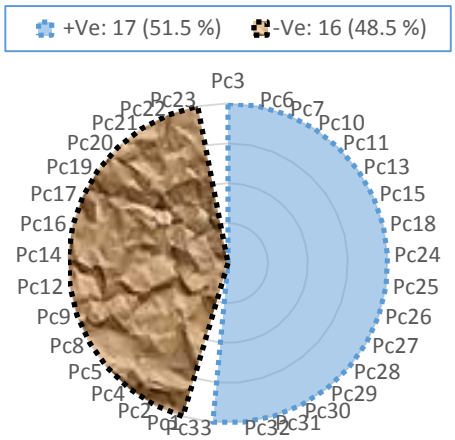

Figure (5): Imipenem-meropenem-EDTA disk synergy test for detection of metallo-β-lactamases in 33 Carbapenemase-resistant Pseudomonas aeruginosa isolates.

One objective of this study was to evaluate the presence of Carbapenemase in 33 Carbapenemase-resistant *P. aeruginosa* isolates and the performance of the modified Hodge test, imipenem-meropenem-EDTA disk synergy test and molecular methods to correctly identify this type of Carbapenemase resistance.

Resistance to Carbapenemase in *P. aeruginosa* is often due to loss of outer membrane proteins, regulation of active efflux pumps, or production of Carbapenemase and MBL. MBLs have been identified from clinical isolates worldwide with increasing Frequency over the past few years, and strains producing these enzymes have been responsible for prolonged nosocomial outbreaks that were accompanied by severe infections [16].

The present study revealed that 11 (38.9%) isolates were modified Hodge's test positive, which optimistically indicates that they may possess the Carbapenemase gene, which mediated the Carbapenemase resistance. This is a phenotypic test which could be used in Najaf hospitals to determine if reduced susceptibility to Carbapenemase is mediated by Carbapenemase production. According to [22] sensitivity and specificity of the modified Hodge test is 100%.

The carbapenem-resistant *P. aeruginosa isolates* by the Kirby-Bauer disk diffusion method from present study were further tested by the imipenem-meropenem-EDTA disk synergy test, and 17 (51.5 %) were found to be MBL producers phenotypically, Figure (5). The procedure was repeated twice to ensure the reproducibility of results. A high proportion of isolates produced MBL was found in Najaf hospitals.

Molecular (genotypic) of Carbapenemase Production

To identify the molecular mechanism of Carbapenemase resistance, the PCR amplification method was employed to identify isolates carrying the Carbapenemase genes. However, to obtain more information on the types of MBLs in the 33 *P. aeruginosa* Carbapenemase-resistant positive isolates in the Kirby-Bauer disk diffusion method, MBLs of these isolates by PCR experiments were analyzed with a series of primers specific for bla_{IMP}, bla_{VIM}, and bla_{NDM}. Presented MBLs genes in these isolates as shown in Table (2).

The MBLs genes presented in 51.5% (17/33) isolates. The bla_{IMP} (578 bp) was the most frequent MBL gene when documented in 15 (45.5%) of the carbapenem-resistant isolates, Figure (6). A bla_{IMP}-type gene alone was detected in 11 (33.33%) and with the bla_{VIM}-type gene in 2 (6.06%), and with *the* bla_{NDM}-type gene in (6.06%) of the isolates, Figure (7). bla_{VIM} type genes were presented in 2 (6.06%). However, results revealed that bla_{IMP}-type genes were the most common MBL gene combination among the isolates. Moreover, 4 (12.12 %) isolates carried a bla_{IMP} -type gene as shown in Figure (8).

No PCR products were produced from carbapenem-resistant *P. aeruginosa* isolates using bla_{IMP}, $bla_{VIM,}$ $bla_{NDM,}$ type genes in 16 (48.48%). PCR primers were also designed, and the reactions were optimized for the class D carpabenemase (bla_{OXA-23}) to study the genetic basis of carbapenem resistance among the 33 *P. aeruginosa* isolates. Results revealed that all of the isolates tested were found to be negative in the PCRs technique for the presence of class D β-lactamases genes as in Table (3).

Table (3): Distribution of various Metallo-β-Lactamase (MBL) genes and their combinations in 33 carbapenem-resistant isolates of *Pseudomonas aeruginosa*.

MBL Genes	No. (%) of Isolates	Isolate Code No.
bla_{IMP}	11 (33.3%)	Pc3, Pc6, Pc7, Pc10, Pc11, Pc13, Pc15, Pc16, Pca18, Pc24, Pc20
bla_{VIM}	2 (6.06%)	Pc13, Pc3
bla_{IMP} + bla_{NDM}	2 (6.06%)	Pc6, Pc7
Bla_{IMP} + bla_{VIM}	2 (6.06%)	Pc13, Pc3

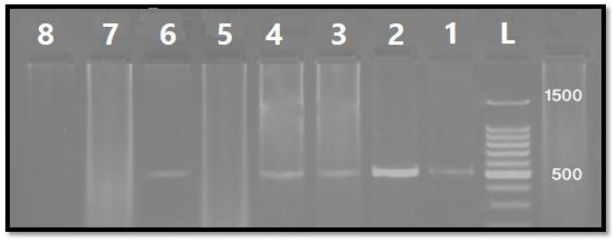

Figure (6): Agarose gel electrophoreses of PCR amplified products of bla_{IMP} gene. The electrophoresis was performed at 60 volts for 2 hr. Lane (L), DNA molecular size marker (l500-bp ladder). Lanes (1, 2, 3, 4, and 6) of

Pseudomonas *aeruginosa* isolates show positive results with *bla*$_{IMP}$ (578 bp).

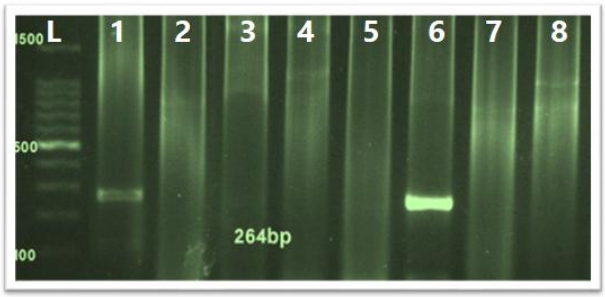

Figure (7): Agarose gel electrophoreses of PCR amplified products for *bla*$_{NDM}$ gene primers. The electrophoresis was performed at 60 volts for 2 hr. Lane (L), DNA molecular size marker (l500-bp ladder), and Lanes (1&6) of *Pseudomonas aeruginosa* isolates show positive results with *bla*$_{NDM}$ (264 bp).

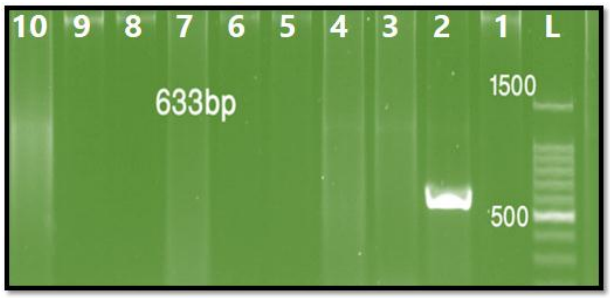

Figure 8: Agarose gel electrophorese of PCR amplified products of *bla*$_{VIM}$ gene primers. The electrophoresis performed at 60 volts for 2 hr. Lane (L), DNA molecular size marker (l500-bp ladder), Lanes (2) of *Pseudomonas aeruginosa* isolates show positive results with *bla*$_{VIM}$ (633 bp).

The emergence of acquired MBLs among *P. aeruginosa* represents an epidemiological risk due to. Firstly, MBLs confer resistance not only to carbapenems but to virtually all β-lactams and are frequently associated with resistance to aminoglycosides; and secondly, genes encoding for MBL enzymes are most commonly carried on mobile genetic elements (integrons, plasmids, transposons) that can spread horizontally among unrelated strains [23]. Hence, accurate identification of MBL-producing strains is very urgently needed. However, PCR gives specific and accurate results [24].

Nine (9) MBL types have been identified in Gram-negative bacilli [25]. The IMP and VIM types worldwide are the most commonly detected MBLs in *P. aeruginosa* [26]. The present study used the imipenem-meropenem-EDTA disk synergy test and EDTA-imipenem microbiological assay to examine the phenotypes of the 33 carbapenem-resistant isolates and found that 17/33 (51.5%) of the isolates were MBL positive. The PCR amplification method was employed to identify isolates that carried the MBL genes (*bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{NDM}$). Among the 33 isolates of carbapenem-resistant *P. aeruginosa*, 17 (51.5%) had the MBL genotype. This result suggests that the most common carbapenemase-type genes in national hospitals were *bla*$_{IMP}$. Similar findings were also reported in Najaf and other world, Japan and Italy, where IMP β-lactamase-producing *P. aeruginosa* strains spread as hospital pathogens [27].

The VIM family currently consists of 23 members [28], occurring mainly in *P. aeruginosa* within multiple-integron cassette structures. PCR analysis confirmed *bla*$_{VIM}$ genes in four 2 (6.06%) of the 33 carbapenem-resistant isolates. The present investigation revealed that *bla*$_{VIM}$ is the second gene encoding MBL among the isolates of *P. aeruginosa* in this study, as shown in Figure (8). The Present study found no belonging to enzymes class D carbapenemase (*bla*$_{OXA-23}$).

The first descriptions of OXA-carbapenemase were identified in *Acinetobacter baumannii* clinical isolates from most continents, both chromosomally and plasmid-mediated No information about the presence of these enzymes in *P. aeruginosa* because of the low prevalence of these enzymes between isolates [28].

Conclusion

The isolates exhibited significant resistance to carbenicillin and piperacillin, while imipenem and meropenem demonstrated lower resistance rates. The presence of

carbapenemase genes, particularly the blaIMP-type gene associated with metallo-beta-lactamase (MBL) production, was prevalent among the isolates, indicating the potential for the spread of carbapenem resistance.

Ethics:

This study was conducted under approval by the medical ethics committee at the University of *Faculty of Sciences, Ilam University, Ilam* Kufa (2020). Verbal and written consent was provided by parents and agreement for publication was obtained from both participants and researchers.

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