History of Medicine, 2023, 9(1): 2315–2321
DOI: 10.17720/2409-5834.v9.1.2023.298

Detection of VIM And IMP Metallo- Beta- Lactamase Genes in Carbapenem Resistant Pseudomonas Aeruginosa Isolated from Different Clinical Infection in AL- Najaf Province

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Received: 12 February 2023 **Accepted:** 4 May 2023

Citation: Almayali EJB, muhana ASAL (2023) Detection of VIM And IMP Metallo- Beta- Lactamase Genes in Carbapenem Resistant Pseudomonas Aeruginosa Isolated from Different Clinical Infection in AL- Najaf Province. History of Medicine 9(1): 2315–2321. https://doi.org/10.17720/2409-5834.v9.1.2023.298

Abstract

Pseudomonas aeruginosa is an opportunistic human pathogen cause most health care-associated infections and it is considered a paradigm of antibiotic resistance development. Carbapenem resistant *P. aeruginosa* has emerged as an important cause of infection in different hospitals worldwide. Present study aimed to investigate the prevalence of multi drug resistant *P. aeruginosa* and to determine frequencies of the main Metallo-β-lactamases (MBLs) genes in isolates collected from Najaf hospitals. The results were as follows: 80 isolates of *P. aeruginosa* out of 230 isolates were Gram-negative bacteria and non-lactose fermenter after growing on MacConkey agar. Forty –eight isolates were resistant to the carbapenem class after conducting a sensitivity test to eighty isolates of *Pseudomonas aeruginosa*. The isolates were tested for susceptibility to 14 antibiotic by the Kirby-Bauer disk diffusion method to *P. aeruginosa* . Overall, the resistant rates for penicillin antibiotics including piperacillin were (83.75%), piperacillin/tazobactam (85 %) , ceftazidime (68.75 %) , cefepime (76.25%). aztreonam (37.5%) , imipenem (60%) and meropenem (55%) , tobramycin (52.5%), gentamicin(55%), amikacin(62.5%). ciprofloxacin (56.25%) , levofloxacin (63.75%), norfloxacin (61.25%) and moxifloxacin (75%). As for the molecular study for the detection of Metallo-beta-lactamase genes of *Pseudomonas aeruginosa* bacteria with carbapenem resistant (48 isolate), it was as follows (bla-VIM , bla-IMP) with the following percentages (10.4%), (22.9%) respectively.

Pseudomonas aeruginosa is Gram-negative bacilli, aerobic non lactose ferment. It is broadly distributed in nature and can adapt to several environments, it can be isolated from any believable source within hospitals (Brooks *et al.*, 2007). *P. aeruginosa* infections are difficult to treat, because of intrinsic resistant to many antibiotics (MDR) and a high risk of emergence of resistance during therapy (Livermore , 2012). It is cause hospital-acquired infections and community acquired, infections with this bacterium have been associated with high morbidity and mortality when compared with other bacterial pathogens (Brusselaers *et al.*, 2011).

P. aeruginosa is opportunistic Gram-negative bacilli bacterium, belonging to the family Pseudomonaceae, that is capable to survive in a wide range of environments (Silby *et al.*, 2011). Antimicrobial agents for suspected cases of *P. aeruginosa* includes, monotherapy and combination therapy; this agent decreases the mortality in patients with severe *P. aeruginosa* infections (Park *et al.*, 2012).

However, *P. aeruginosa* infections treatment has become a great contest due to the capability of this bacterium to resist many of the available antibiotics (Lister *et al.*, 2009).The World Health Organization (WHO), has recently recorded carbapenem-resistant *P.aeruginosa* as one of three bacterial species, in which there is a serious need for the development of new antimicrobial agents to treat infections (Tacconelli *et al.*, 2019).

P. aeruginosa demonstrations resistance to variation of antibiotics, including quinolones, β-lactams, and aminoglycosides (Hancock and Speert, 2000). The main mechanisms of *P. aeruginosa* used to counter antibiotic can be classified into acquired, intrinsic, and adaptive resistance. *P. aeruginosa* that intrinsic resistance includes, expression of efflux pumps that eject antibiotics out of the cell, low outer membrane permeability, and antibiotic-inactivating enzymes. The acquired resistance of *P. aeruginosa* can be realized by either mutational changes or horizontal transfer of resistance genes (Breidenstein *et al.*, 2011). *P. aeruginosa* adaptive resistance includes, formation of biofilm in the lungs of infected patients, where the biofilm helps as a diffusion barrier to perimeter antibiotic access to the bacterial cells (Drenkard , 2002). It can acquire resistance genes from other organisms via transposons, plasmids, and bacteriophages (Lambert *et al.*, 2002), and the normally happening chromosomal AmpC β-lactamase (Nordmann *et al.*, 2011).

Genetic mutations decrease cell wall and membrane permeability to antibiotics. These mutations happen frequently in Gram-negative bacteria and resistance to β-lactam antibiotics, chloramphenicol, aminoglycosides, and tetracycline may be partly attributed to the decreased uptake (Strateva and Yordanov ,2009).

Materials and methods

Specimens' collection

From November 2020 to September 2021, 850 clinical specimens were collected from patients suffering from different clinical infections from hospitals in Al –Najaf province to isolates *Pseudomonas aeruginosa* .

Specimens Culture and biochemical test

After collecting the specimens using swabs, they were cultured on the commonly used media(MacConkey) which are initially based on isolation and initial diagnosis. And then adopting the biochemical tests from them IMVIC test , catalase test , coagulase and oxidase , In addition to using VITEK-2 Compact System to confirm the diagnosis Identification (MacFaddin,2000)(Pincus, 2006).

Antimicrobial Activity

Antibiotic sensitivity testing is performed by placing a variety of antibiotic discs with known doses on Muller Hinton media, evaluating the results after 24 hours, calculating the diameter of the inhibition zone, and comparing the results to CLSI 2021.

DNA extraction

Genomic DNA was extracted by using a commercial total DNA extraction kit (Favorgen, Taiwan).

Molecular identification

Gel electrophoresis was used for detection of DNA by UV transilluminator. The PCR assay was performed to detect the (16 s rRNA , bla-VIM, bla-IMP*)* genes for *Pseudomonas aeruginosa* .

Isolation and Identification of Pseudomonas aeruginosa Isolates

Identification of *Pseudomonas aeruginosa* was first made by the bacteriological methods including colonial morphology, Gram's stain, and the biochemical tests (Table 1). Characteristics of *Pseudomonas aeruginosa* were subjected to biochemical tests for identification, and finally identified by Vitek2-automated system . Only 80 isolates belonged to genus *Pseudomonas aeruginosa* .

Pseudomonas aeruginosa by production pyocyanin and giving strongly oxidase positive , and give indole (Indole test is used to determine the ability of an organism to split amino acid tryptophan to form the compound indole, vogues procures (is a test used to detect acetoin in a bacterial broth culture) , methyl red (test detects the production of sufficient acid during the fermentation of glucose , and urease (Urease broth is a differential medium that tests the ability of an organism to produce an exoenzyme, called urease, that hydrolyzes urea to ammonia and carbon dioxide. The broth contains two pH buffers, urea, a very small amount of nutrients for the bacteria, and the pH

indicator phenol red) negative result this agrees with (Koneman *et al.*,2006).

Table (1): Morphological and biochemical tests of Pseudomonas aeruginosa isolates.

Test	Result
Gram stain	Negative
Shape	Rods
Capsule	Non – capsulated
Catalase	Positive
Oxidase	Positive
Motility	Positive
Indole test	Negative
Methyl red	Negative
Voges-Proskauer	Negative
Citrate	Positive
Urease	Negative
TSI	Alkaline / Alkaline
Lactose fermenter	Non fermenter

All 80 isolates tests gave positive bands in the PCR assay and gave expected size of PCR products. PCR products from isolates collected from different source were obtained with primer pairs (specific rRNA as shown in (Figure 1).

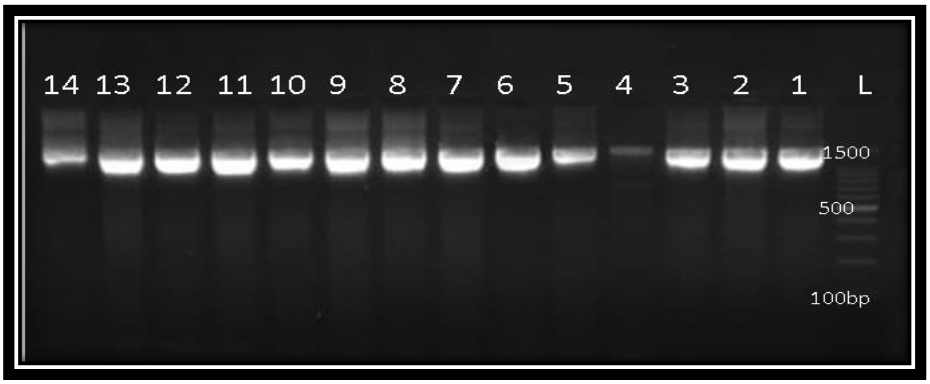

Figure:(1): Ethidium bromide-stained agarose gel electrophoresis of PCR products from extracted total DNA of Pseudomonas aeruginosa using primer 16s rRNA with product 1500 bp. The electrophoresis was performed at 70 volt for 1- 1.5 hr. (L), DNA molecular size marker (100 bp ladder). (all isolate show positive results with specific 16s rRNA for Pseudomonas aeruginosa) .

This sequence is used to diagnose *Pseudomonas aeruginosa* bacteria based on 16S rRNA, this agree with Amoon *et al.*, (2018)16S rRNA gene sequence analysis is a robust tool for characterization of new pathogens in clinical specimens with suspected bacterial disease. Forty bacterial isolates were obtained from different clinical specimens (wound, urine and sputum) using enrichment selective media and biochemical tests to characterize and identify the bacteria as *P. aeruginosa.* DNA was extracted from *P. aeruginosa* . The amplified PCR products were sequenced. The identity and similarity of the nucleotide sequence of the isolated strains was detected by comparing them with published sequences using BLASTn.

In current study , the highest percentage (37.3%) of *Pseudomonas aeruginosa* was isolated from burn swabs compared to other sites of infections (Figure 2). Other isolates were recovered from urine sample (18.8%), wound swabs (18.8%) , stool (8.8%), throat (7.5%) , ear (6.3%) and blood sample were (2.5 %), while no isolate was recovered from CSF samples.

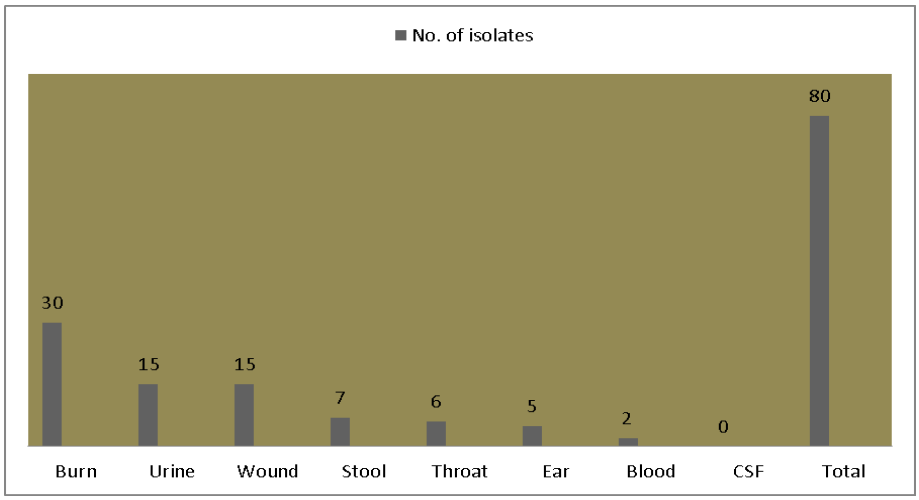

Figure (2): Distribution of Pseudomonas aeruginosa obtained from different clinical samples according to the sample source.

Pseudomonas aeruginosa is one of the most common pathogens implicated with a wide variety of serious invasive diseases in humans such as pneumonia and sepsis. It is almost impossible to prevent exposure to *P. aeruginosa* because it can be found anywhere. This opportunistic pathogen occasionally migrates from its natural environment and causes disease in humans (Hong *et al.,* 2015).

In previous study, Al-Muhannak (2010) found that *P. aeruginosa* and *Klebsiella* spp. were the most frequently isolated Gram-negative pathogens (30.5% each) in Najaf hospitals. In addition, Belal (2010), Al-Shara (2013), and Resol (2015) reported 26.1%, 18.8%, and 5.2% isolates of *P. aeruginosa* from Najaf hospitals, respectively.

P. aeruginosa is one of the most important causes of infection in burn patients worldwide, including Iraq and documented as the most prevalent isolated bacteria in these patients .Burn infections caused by MDR *P. aeruginosa* have been associated with high morbidity and mortality rates worldwide .Previous study performed in burn units in Iraq, demonstrated a high occurrence of *P. aeruginosa* isolates recovered from burn infections (Al-Dahmoshi, 2015).

Microbial infections remain an urgent threat to human health, specifically with the increasing antimicrobial resistance mechanisms. The opportunistic human pathogen *Pseudomonas aeruginosa* successfully establishes diseases in patients with compromised immune systems and those suffering from severe conditions. The development of diagnostic platforms for rapid detection, reliable confirmation of infection, and continuous monitoring of *P. aeruginosa* is a critical challenge of clinical importance, especially in terms of tailoring effective treatment strategies. During infection stages, this pathogen produces various virulence metabolites, which promote virulence by interfering with cellular functions in immune cells, allowing *P. aeruginosa* to successfully establish infectious colonies that proliferate (Simoska and Stevenson, 2022).

Also in previous studies , One of these particularly problematic microorganisms is the opportunistic human pathogen *Pseudomonas aeruginosa,* which remains one of the leading causes for the significant increase in mortality and morbidity rates (Alatraktchi *et al.*,2016). Namely, this Gram-negative bacterium is most commonly associated with severe infections, including the urinary tract, the respiratory tract (e.g., cystic fibrosis), as well as the central nervous and vascular systems (Lyczak *et al.*,2000) . Furthermore, high levels of *P. aeruginosa* cells frequently colonize chronic wound infection sites (e.g., leg ulcers, diabetic foot ulcers, burns), causing wound inflammation and delays in the healing processes . Thus, the development and advancement of selective, sensitive, and cost-effective methods for fast detection, reliable identification, and real-time monitoring of *P. aeruginosa* are necessary for successfully selecting effective treatments and intervention strategies (Hudu *et al.*, 2016).

Antibiotic susceptibility test of P. aeruginosa

In the current study , the ability of the 80 *P. aeruginosa* clinical isolates to grow in the presence of antibiotics was

tested. Preliminary susceptibility screening of the isolates was performed using the Kirby-Bauer disk diffusion method according to CLSI (2021) guidelines, which included 14 anti-pseudomonal agents from six antimicrobial categories. The results of drug susceptibility testing are shown in Table (2). Overall, the resistant rates for penicillin antibiotics including piperacillin were 83.75% . The resistant rate for β-lactams/ β-lactamase inhibitor combination antibiotic including piperacillin/tazobactam was 85 % . The rates of resistance to the third generation of cephalosporins were as follows: ceftazidime 68.75 % . Furthermore, 76.25% of the isolates were resistance to fourth generation cephalosporins (cefepime). The isolates had relatively low resistance to monobactams, with a resistant rate of 37.5% to aztreonam. According to the susceptibility results for the carbapenems, 60% of the evaluated isolates exhibited resistance to imipenem and 55% of the isolates showed resistance to meropenem. The overall prevalence of resistance to aminoglycosides including, tobramycin, gentamicin, and amikacin were 52.5% , 55%, and 62.5% respectively. Resistance to fluorinated quinolones was as follows: ciprofloxacin 56.25% , levofloxacin 63.75%, norfloxacin 61.25% and moxifloxacin 75%.

In previous studies on the resistance of these bacteria, *Pseudomonas aeruginosa* exhibit multiple resistance mechanisms to antibiotics including decreased permeability, expression of efflux systems, production of antibiotic inactivating enzymes and target modifications. *P. aeruginosa* exhibits most of these known resistance mechanisms through both intrinsic chromosomally encoded or genetically imported resistance determinants affecting the major classes of antibiotics such as β-lactams, aminoglycosides, quinolones and polymyxins (Bassetti *et al.*,2018).

Eight categories of antibiotics are mainly used to treat *P. aeruginosa* infections including aminoglycosides (gentamicin, tobramycin, amikacin, netilmicin), carbapenems (imipenem, meropenem), cephalosporins (ceftazidime, cefepime), fluoroquinolones (ciprofloxacin, levofloxacin), penicillin with β-lactamase inhibitors (BLI) (ticarcillin and piperacillin in combination with clavulanic acid or tazobactam), monobactams (aztreonam), fosfomycin and polymyxins (colistin, polymyxin B). The emergence of MDR, XDR and PDR strains occurs in a timely fashion by the modification of regulatory mechanisms controlling the expression of resistance determinants, by mutations, alteration of membrane permeability, and horizontal acquisition of antibiotic-inactivating enzymes or enzymes inducing target modifications. Noteworthy, is the multi-resistance of many strains conferred by simultaneous production of these mechanisms (El Zowalaty *et al.*, 2015).

Table (2): Antimicrobial susceptibility rates among P. aeruginosa isolated from clinical samples (n= 80)

Antibiotic	Antibiotic sensitive test result			Resistant (%)
	R	I	S	
Piperacillin	67	3	10	83.75%
Piperacillin/tazobactam	68	2	10	85 %
Ceftazidime	55	6	19	68.75 %
Cefepime	61	3	16	76.25%
Aztreonam	30	5	45	37.5%
Imipenem	48	4	28	60%
Meropenem	44	5	31	55%
Tobramycin	42	8	30	52.5%
Gentamicin	44	7	29	55%
Amikacin	50	7	23	62.5%
Ciprofloxacin	45	5	30	56.25%
Levofloxacin	51	2	27	63.75%
Norfloxacin	49	1	30	61.25%
Moxifloxacin	60	0	20	75%

R=resistant , I=intermediate , S=sensitive

Molecular screening of MBL producers to P. aeruginosa

All 48 carbapenem resistant *P. aeruginosa* isolates were screened by conventional PCR for potential gene determinants encoding MBL using a specific primers for Ambler class B MBL (bla -VIM, bla-IMP) The prevalence of isolates that carried MBL genes is shown in Figure(3 and 4) .

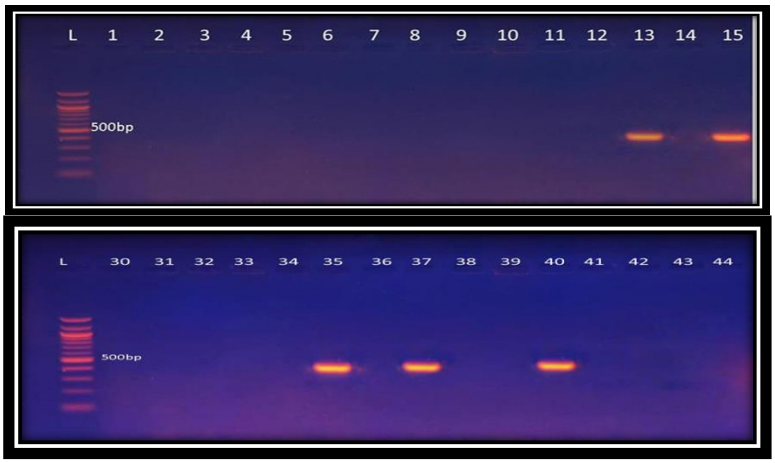

Figure:(3): Ethidium bromide-stained agarose gel electrophoresis of PCR products from extracted total DNA of Pseudomonas aeruginosa using primer bla VIM with product 390bp. The electrophoresis was performed at 70 volt for 1- 1.5 hr. (L), DNA molecular size marker (100 bp ladder). (13,15,35,37,40 isolate show positive result).

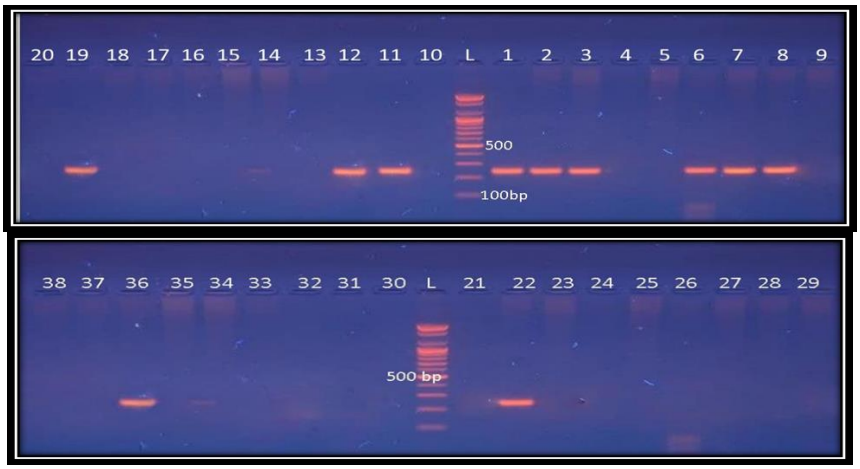

Figure:(4): Ethidium bromide-stained agarose gel electrophoresis of PCR products from extracted total DNA of Pseudomonas aeruginosa using primer bla IMP with product 232 bp. The electrophoresis was performed at 70 volt for 1- 1.5 hr. (L), DNA molecular size marker (100 bp ladder). (1,2,3, 6,7,8,11,12,19,22,36 isolate show positive result).

In previous study Particularly, the production of carbapenemases in *P. aeruginosa* is important since CRPA has been rapidly spreading as a consequence of the acquisition of carbapenemase genes through mobile genetic elements. So far, class A, B, and D carbapenemases have been identified in *P. aeruginosa*, and class B Metallo-β-lactamase (MBL) enzymes, including Verona Integron-encoded Metallo-β-lactamase (VIM), imipenemases (IMP), and New Delhi metallo-β-lactamase (NDM), are the most prevalent carbapenemases produced by *P. aeruginosa*(Yoon and Jeong , 2021).

The carbapenem resistance rate of the isolated *P. aeruginosa* was 46.7% (59/124). A total of 54 (91.5%) out of the 59 CRPA isolates were identified as multidrug-resistant. The majority of the CRPA isolates (81.4%, 48/59) harbored carbapenemase genes, such as blaIMP-6 or blaNDM-1.The studied CRPA isolates simultaneously harbored 10 to 14 virulence factors of the 16 virulence factors examined. Nine virulence factor genes (toxA, exoT, plcH, plcN, phzM, phzS, lasB, aprA, and algD) were identified in all CRPA isolates (Park and Koo, 2022).

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