Khalij Libya Journal of Dental and Medical Research. 2026;10(1):63-69
https://doi.org/10.47705/kjdmr.261011
eISSN:*2708-888X*

Original article

PCR-Based Detection of VIM Carbapenemase Genes in Clinical *Pseudomonas aeruginosa* Isolates from Libyan Hospitals

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Abstract
Pseudomonas aeruginosa (P. aeruginosa) is the commonest human pathogen recognized for its ubiquity, its intrinsically advanced antibiotic resistance mechanisms, and its association with serious illnesses, especially hospital-acquired infections. An interest has centered on the emergence of strains with VIM (Verona integron-encoded metallo-β-lactamase). As there is little information on the detection of such genes in P. aeruginosa from patients of the Middle East and Arab countries, including Libya, such information needs to be further investigated. However, the occurrence of VIM genes among P. aerogenousa clinical isolates in Libyan hospitals was investigated in this study. To achieve this goal, a total of 106 P. aeruginosa isolates had been collected from the stocks of the well-known teaching hospital in Tripoli, namely the Burn and Plastic Surgery Center (BPSC), for a period of 12 months between 2016 and 2017. Isolated organisms were identified to the species level and tested for their susceptibility to a variety of antimicrobial agents. The MBL-producing P. aeruginosa isolates were screened for the blaVIM gene using PCR-based methods. The results of the antibiotic susceptibility testing revealed that all isolates except for colistin were found to be resistant to the tested antibiotics to varying degrees. The VIM-positive isolates among MDR isolates were 10.7%, and among XDR isolates was 1.3%. The VIM-positive isolates (20%) had significantly higher rates of resistance to certain antibiotics compared to VIM-negative isolates (80%). The high rate of antibiotic resistance among P. aeruginosa strains expressing the blaVIM gene is alarming and can be responsible for serious infections, especially among hospitalized patients; therefore, appropriate infection control measures and guidelines need to be established to reduce these infections among patients.
Keywords. *Pseudomonas Aeruginosa, Multidrug-Resistant (MDR) And Extensively Drug-Resistant (XDR), Carbapenem Resistance, VIM Metallo-B-Lactamase, PCR Detection.*

Received*: 05/12/25*
Accepted*: 04/02/25*
Published*: 12/02/26*

Introduction

Pseudomonas aeruginosa is the commonest human pathogen and is a multidrug-resistant pathogen recognized for its ubiquity, its intrinsically advanced antibiotic resistance mechanisms, and its association with serious illnesses, especially hospital-acquired infections such as ventilator-associated pneumonia, sepsis, and urinary tract infection. Chronic infection of the lower respiratory tract with *P. aeruginosa* is prevalent among patients with cystic fibrosis. These patients may present with chronic productive cough, anorexia, weight loss, wheezing, and tachypnea [1]. MDR *P. aeruginosa* has been described in immunocompromised patients, mainly with cystic fibrosis or neoplastic diseases, and patients in the ICU. However, clones are spreading into new geographic areas, and susceptible strains are acquiring resistance genes. New extended-spectrum *β*-lactamases and carbapenemases are emerging, leading to pan-resistant strains [2-4].

An interest has centered on the emergence of strains with IMP (Imipenemase) and VIM (Verona integron-encoded metallo-β-lactamase). These enzymes hydrolyze most β-lactams, including carbapenems, and are encoded by integrons that often also specify aminoglycoside 6′-*N*-acetyltransferases. IMP and VIM type MBLs are spread through all continents after their first identification in Japan and Italy, respectively, while other metallo-enzymes have been detected sporadically [5,6,7,8,9]. However, for further work, the occurrence of VIM genes among *P. aerogenousa* clinical isolates in Libyan hospitals is investigated in this study. The most clinically important MBL families belong to 4 families, VIM, IMP, SPM, and NDM., which are located within a variety of integron structures, where they have been incorporated as gene cassettes. When these integrons become associated with plasmids or transposons, transfer between bacteria is readily facilitated [10,11,12,13].

The VIM enzymes were first described in the late 1990s in Verona, Italy (Verona integron-encoded metallo β-lactamase), and have since spread throughout the globe. These enzymes were initially found in *P. aeruginosa*, but their association with class 1 integrons, along with reports locating them in different types of MGEs, has likely contributed to their dissemination to many different bacterial species, becoming a major concern around the globe [1,11].

The VIM-1 carbapenamase, found in a nosocomial *P. aeruginosa* strain isolated at the Verona University Hospital, Italy, in 1997, is the first representative of a new family of acquired MBLs. *bla* VIM-1 is a part of a

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gene cassette inserted in the class 1 integron, which carries multiple resistance encoding genes. In 2004–2005, *P. aeruginosa* clinical isolates producing VIM-1 were detected from different French hospitals. However, the VIM-2 was originally identified in a *P. aeruginosa* bloodstream isolate from a patient with neutropenia in Marseille (South France) [14]. Although there are more than 16 VIM types, in *P. aeruginosa*, the VIM-2 is found to be the most widespread MBL that is associated with the localization of its encoding gene. Integron-located resistance genes provide them with an increased potential for expression and dissemination. Several class 1 integrons have been found in transposons, which enables the integrons to be transposed [15,16]. The objective of this study is to determine the occurrence of VIM genes among *P. aeruginosa* from clinical isolates in Libyan hospitals.

Methods

Study Design

The present study was carried out at the National Center for Disease Control (NCDC) and at the research laboratories of the Microbiology Department, Faculty of Medicine.

Sample collection and storage

A total of 106 clinical isolates of *P. aeruginosa* were collected from the Burn and Plastic Surgery Center (BPSC) over 12 months between September 2013 and September 2014. The identified isolates were archived at NCDC laboratories and kept for long- term storage at – 60°C as a reference; they are provided for academic use and research purposes.

Identification of isolates

A total of 75 non-duplicate nonconsecutive clinical isolates of *P. aeruginosa* were identified by conventional method such as colony characteristics on MacConkey agar for selective growth and chromogenic media for the confirmation of distinctive pigment production. The latter identifies the typical colonies with blue-green diffusible pigment of pyocyanin, and grape odor was indeed further confirmed by the oxidase test before introducing the BD Phoenix Automated Microbiology System. All these steps were performed after the identification using the Gram staining technique.

Quality control isolates

E. coli ATCC 25922, *K. pneumoniae* ATCC 700603, and *P. aeruginosa* NCTC 10662 are used as controls.

Antimicrobial Susceptibility Testing

The BD Phoenix Gram-negative antimicrobial susceptibility testing card was used to determine the susceptibility of *P. aeruginosa* isolates to different antimicrobial agents. BD Phoenix provides AST results for antimicrobials as susceptible (S), Intermediate (I), or Resistant (R), and are interpreted according to CLSI criteria. In the present study, Intermediate (I) and Resistant (R) categories were combined as resistant.

VIM Enzyme DNA Amplification

PCR analysis for the Metallo-β-lactamase gene VIM was carried out using the primers, which are listed below in (Table 1).

Table (1). PCR Primers sequences used for VIM detection in P. aeruginosa.

Primer	Sequence of primer 5'-3'	Size of product (bp)
VIM-Forward	GTGGCAACGTACGCATCAC	744bp
VIM-Reverse	CCATAGAGCACACTCGCAGA	

Molecular method

PCR analysis was performed on all isolates, and the extraction of DNA was done by using a simple boiling method [17].

Statistical analysis

Susceptibility data were compared using the SPSS 20 statistical package for the social sciences program. Chi-square test is used, and *P*-values of ≤ 0.05 were considered statistically significant.

Results

Detection of the VIM gene

To determine the presence of the *bla*VIM encoding gene on all *P. aeruginosa* clinical isolates, PCR was performed. The results of the antimicrobial susceptibility tests for the VIM gene are summarized in (Table 2) and showed that the VIM-positive strains were 27/75 (36%) and the VIM-negative strains 48/75 (64%).

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eISSN:2708-888X

However, all samples of chloramphenicol, cefutaxime, ertapenem, ampicillin, amoxacillin-clavulanate, and cefuroxime were found to be completely resistant.

Table (2). Antimicrobial susceptibility tests for the VIM gene

Antibiotics	VIM- negative		VIM- positive		P-value	OD
	Count (48)	N % (64%)	Count (27)	N % (36%)		
Gentamicin	21	28.4%	6	8.1%	0.19	0.51
Ertapenem	48	64.0%	27	36.0%	1.00	1.00
Imipenem	18	24.0%	5	6.7%	0.21	0.49
Meropenem	16	21.6%	5	6.8%	0.30	0.56
Ceftazidime	24	32.0%	9	12.0%	0.38	0.67
Azetronam	30	40.0%	13	17.3%	0.52	0.77
Piperacillin	9	12.0%	4	5.3%	0.72	0.79
Colsitin	0	0.0%	1	1.3%	0.31	5.29
Ciprofloxacin	21	28.0%	9	12.0%	0.56	0.76
Levofloxacin	26	34.7%	12	16.0%	0.64	0.82
Amikacin	8	10.8%	3	4.1%	0.57	0.67
Ampicillin	48	64.0%	27	36.0%	1.00	1.00
Amoxicillin-Clavulanate	48	64.0%	27	36.0%	1.00	1.00
Cefuroxime	48	64.0%	27	36.0%	1.00	1.00
Chloramphenicol	48	64.0%	27	36.0%	1.00	1.00
Cefotaxime	48	64.0%	27	36.0%	1.00	1.00
Ceftriaxone	25	34.2%	13	17.8%	0.85	0.92

Distribution of the VIM gene in carbapenems

The distribution of the VIM gene in carbapenems was found to be with a percentage of 36% of VIM-positive and 64% of VIM–negative (Table 3). Imipenem showed a resistance of 6.7% relating to VIM-positive and a resistance of 24% relating to VIM - negative. Similarly, meropenem showed a resistance of 6.8% relating to VIM-positive and a resistance of 21.6% relating to VIM - negative (Table 4 and Figures 1 and 2).

Table (3). Frequencies and rates of antimicrobial agents

Antimicrobial agents		Count	Percentage (%)
Imipenem	R	23	30.7%
	S	52	69.3%
Meropenem	R	21	28.4%
	S	53	71.6%
VIM	Negative	48	64.0%
	Positive	27	36.0%

Table (4). Distribution of the VIM gene in relation to the resistance pattern in carbapenems

Carbapenems		VIM			
		Negative		Positive	
		Count	Percentage (%)	Count	Percentage (%)
Imipenem	R	18	24.0%	5	6.7%
	S	30	40.0%	22	29.3%
Meropenem	R	16	21.6%	5	6.8%
	S	32	43.2%	21	28.4%

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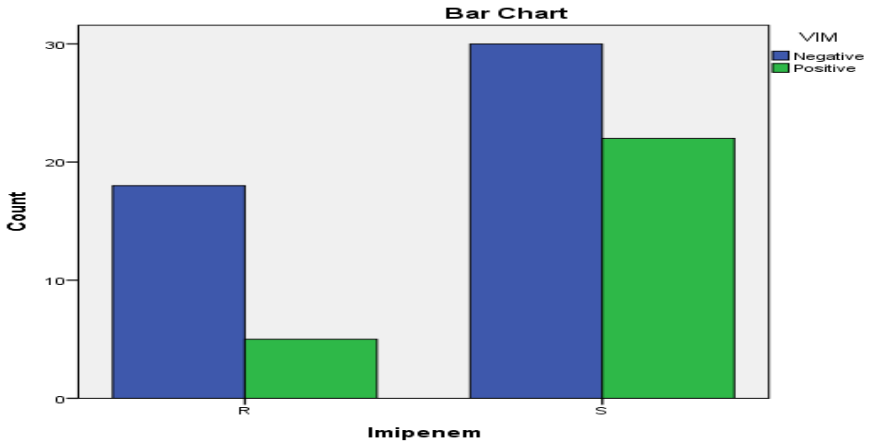

Fig. (1). Imipenem distribution of VIM gene count

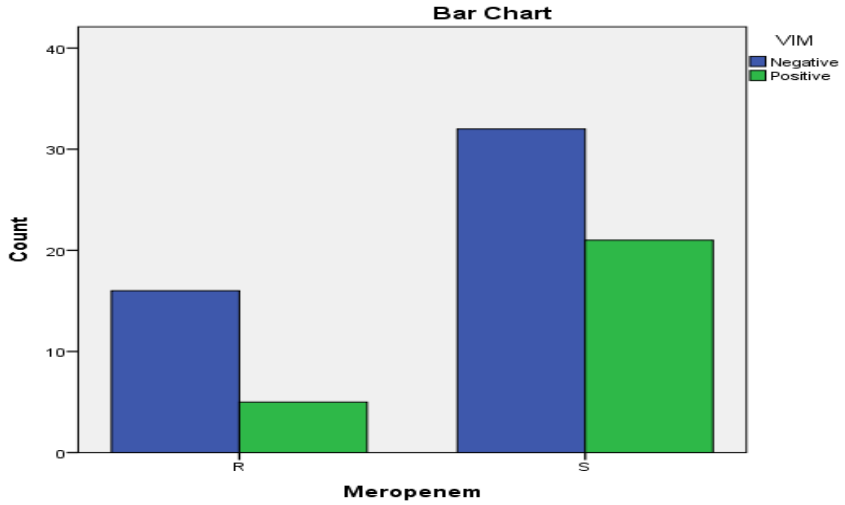

Fig. (2). Meropenem distribution of VIM gene count

VIM gene distribution in relation to the MDR and XDR pattern

In relation to the VIM gene distribution, the frequency of multidrug-resistant strains among isolates was found to be variable, with the VIM-positive isolates among MDR isolates was 10.7% and among XDR isolates was 1.3% (Table 5 and Figures 3 and 4).

Table (5). VIM gene distribution in relation to the MDR and XDR pattern

Isolates		VIM			
		Negative		Positive	
		Count	Percentage (%)	Count	Percentage (%)
MDR	Non-MDR	30	40.0%	19	25.3%
	MDR	18	24.0%	8	10.7%
XDR	Non-XDR	36	48.0%	26	34.7%
	XDR	12	16.0%	1	1.3%

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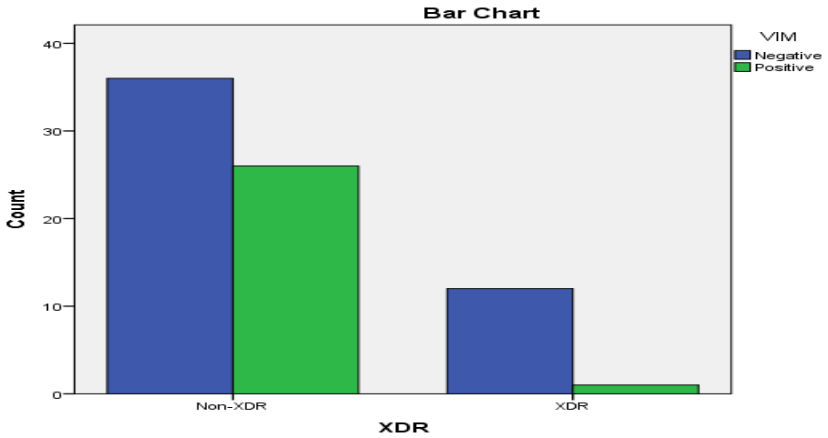

Fig. (3). XDR pattern of the VIM gene count

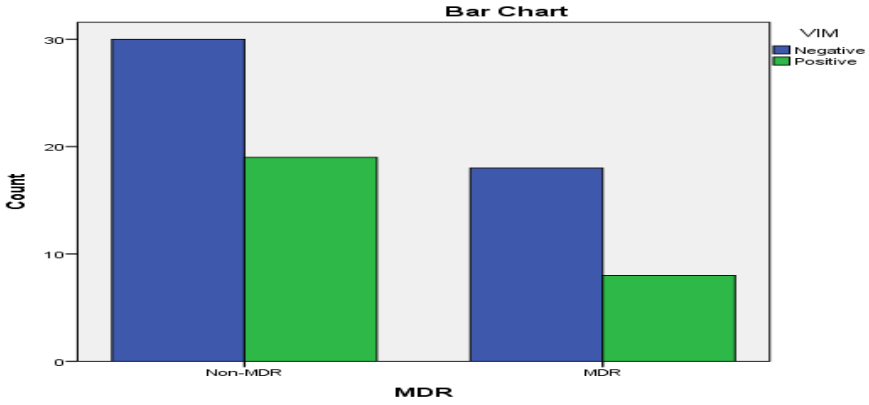

Fig. (4) MDR pattern of the VIM gene count

Discussion and Conclusion

Discussion

P. aeruginosa is responsible for prolonged treatment and acute infections [18]. The rate of imipenem-resistant *P. aeruginosa* isolated from different healthcare settings in Libya has increased consistently from 8.3% in 2012 to 36% in 2015 [19,20]. High rates of resistance to commonly used carbapenem agents in Libya were also observed in this study (imipenem 30.7% and meropenem 28.4%). This can be explained in part by the increase in consumption of these antimicrobial agents in the last decade, leading to a selective pressure of antibiotics on *P. aeruginosa*, and consequently, the bacteria modify their resistance mechanisms.

By analyzing the frequencies and rates of antimicrobial resistance in the present study, imipenem showed a resistance rate of 30.7%. This finding is generally in agreement with other data seen in Egypt, which reported that imipenem-resistant strains was 29% [21] and 30% of *P. aeruginosa* isolates harbored a resistant gene [22,23].

Together with this study, many studies [9,16,20,24,25,26,27] have reported that the MBLs were found to be emerged in the carbapenem resistance in *P. aeruginosa,* and hence the VIM (*blaVIM*) appears to be an important MBL gene in MBL-producing isolates.

The VIM gene in *P. aeruginosa* is reported to be the most common gene among several genes encoding the MBLs [18]. In 2014, Castanheira and co-workers found that 20% of the isolates were positive for MBL, with the VIM-type enzymes [28]. In this study, the prevalence of the VIM gene was found to be 36%, which is similar to the finding of an Iranian study, which reported that 30% of *P. aeruginosa* isolates harbored the *blaVIM* [22].

The prevalence of the VIM gene that was seen in this study may be explained by the fact that these strains were only isolated from patients hospitalized in a burn hospital for a long time, intubated, and had undergone antibiotic therapy, mainly a combination of carbapenems, aminoglycosides, and/or fluoroquinolones. In addition, the majority of these isolates were recovered from burn patients who had skin grafting. This indicates the presence and propagation of the above-mentioned gene under such circumstances.

https://doi.org/10.47705/kjdmr.261011
eISSN:*2708-888X*

Conclusion

This study proved that the majority of *P. aeruginosa* strains were resistant to various classes of antibiotics. The high incidence of carbapenem resistance among *P. aeruginosa* VIM producers is very alarming and can be responsible for serious infections, especially in Libyan hospitals. It appears from this study that antibiotic resistance may be considered a medical threat, limiting the treatment options in Libyan hospitals. Finally, it is suggested that it is important to adopt and implement continuous surveillance programs for such organisms to assess the effectiveness of current control strategies, as well as the formulation of new ones.

Limitation of the study

This study has limitations, of which the most important is the limited data about the specimens' source, e.g., wound, blood, or urine, and demographic data regarding patient gender, age, the treatment received in the hospital, intubated or not, and whether they had undergone antibiotic. This study has limitations, of which the most important is the limited data about the specimens' source, e.g., wound, blood, or urine. Because of the scarce information about the participants, it is difficult to determine how the variation in participation has influenced the study outcome.

Conflict of interest. Nil

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