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DETECTION OF *bla*-VIM1GENE IN CARBAPENEM-RESISTANT *Pseudomonas aeruginosa* ISOLATED FROM CLINICAL SAMPLES IN WASIT PROVINCE HOSPITALS.

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ABSTRACT

The present study investigated the presence of *bla*-VIM1in clinical isolates of *Pseudomonas aeruginosa*. During the period from November 2017 to February 2018, a total of two hundred patients admitted to (Al-Zahraa Teaching Hospital, Al-Karama Teaching Hospital and Al-Kut Hospital for Gynecology, Obstetrics and Pediatrics) in Wasit province. One hundred and three of isolates were diagnosed as *P. aeruginosa*. High prevalence of *P. aeruginosa* isolates were detected in burn swab samples 70 (35%) followed by sputum 12 (6%) and ear 11 (5.5%). All 103 *P. aeruginosa* isolates were primarily screened for carbapenems - resistance, 36 (34.95%) were resistant to carbapenems. Carbapenems resistant isolates were underwent antimicrobial susceptibility to 14 antibiotics using Kirby-Bauer disk diffusion method. The phenotypic and molecular methods of carbapenem resistance were investigated. Carbapenems resistant isolates were identified by the Double disc synergy test, which detects the probability of isolates able to produce Metallo-beta- lactamases (MBL). Out of the isolates 32 (88.89%)were positive. Imipenem-EDTA combined disk method showed 30 (83.33%) of isolates possessed ability to produce the Metallo-beta-lactamases. In addition , the Modified Hodge test (MHT) showed the ability of isolates to produce Carbapenemases enzyme. 16 (44.44%) of the isolates were positive to Modified Hodge test. Carbapenemase gene were detected by PCR technique. The results demonstrated that out of 36 carbapenems resistant *Pseudomonas aeruginosa* (CRPA) isolates 34 (94.44%) were positive to *bla*-VIM1 gene.

INTRODUCTION

Pseudomonas aeruginosa is an aerobic Gram-negative rod-shaped. It is widely distributed in nature and can adapt to many environments, it can be isolated from nearly any conceivable source within hospitals (1). It is an important cause of both community and hospital-acquired infections. Infections with these bacteria have been associated with high mortality and morbidity when compared with other bacterial pathogens (2) *P. aeruginosa* infections are clinical problem, it's a difficult to treat because of high resistant to many antibiotics (Multi-drug resistant) and a high risk of emergence of resistance during therapy (3).

Beta-lactam as antibacterial agent are broadly used to treat diseases caused by Gram-negative Pathogens However, the adequacy of these medications is lessened impressively because of the presence of extended-spectrum beta lactamases (ESBLs) and the consequent emergence of multi-drug resistant (MDRs) strains (3).

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 (4) They include meropenem and imipenem, which are among the few therapeutic options still available for treating infections caused by *P. aeruginosa* (5). Carbapenems are considered to be antimicrobial agents of choice and are frequently used for the treatment of hard-to-manage *P. aeruginosa* infections. However, carbapenem resistance in *P. aeruginosa* has been reported to increase steadily over the years across the world, but the relative contribution of different carbapenems resistance mechanisms is not well established (6,7). The goal of this research was to detect the presence of *bla*-VIM1 producers in isolates of *P. aeruginosa* isolates producer between clinical *P. aeruginosa* isolates in Wasit hospitals. Also, study the relationships between the presence of resistance genes *bla*-VIM1and the sensitivity to ten antibiotics.

MATERIALS AND METHODS

Collection of samples

Over four months from November 2017 to February 2018, different samples including (burn swab, ear swab, urine, sputum and wound swab) from two hundred patients admitted to (Al-Zahraa Teaching Hospital, Al-Karama Teaching Hospital and

Al-Kut Hospital for Gynecology, Obstetrics and Pediatrics) in Wasit province were enrolled in this study.

Identification of *P. aeruginosa*

In the case of swab samples, two swabs were taken from each patient, while sputum and urine were divided directly into two parts, the first swab was prepared for stained smear preparation (Gram stain), and the other was used for culturing on different culture media for further isolation and characterization of the causative agents. The isolated bacteria were identified by standard laboratory methods and API20E system (BioMerieux), *P. aeruginosa* were isolates in Brain-Heart Infusion (BHI) broth containing 15% glycerol, and the tubes were stored in freezing at -20 C° (8).

Antimicrobial susceptibility testing

Resistance patterns of the *P. aeruginosa* isolates to different antibiotics was determined using disk diffusion test (Kirby-Bauer) on Muller Hinton agar media (9), the antibiotic discs used in this study were Levofloxacin (5 µg), Meropenem (10 µg), Imipenem(10 µg), Aztreonam (30 µg), Ceftazidime (30 µg), Amikacin (30 µg), Gentamicin (30 µg), Ciprofloxacin (10 µg), Piperacillin (10 µg), Colistin sulphate (25 µg). The standard isolate from central public health laboratory *E. Coli* ATCC25922 was used as a negative control. The resulting zones of inhibition were measured and compared with the break points standard value of Clinical Laboratory Standards Institute CLSI (9). The minimum inhibitory was determined by Vitik2 - compact system AST card, and standard agar dilution method (10) according to the CLSI (9).

Phenotypic detection of Metallo-β-lactamases (MBL)

All imipenem and meropenem-resistant isolates were examined for MBL production using the IMP-EDTA double disk synergy test as described by (11), furthermore, Modified Hodge test (MHT) was used for detection of Carbapenemases production *P. aeruginosa* isolates according to CLSI guidelines using 10 µg meropenem susceptibility disk, which was placed in the center of the test area. *P. aeruginosa* was streaked in a straight line from the edge of the disk to the edge of the plate. The plate was incubated overnight at 37 C° in ambient air for 16-24 hours. After 24 hours, MHT positive test showed a clover leaf-like (12).

DNA Extraction and polymerase chain reaction (PCR) amplification

In this study, both plasmid DNA and chromosomal DNA were extracted, plasmid DNA was extracted according to (13), while chromosomal DNA was extracted by Genomic DNA Mini Kit (Genaid) according to company instructions. All carbapenem-resistant isolates were screened by standard PCR conventional using specific primers for *bla*NDM-1 gene as shown in table (1). PCR reaction tubes were transferred into thermal cycler (eppendroff,Germany) that was programmed as following: initial denaturation for 4 mints at 95 C°, 30 cycles were performed (the conditions for each cycle were: 30 sec. at 94 C°, 1mints. at 56 C° and 90 sec. at 72 C°), and final extension at 72 C° for 5 mints. Amplified products were electrophoresed on 1.5% agarose for 90 mints at 5 V/cm.

Table 1. Sequences of primer that used in the detection blaNDM-1 gene

Gene		Nucleotide sequences (5'→3')	Products size bp	References
***bla*-VIM1**	**F**	**AGT GGT GAG TAT CCG ACA G**	261	(14)
	R	**ATG AAA GTG CGT GGA GAC**		

Statistical Analysis

Statistical analysis was performed with Graph Pad Prism version 6 software, percentages were used for the comparison between samples of the study. Data analysis was done using Chi-square for the comparison of categorical data.

RESULTS

A total of two hundred samples were recorded in this study which include, burn swabs (n=105, 52.50%), ear swabs (n=19, 9.50%), wound swab (n=36, 18.00%), sputum from patients with lower respiratory tract infection (n=7, 3.50%) The patient's ages ranged from one to greater than 61 years table (2).

Table 2. Distribution of *Pseudomonas aeruginosa* isolates according to age groups

Age groups	*P. aeruginosa*	Others bacteria	Negative	Total
1-10 yr.	11	10	2	23
11-20 yr.	10	9	2	21
21-30 yr.	20	12	4	36
31-40 yr.	27	13	2	42
41-50 yr.	11	14	3	28
51-60 yr.	9	7	4	20
≥61 yr.	15	13	2	30
Total	**103**	**78**	**19**	**200**

One hundred and eighty one bacterial species were isolated from these samples with the rate of *P. aeruginosa* (n=103, 51.50%) followed by *E. coli* (n=28, 14.00%) and the lowest percentage were *K. pneumoniae* (n=2, 1.00%). There is non-significant association between Pseudomonas infections and age groups.

Antimicrobial susceptibility test

The results of antibiotic susceptibility test for isolated *P. aeruginosa* indicated different antibiotic profiles as shown in table (4). In total, 55.5% (n=103) resistance to the third generation ceftazidime 57.28% of the isolates exhibited resistance to the fourth generation cefepime. While the resistance to monobactams, aztreonam was 51.46%. The highest resistance percentage was found against gentamicin (91.26%). According to the results of the fluoroquinolones susceptibility testing, 83.50% and 60.19% of the isolates were resistant to ciprofloxacin and levofloxacin, respectively table (3).

Table 3. Susceptibility patterns of *Pseudomonas aeruginosa* to different antibiotics

Antibiotic	Sensitive (S)		Intermediate (IR)		Resistant (R)	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
Imipenem	67	65.05	0	0	36	34.95
Meropenem	67	65.05	0	0	36	34.95
Ciprofloxacin	12	11.65	5	4.85	86	83.50
Levofloxacin	38	36.89	3	2.91	62	60.19
Amikacin	9	8.74	6	5.83	88	85.44
Gentamicin	5	4.85	4	3.88	94	91.26
Cefepime	42	40.77	6	5.83	55	53.40
Ceftazidime	39	37.86	5	4.85	59	57.28
Aztreonam	40	38.83	10	9.71	53	51.46
Piperacillin	17	16.50	16	15.53	70	67.96
Piperacillin/Tazobactam	50	48.54	8	7.77	45	43.69
Colistin	102	98.03	0	0	1	0.97
Ticarcillin/Clavulanic acid	47	45.63	5	4.85	51	49.51
Ticarcillin	40	38.83	7	6.80	56	54.37

Phenotypic detection of MBLs

From 36 of *P. aeruginosa* carbapenem-resistant isolates, MHT revealed 16 (44.44%) were positive showing their ability to produce carbapenemases figure (1), moreover, double disc synergy indicate that in 32 (88.89%) isolates figure (2), and 30 (83.33%) isolates were positive to Imipenem-EDTA combined disk test figure (3). Those isolates, which were found MBL positive by Double disc synergy test , Imipenem-EDTA combined disk test and were also found to be MBL positive MHT table (4).

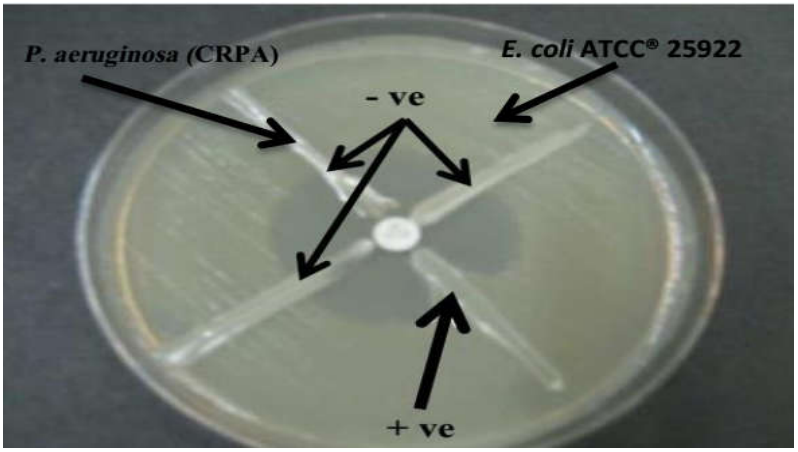

Figure 1. The Modified Hodge test (MHT) of carbapenemase production

-ve =Negative carbapenemase production.

+ve = Positive carbapenemase production.

Figure 2. Combined disk test for *P. aeruginosa (CRPA)* showed positive test for MBLs production using imipenem and imipenem with EDTA. 1- Imipenem without EDTA. 2- Imipenem with EDTA.

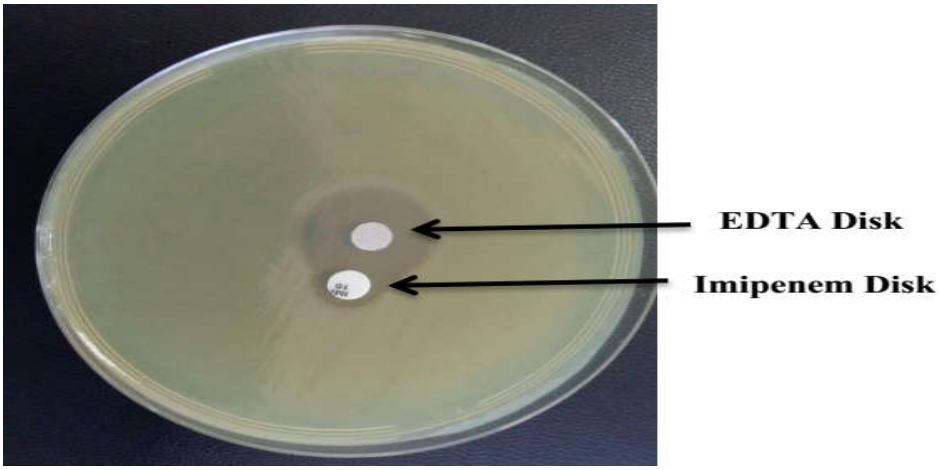

Figure 3. Double disc synergy test for *P. aeruginosa* (CRPA) showed positive test for MBLs production using imipenem disk and EDTA disk.

Table 4 . Phenotypic test for carbapenemase production

Test	Positive	Negative
Double disc synergy test	32 (88.89%)	4 (11.11%)
Imipenem-EDTA combined disk method	30 (83.33%)	6 (16.67%)

modified Hodge test	16 (44.44%)	20 (55.56%)

PCR screening for NDM-1 encoding gene

PCR was carried out by using specific primers for VIM-1 and performed on all the Carbapenems-resistant isolates for generation of specific amplification band with certain molecular weight that were 261 bp fragment which represented *bla*-VIM1gene. The results showed MBL gene *bla*-VIM1 (261 bp) was detected in 34 (94.44%) of the carbapenem-resistant isolates on plasmid DNA, while MBL gene *bla*-VIM1*gene* not found on chromosomal DNA figure (1).

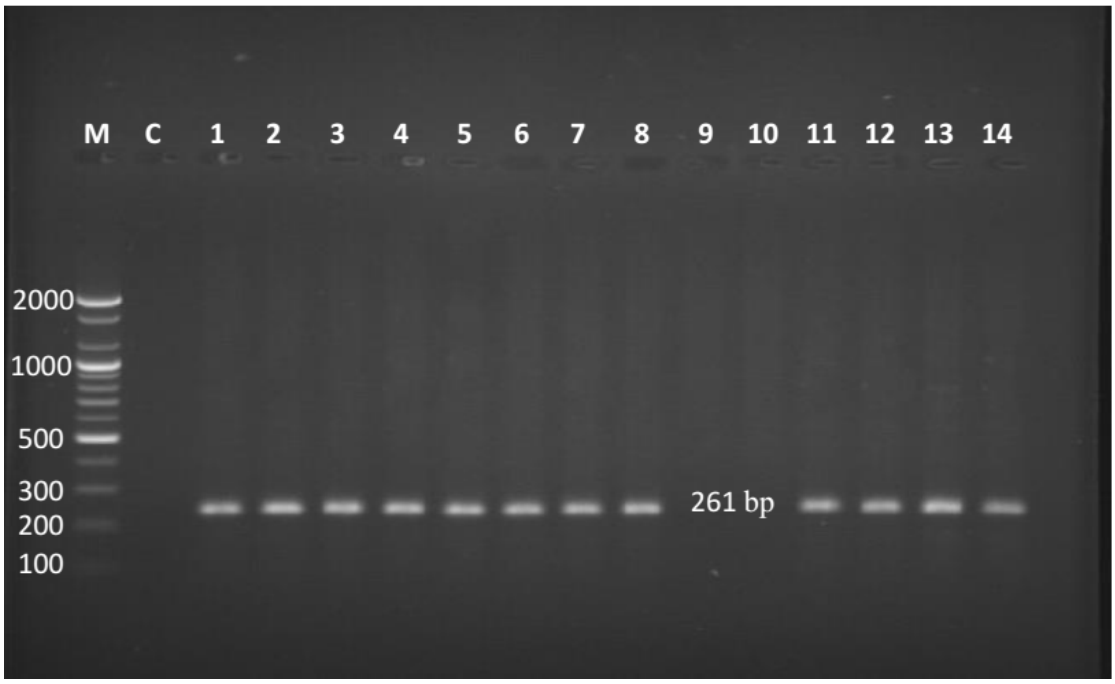

Figure 1. Gel electrophoresis of amplified plasmid DNA for detection of MBL bla-NDM1 gene (261bp) using PCR with specific primers; 1,5% Agarose for 90 minutes at 70 V\cm. Lane M: Marker DNA ladder Size (100bp), Lane C: Negative control and Lanes (1-14) positive for *bla*-VIM1 (٢٦١ bp) except (٩,١٠)

DISCUSSION

The current emergence of *P. aeruginosa* carbapenem-resistant represents a major threat to the clinical approach because it exhibits intrinsically decreased susceptibility to a range of antimicrobials and possesses a great ability to develop resistance to multiple classes of agents (15). Among two hundred samples were

recorded in this study, the mean age of the patients were 36.61 years. Results of current study revealed that, there is non-significant (P > 0.05) association between Pseudomonas infections and age. It is noteworthy to mention that result was disagreed with a study conducted by Magliano *et al*. (16) who was reported the high rate of *P. aeruginosa* infection among age group (≥ 60 years). In the present study, *P. aeruginosa* has been the predominant bacterial isolated among study group followed by *E. coli* (14%) and the lowest percentage were *K. pneumoniae* (1%). These findings are compatible with study conducted in Egypt (17), the present study is incompatible with a study in Baghdad (18) who found that *Acinetobacter baumannii* (31%) is even more common than *P. aeruginosa* (12%), and study conducted in Baghdad (19), who reported that *S. aureus* (30%) was the most common agents, then *P. aeruginosa* (14.6%) this difference in results can be attributed to sample difference and kind of test used in isolation and diagnosis of different bacterial species.

Carbapenems are a class of β-lactam antibiotics with good antimicrobial activity against *P. aeruginosa* but the arises and spread of acquired carbapenem-resistance in this species have challenged the success of therapeutic and control efforts (20). Result in current study showed there was no difference found in activity of imipenem and meropenem to *P. aeruginosa* (both of them have the same percentage 34.95% resistant, respectively), which disagree with Gupta *et al*. 2006 who found that the imipenem had a better activity than meropenem (21). Furthermore, current finding indicated that higher resistance against imipenem and meropenem have compared with study in Najaf (22), who reported that the resistance rate was 7.4% and 14.8%, respectively. The percentage of fluoroquinolone-resistant isolates was 83.50% and 60.19% of isolates resistant to ciprofloxacin and Levofloxacin, respectively identified in this study is considerably higher than that reported in study conducted in Najaf Hospitals, in which resistance were 73.4 % for ciprofloxacin and 55.5 % for Levofloxacin (22) also it is in harmony with previous study in Najaf (23). Fluoroquinolone resistance among *P. aeruginosa* isolates looks to be increasing in the Wassit hospitals, perhaps because of high increasing fluoroquinolone use, and the lack of adherence to approved infection control practices by hospitals. The *P. aeruginosa* isolates were most resistant to amikacin (85.44%) and gentamicin (91.26%), the resistance rate was higher when compared with other study reported (22) in Najaf, who revealed that only 64.8% of the *P. aeruginosa* isolates resistant to

this antibiotic. However, the findings of *P. aeruginosa* antibiogram in the present study disagree with a study done in United States of America (24). Results in the current research, showed that 51.46% of the *P. aeruginosa* isolates were resistant to aztreonam, Present findings are higher than previous study done by Abdullah, who showed low rate of aztreonam resistance among *P. aeruginosa* clinical isolates (25). Colistin resistance is not dependent upon bacterial metabolic activity and acquired resistance is rare (26). In present study, the resistance of the isolates to Colistin was 2.78%, this result disagreed with the study in Turkey, who mentioned that all multidrug-resistant strains were 100% susceptible to Colistin (26). The present investigation showed that Colistin was only antibiotic that may remain highly active against carbapenem resistance *P. aeruginosa* (CRPA) isolates, these results accepted with study in Iran (27). This might be explained by the high cost of Colistin and limited use out of the hospitals. The high rate of resistance observed in *P. aeruginosa* isolates in this study, may be explained by incorrectly prescribed antibiotics, extensive of antibiotics in animal food which in turn transfers to humans by meat and egg consumption, and availability of few new antibiotics.

The production of MBLs is the most common mechanism for carbapenem resistance in *Enterobacteriaceae* and *P. aeruginosa* isolates (28,29).The resistant isolates were tested by MHT revealed 16 (44.44%) were positive isolates, in addition double disc synergy test showed that 32 (88.89%) isolates out of 36 *P. aeruginosa* (CRPA) were positive. Present study revealed that these two tests may be useful in screening for MBL, but these tests cannot be routinely performed in all national laboratories. The current results showed that 94.44% percentage of *P. aeruginosa* (CRPA) isolates have *bla*-VIM1 gene in plasmid DNA, the percentage of *bla-VIM1*gene in the current study was higher than previous study in Saudi Arabia was noted VIM appeared in 29.4% of our isolates (30). In another study in Turkey, 100 *P. aeruginosa* isolates were collected from patients in a Turkish university hospital 1% isolate was found to carry *bla*-VIM gene (31).

When compared to previous studies in Iran, current finding indicated that the rate of *bla*VIM-1 was higher in this study compared with previously reported by Mahmood (32), who found the rate was 18%. The *bla* VIM genes are located in class 1 integrons as a gene cassettes and have been identified on plasmid with different

replicon types (33), increasing the possibility of dissemination and linkage to other antibiotic resistance genes. However, this constitutes the first report on prevalence and detection of *bla*-VIM1 gene in Wasit province.

CONCLUSION

We concluded Rate of occurrence of *bla*-VIM1producers was highest among carbapenem- resistant *Pseudomonas aeruginosa* isolated from clinical samples in Wassit Province hospitals. Therefore, the detection of *bla*-VIM1 positive *P. aeruginosa* isolates in this study indicates importance of strengthening surveillance to prevent the nosocomial infection and dissemination of VIM1 in Wassit.

الكشف الجزيئي عن جين الميتالوبيتالاكتاميز (*bla*VIM-1) في جرثومة *Pseudomonas aeruginosa* المقاومة لمضادات الكاربابينيم والمعزولة من مرضى مستشفيات محافظة واسط

زياد خلف حسين ، اسراء جبار شمخي

الخلاصه

تهدف الدراسة الحالية الى التحري عن موروث الميتالوبيتالاكتاميز *bla*VIM-1 بين عزلات بكتريا *Pseudomonas aeruginosa* تم الحصول على ١٠٣ عزلة بكتيرية تابعة لبكتريا *Pseudomonas aeruginosa*من مجموع ٢٠٠ عينة سريرية ومن ثلاث مستشفيات رئيسية في محافظة واسط للفترة من تشرين الثاني ٢٠١٧ الى شباط ٢٠١٨. ان اكثر عزلات *Pseudomonas aeruginosa* تم عزلها من عينات الحروق (35%) 70 تلهتا عينات الجروح (6%) 12 والاذن (5.5%) 11.

وقد اظهر المسح الاولي لـ ١٠٣ عزلة *Pseudomonas aeruginosa* مقاومة للمضادات الحيوية من عائلة الكاربابنيم وعددها ٣٦ عزلة. اختبرت حساسية العزلات المقاومة لمضادات الكاربابنيم للمضادات الحيوية بطريقة انتشار القرص.

تم دراسة الطرق المظهرية للعزلات المقاومة للكاربابنيم .بالأضافة الى انهُ تم تحديد العزلات المقاومة للكاربابنيم بواسطة اختبار (Double disc synergy test) في الكشف عن قابلية العزلات على انتاج انزيمات البيتا لاكتاميز(Metallo-beta-lactamases (MBL ، وكان من بين العزلات ٢٣(٨٨.٨٩%) موجبة لهذا الفحص. وكذلك طريقة فحصImipenem-EDTA combined disk . اظهرت ٣٠ (٨٣.٣٣%) من العزلات تمتلك القدرة على انتاج انزيمات البيتالاكتاميز .بالاضافة الى ذلك اظهر اختبار Modified Hodge test قدرة العزلات على انتاج انزيم الكاربابنيميز حيث اظهرت ١٦ (٤٤.٤٤%) من العزلات ايجابية لهذا الفحص.

تم الكشف عن جين *bla*-VIM1 بواسطة تقنية سلسلة تفاعل انزيم البلمرة (PCR) حيث اظهرت النتائج للـ ٣٦ عزلة *Pseudomonas aeruginosa* مقاومة للكاربابنيم (CRPA) ، (٩٤.٤٤%)٣٤ عزلة كانت موجبة لجين *bla*-VIM1 عزلة موجبة لجين

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