Dawood and Alkazaz *Iraqi Journal of Science, 2024, Vol. 65, No. 9, pp: 5016-5025*
DOI: 10.24996/ijs.2024.65.9.18

ISSN: 0067-2904

Evaluation of the Expression of *SIM* and *NDM* Genes in *Pseudomonas Aeruginosa* Isolated from Clinical Sources

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Received: 4/6/2023 Accepted: 20/8/2023 Published: 30/9/2024

Abstract

Pseudomonas aeruginosa has recently been labeled a major threat to public health due to its resistance to almost all commonly used antibiotics. Many factors have been suggested for *P. aeruginosa's* antibiotic resistance. The goal of this study is to find out what role SIM and NDM, which are related to carbapenem resistance, play in P. aeruginosa isolates from local clinical sources. In this study, out of 110 different clinical specimens, 50 were identified as *P. aeruginosa* from hospitalized patients. All of the isolates were characterized based on the biochemical test and confirmed using the VitekII compact system. P. aeruginosa isolates were tested for antibiotic susceptibility using 8 antibiotics, including: amikacin, tobramycin, ciprofloxacin, levofloxacin, imipenem, meropenem, piperacillin/tazobactam, ticarcillin, and clavulanate. Susceptibility testing results revealed that every isolate was highly resistant to Piperacillin/Tazobactam, with lower resistance to Ticarcillin/clavulanate (TCC). Using the broth dilution method, the minimum inhibitory concentration (MIC) of P. aeruginosa isolates resistant to meropenem ranged from 32µg/100µl to 128 µg/100µl. The EDTA combined disc test was used to detect the ability of P. aeruginosa isolates to produce carbapenemase, and the results showed that all isolates were carbapenemase producers. Additionally, conventional PCR confirmed the identification of *P. aeruginosa* using 16S. Real-time PCR was adopted to assess the expression of the NDM and SIM genes in 25 of the identified P. aeruginosa isolates. The mean of gene expression results for NDM showed increased expression compared to the control sample of 1.74, while the SIM gene showed expression of 0.95. These genes, SIM and NDM in class B (which are important for resistance in Pseudomonas aeruginosa), result from chromosomal changes that mutate the membrane permeability flow pump, causing excessive expression.

Keywords: Antibiotics resistance, carbapenem resistance genes, MIC, Real-Time PCR

تقييم تعبير جينات *SIM* و *NDM* في بكتريا الزائفة الزنجارية المعزولة من المصادر السريرية

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الخلاصة

تم تصنيف بكتريا الزائفة الزنجارية مؤخرًا على أنها خطر كبير على الصحة العامة بسبب مقاومتها لجميع المضادات الحيوية تقريبًا. هناك العديد من العوامل التي تساهم في تطوير البكتريا لهذه الصفة. الهدف من الدراسة هو التأكد من حدوث عزلات وبائية من بكتريا الزائفة الزنجارية التي تحمل جينات المقاومة لل

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carbapenem بالإضافة إلى نشاط هذه الجينات .تم جمع في هذه الدراسة 110 عينة سريرية مختلفة، وتم التأكد من 50 عينة على انها بكتريا الزائفة الزنجارية من المرضى في المستشفى. ثم؛ تم توصيف جميع العزلات باستعمال الاختبار البيوكيميائي والتأكد من ذلك باستعمال نظام VitekII تم اختبار جميع العزلات البكتيرية لثمانية انواع من اقراص المضادات الحيوية منها: Amikacin, Tobramycin, Ciprofloxacin, Levofloxacin, Imipenem Meropenem, Piperacillin /Tazobactam, Ticarcillin clavulanate.. وفقا لاختبار الحساسية الجرثومية أظهرت جميع العزلات مقاومة عالية للبيبيراسيلين ومقاومة أقل للتيكارسيلين. تم تحديد الحد الأدنى للتركيز المثبط فقط لعزلات *Pseudomonas aeruginosa* المقاومة لـ Meropenem باستعمال طريقة تخفيف المرق. أظهر MIC أن هذه البكتيريا كانت مقاومة لـ Meropenem بتركيز يتراوح بين 32 ميكروغرام / 100 ميكرولتر إلى 128 ميكروغرام / 100 ميكرولتر. وتم استخدم اختبار EDTA combined disc لتحديد قدرة عزلات *P.aeruginosa* على انتاج انزيم carbapenemase واظهرت النتائج بان جميع العزلات كانت منتجة لل carbapenemase ثم تم استخدام PCR التقليدي للكشف عن العزلات باستخدام 16 لإثبات أن البكتريا *p. aeruginosa* تم اجراء تفاعل البلمرة الكمي لتحديد تعبير الجينات NDM و*SIM* لخمسة وعشرين عزلة من عزلات *P. aeruginosa* المشخصة وكان متوسط نتائج التعبير الجيني لـ *NDM* أظهر تعبيرًا زائدًا مقارنة بعينة السيطرة هو 1.74 بينما اظهر جين *SIM* تعبيرا (0.95 وان هذه الجينات **_SIM_ and _NDM_** في الفئة B (التي تعتبر مهمة للمقاومة في الزائفة الزنجارية ناتجة عن التغيرات الكروموسومية والتي تحور مضخة تدفق نفاذية الغشاء مسببة الإفراط في التعبير.

الكلمات المفتاحية: مقاومة المضادات الحيوية ، جينات مقاومة الكاربابينيم ، التركيز المثبط الادنى ، تفاعل البلمرة الكمي

1. Introduction

A common Gram-negative bacterium of the *Pseudomonadaceae* family, *Pseudomonas aeruginosa*, can thrive in a variety of conditions [1]. Due to this bacteria's great resistance to a variety of medications, treating its illness is quite difficult [2]. One of *P. aeruginosa's* most notable traits is its exceptional ability to evolve chromosomal alterations that confer antimicrobial resistance to almost all antipseudomonal treatments [3]. Strong biofilms produced by *Pseudomonas aeruginosa* are known to be prevalent in the environment and to have a considerable impact on human life in both positive and negative ways [4]. *Pseudomonas aeruginosa* biofilms have a matrix that mostly consists of polysaccharides, proteins, extracellular DNA, and lipids. The composition of the matrix depends on the strain as well as the development circumstances and the age of the biofilm [5]. When they target the extracellular matrix, a number of virulence factors may produce pathogenicity that promotes adhesion and/or interferes with host cell signaling pathways. *P. aeruginosa* has the ability to infect an organism and its immune system with a number of diseases, making infections nearly impossible to treat [6]. It also has a number of virulence factors that are frequently present, particularly exotoxin A and exotoxin S, which are regulated by cell-to-cell signaling patterns. Exotoxin A also prevents protein synthesis from occurring [7]. *P. aeruginosa* is one of the three most common bacteria causing healthcare-associated respiratory infections, and it is currently resistant to several classes of treatment [8]. Carbapenems are thought of as first-line treatments for severe *P. aeruginosa* infections. These genes are typically encoded by mobile genetic elements that can spread horizontally across Gram-negative bacteria, such as plasmids, transposons, and integrons [9]. The metallo-lactamases (MBL) of Class B, such as Seul imipenemase (*SIM*) and New Delhi metallo-lactamase (*NDM*), are among the numerous carbapenemases that have been discovered in *Pseudomonas* species [10]. The aim of the study is to ascertain the incidence and epidemiology of *P. aeruginosa* isolates carrying the carbapenem gene as well as the gene's activity.

2. Materials and Methods

2.1 Bacteria isolation and identification

A total of 110 bacterial samples were collected, including wound, ear, urine, and burn samples, from individuals who were admitted to different hospitals in Baghdad city: the educational laboratory/medical city, Baghdad teaching hospital, Burns specialist hospital, and Ghazi Al-Hariri Hospital. Utilizing both the Vitek 2 compact system and culture media with biochemical testing, all isolates were correctly diagnosed.

2.2 Antibiotic susceptibility test

All of the bacterial isolates were tested for antibiotic susceptibility using 8 different antimicrobial agents, which are: Amikacin (30µg/disc), Tobramycin (10µg/disc), Ciprofloxacin (5µg/disc), Levofloxacin (5µg/disc), Imipenem and Meropenem (10µg/disc), Piperacillin/Tazobactam (10/100µg/disc), and Ticarcillin/clavulanate (75/10µg/disc). After the bacterial suspension was adjusted to 0.5 McFarland by measuring the optical density with a spectrophotometer, the bacterial suspension was spread on the surface of Muller Hinton agar by using a cotton swab, and the antibiotic discs were placed on the surface of the agar and incubated. The results were interpreted according to the guidelines of CLSI (2022) [11].

2.3 EDTA combined disc test (phenotype detection)

This test, which helps in the detection of the genes (*NDM*, *SIM*) [12], was carried out following the instructions given by Galani et al., in which the inoculum was adjusted to 0.5 McFarland before the bacterial specimen was transferred from the inoculum to the Muller Hinton agar. Following the spread of bacteria on the agar, imipenem and imipenem+EDTA were added to the agar surface, incubated for 18 hours at 37 ^{o}C, and the results were analyzed using the CLSI (2022) guidelines [11].

2.3 Extraction of Genomic DNA

By using the Easy Pure® Bacteria Genomic DNA Kit (TRANS Gen Biotech/China), genomic DNA was extracted. Genes were detected in 16S rRNA by conventional PCR using the primers listed in Table 1.

Table 1: All primers used in this study

Gene name	Primer sequence 5′→3′		References
SIM	F	TACAAGGGATTCGGCATCG	[12]
	R	TAATGGCCTGTTCCCATGTG	
NDM-1	F	ACCGCCTGGACCGATGACCA	[12]
	R	GCCAAAGTTGGGCGCGGTTG	
16S-rRNA	F	GAGCGGATAACAATTTCACACAGG	[13]
	R	CGCCAGGGTTTTCCCAGTCACGAC	
16S-PCR	F	TGCCTGGTAGTGGGGGATAA	[12]
	R	GGATGCAGTTCCCAGGTTGA	

The ® PCR Super Mix was prepared, and a reaction volume of 25 µl was used for this process. The components of the reaction included 2×EasyTaq® PCR Super Mix (12.5µl), Forward Primer (1 µl), Reverse Primer (1µl), Template DNA (5µl) and Nuclease-Free Water (5.5µl). Primers shown in Table 1 were used for the detection of 16S by the extraction of the DNA of *Pseudomonas aeruginosa*. The steps of PCR included: Stage 1: initial denaturation at 94 ºC for 5 min in 1 cycle; Stage 2: denaturation at 94 ºC for 30 sec in 35 cycles; Stage 3: annealing at 58 ºC for 30 sec in 35 cycles; Stage 4: extension at 72 ºC for 1 min in 35 cycles. The final extension phase was performed at 72 ºC for 5 minutes before the amplification was completed. A 2% agarose gel containing ethidium bromide was used to identify the PCR results, and the gel was afterwards analyzed under a UV light source.

2.4 Extraction of RNA

For gene expression, complementary DNA (cDNA) was synthesized from mRNA using the Easy Script® One-Step gDNA Removal and cDNA Synthesis Super Mix Kit (cDNA). RNA was extracted using the TransZol Up Plus Kit (TRANS/China).

2.5 Complementary DNA synthesis from mRNA

Complementary DNA (cDNA) was synthesized using the Easy Script® One-Step gDNA Removal and cDNA Synthesis Super Mix Kit to produce cDNA from mRNA. After mixing all the necessary components, three stages of conventional PCR were used to transform mRNA into cDNA. Random primers were annealed to mRNA during the first stage, which started at 25 °C for 10 minutes. The reaction mixture was then moved on to step two to allow the enzyme reverse transcriptase to transform mRNA into cDNA, where the reaction temperature was raised to 42 °C for 30 minutes. Step three of the reaction involved raising the temperature to 85 °C for five minutes to stop the reaction.

2.6 Determination of Minimum inhibitory concentration (MIC)

Using a broth microdilution technique, the MICs of Meropenem for P. aeruginosa were calculated. The antibiotic was dissolved in Muller-Hinton broth before being transferred to the microdilution plate. about 16, 32, 64, 128, and 256µg/100µl as the initial concentration. The result of MIC was interpreted according to the guidelines of CLSI (2022) after 18 hours of growth [11].

2.7 Gene Expression by RT-PCR

Gene expression analysis using RT-PCR was carried out on 25 RNA samples in accordance with their resistance. SYBR green, a fluorescent dye that can only bind to double-strand DNA and emit light after doing so, was utilized in this approach. Real-time PCR allows for the measurement of fluorescence light. In this stage, 10^1 of a 2x QPCR master mix, the procedure was performed on a Corbett Realtime PCR System using Syber Green. Reaction components included 2×EasyTaq® PCR Super Mix (10µl), cDNA (3µl), primers (2µl), and nuclease-free water (5 µl). The thermal cycle conditions of genes are: (1) denaturation to 95°C in 10 sec; (2) annealing to 64 °C for SIM and 60 °C for NDM for 30 sec. After a minute-long dissociation phase at 95 °C, the amplification finally came to an end.

3. Results and Discussion

3.1 Identification of P. aeruginosa

In this study, the total number of specimens was 110, of which only 50 were diagnosed as *Pseudomonas aeruginosa* using the Vitk2 device, culture media (MacConkey agar, Blood agar, and cetrimide agar), and biochemical tests (oxidase, catalase, citrate, and indole tests).

According to the type of clinical source, the fifty isolates were distributed as follows: 40% from burn swab samples, 30% from wound swabs, 20% from ear discharge, and 10% from urine. Figure 1 shows the distribution of *P. aeruginosa* isolates according to clinical sources.

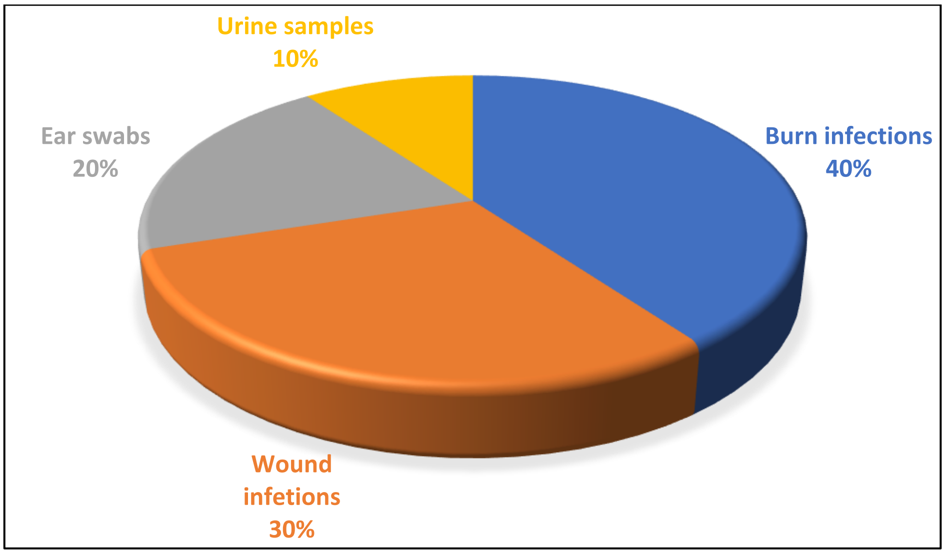

Figure 1: Distribution of *P. aeruginosa* isolates according to clinical sources

3.2 Antibiotics susceptibility test

The result of the test of susceptibility to antibiotics revealed that, out of 50 *P. aeruginosa* isolates, 36, 38, 96, 40, 56, 50, 96, and 32% were resistant to Ciprofloxacin, Levofloxacin, Piperacillin/Tazobactam, Tobramycin, Imipenem, Meropenem, Amikacin, and Ticarcillin/clavulanate (Figure 2).

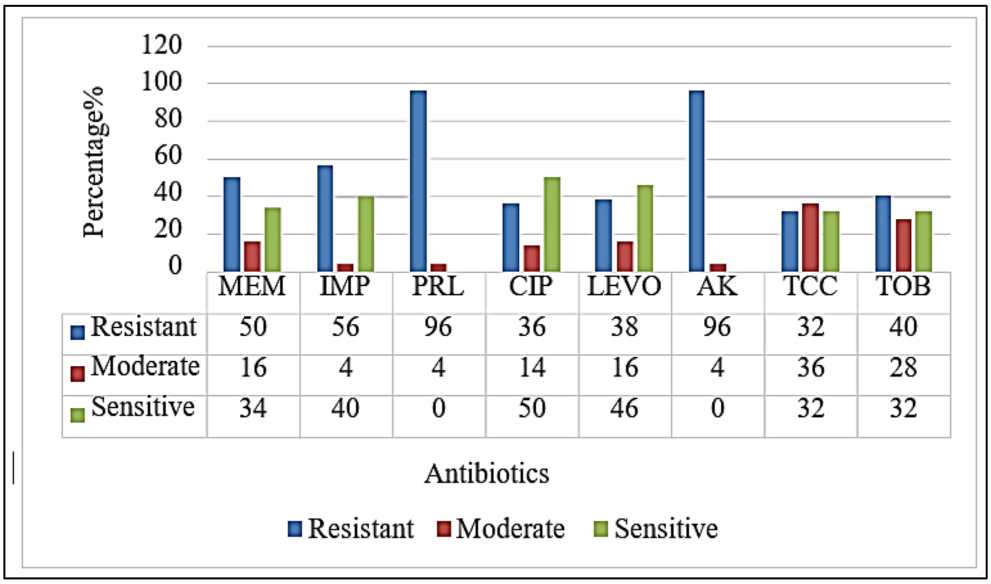

Figure 2: The patterns of antibiotic resistance of *P. aeruginosa* to Amikacin (AK), Tobramycin (TOB), Ciprofloxacin (CIP), Levofloxacin (LEV), Imipenem (IMP), Meropenem (MEM), Piperacillin/Tazobactam (PRL), and Ticarcillin/clavulanate (TCC)

The growth and spread of multidrug-resistant (MDR) strains of P. aeruginosa are considered major health problems for many reasons. P. aeruginosa is a leading cause of death from infection, especially in hospitals and among those with impaired immune systems [14]. It has been reported [15] that all isolates of P. aeruginosa are resistant to tobramycin and gentamycin. Also, most of them were also resistant to tobramycin (77%), whereas nonsusceptibility rates were lower for amikacin (7%) and nearly all (>95%) of the isolates were non-susceptible to piperacillin-tazobactam, ceftazidime, imipenem, and meropenem, in the study reported by [16]. The current work corresponds to [17], who reported that 77.5, 65, and 55% of isolates were resistant to meropenem, gentamycin, and imipenem, respectively. However, in the investigation conducted by [18], the rates of resistance to imipenem were 98%. Other researchers reported the most prevalent rate of resistance to gentamicin (88.5%) and showed that imipenem was the most effective antibiotic against P. aeruginosa [19]. P. aeruginosa's rapid evolution into a widespread multidrug-resistant organism is thought to be a serious issue that can be explained by a number of theories, one of which is the improper use of antibiotics, which makes these bacteria the subject of research [20].

3.3 PCR amplification for detection of 16S- rRNA

The traditional PCR was carried out satisfactorily. In this study, 505-bp bands were amplified as 16S, and 16S rRNA was used to confirm that the 25 isolates used in real-time PCR were indeed P. aeruginosa. This gene is regarded as one of the most important genes because it has hyper-constant sequencing. It also plays a fundamental role in molecular identification and classification and can provide species-specific signature sequences useful for identifying all types of bacteria [20]. The results are shown in Figure 3. Khalifa et al. (2019) reported that 7 out of 9 isolates were identified as Pseudomonas aeruginosa using PCR after performing different conventional examinations [21]. In addition, Al-Tememe and Abass (2022) reported that all ten examined isolates of P. aeruginosa confirmed the detection of the 16S-rRNA gene using PCR [22].

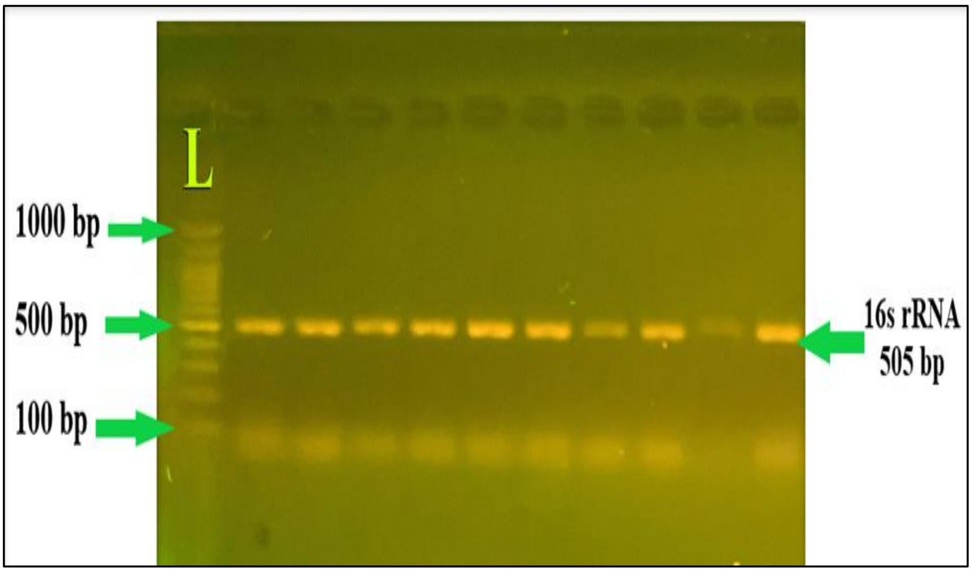

Figure 3: Gel electrophoresis for the *16S rRNA* gene (Agarose 2%, at 100 volts, 60 min.) visualized under UV light after staining with ethidium bromide.

3.4 Minimum inhibitory concentration

Imipenem and Meropenem, two isolates of bacteria resistant to carbapenem antibiotics, were the only ones for which the lowest inhibitory concentration was established. The findings indicated that the MICs of Meropenem against clinical isolates of MDR *P. aeruginosa* ranged from 32µg/100µl to 128 µg/100µl. The minimum inhibitory concentration of Meropenem was determined by subjecting some resistant and susceptible *P. aeruginosa* isolates to the antibiotic's pressure to ascertain the gene expression of *SIM* and *NDM* and its relationship to Meropenem resistance.

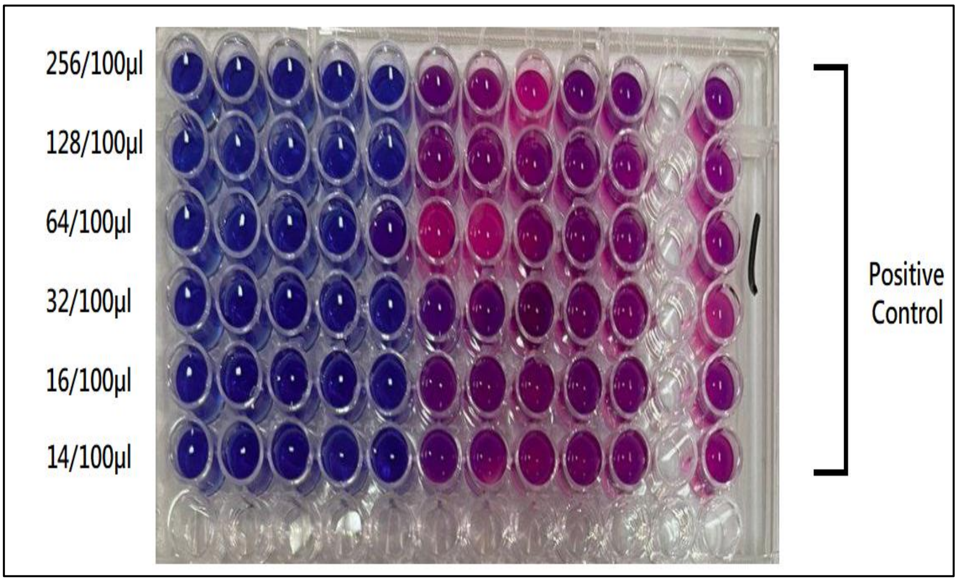

Figure 4: Microdilution plate indicating that the growth of *P. aeruginosa* was inhibited at 32µg/100µl ,64 µg/100µl and 128 µg/100µl.

It has been reported that the MIC of Meropenem in 6 (32%) isolates of *Pseudomonas aeruginosa* was 4 µg/µl, when used as monotherapy [23]. In addition, [24] reports that MIC values of Meropenem were 64 and 4 µg/µL for two MDR *P. aeruginosa* isolates. Among 22 isolates, 18 (81.8%) were found to be MBL producers by phenotypic method, and the MIC range of meropenem was 8 to >32 µg/µl [25]. The range of MIC for meropenem was between 0.5 and ≥ 64 µg/µL. Out of 430 isolates, 352 (81.9%) had a MIC value of ≤ 2 µg/µL (sensitive MIC value) [26].

3.5 Phenotype detection

The combined disc synergy test (CDST) was performed on 25 isolates because all other isolates were equally resistant to the antibiotics used. The results showed that these 25 isolates were resistant to imipenem but became sensitive when imipenem and EDTA were added, suggesting that all isolates of *P. aeruginosa* are producers of carbapenemase, as shown in Figure 5. This indicates that these bacteria produce genes that make them resistant to carbapenem.

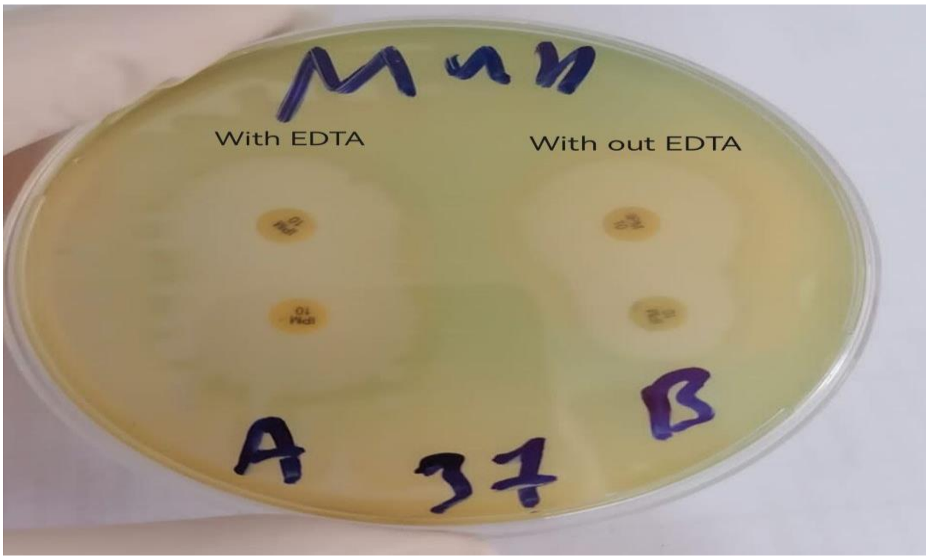

Figure 5: EDTA combined disc test

In the study conducted by [27], only 4 (5.33%) out of 75 *P. aeruginosa* isolates showed a positive result for the mentioned test. As the CDST is more sensitive for detecting MBL-generating isolates, 20 (13.3%) of the *P. aeruginosa* isolates tested positive for resistance to imipenem, of which 12 (8%) [28]. Regular clinical laboratory methods require the use of an MBL inhibitor (such as EDTA) to identify Pseudomonas isolates that produce MBL. While polymerase chain reaction (PCR) is considered the gold standard for MBL detection, it is generally only available at reference labs due to its high cost [29].

3.6 Expression of SIM and NDM genes

Real-time PCR was used to assess the gene expression of *SIM* and *NDM*. SyBR green was the dye used. Gene expression was measured as CT (Cycle Threshold), where higher CT values indicate lower gene expression and lower CT values indicate higher gene expression. In this study, the 25 isolates were confirmed by molecular detection using 16S, and all of the isolates were confirmed to be *P. aeruginosa*. RNA expression analysis was achieved using real-time methods. Reference or housekeeping genes have historically been used in PCR to regulate sample-to-sample variation [30]. Genotypic expression was performed using the RT-PCR technique to detect *SIM* and *NDM* genes among 25 *P. aeruginosa* isolates by extraction of RNA. The current results show an amplification curve, indicating that the *P. aeruginosa* 16s housekeeping gene was obtained in the 25 samples. The expression level of *SIM* and *NDM* genes among the 25 *P. aeruginosa* isolates was assessed using cDNA. The expression of target genes was normalized to the 16S gene as a housekeeping gene. Our results indicate that the expression level of the SIM gene was upregulated in carbapenem-resistant isolates compared to the control (1.00), which is 4.33. The expression level of the NDM gene was upregulated in carbapenem-resistant isolates compared to the control (1.00), which is 6.03. These results suggest that other undiscovered factors or pathways likely contribute to the upregulation of SIM and NDM in class B of the carbapenem group. These factors are important because resistance in P. aeruginosa is induced by chromosomal changes that modify the membrane permeability efflux pump. Parallel investigations from several countries have shown that oprD gene alterations, as well as the synthesis of the metallo-lactamases SIM and NDM, are the primary causes of carbapenem resistance in P. aeruginosa [31].

Table 2: Expression of average folding to genes (*NDM*, *SIM*) comparison of control

	Gene	**folding for**	***SIM* gene**		
Isolate	**Ct *SIM***	**Ct *rRNA16S***	**Δct**	**ΔΔct**	**Fold**
Carbapenem -resistant	23.37545	22.45818	0.917273	0.529509059	4.33
Carbapenem -sensitive	24.88	21.85	3.03	0.122427537	1.00
	Gene	**folding for**	***NDM* gene**		
	Ct *NDM*	**Ct *rRNA16S***	**Δct**	**ΔΔct**	**Fold**
Carbapenem -resistant	25.0055	22.477	2.5285	0.173318793	6.03
Carbapenem -sensitive	26.97	21.85	5.12	0.028755864	1.00

Conclusions

The class B *NDM* and *SIM* carbapenem genes appeared to be widely distributed among clinical isolates. *P. aeruginosa* isolates showed the strongest resistance to several antibiotics. The extensive distribution of genes in *P. aeruginosa* may pose a serious threat to efforts to find efficient antimicrobial therapies.

Ethical clearance

This research was ethically approved according to the approval with reference number CSEC/0123/006 issued by the Ethical Committee of the University of Baghdad, College of Science.

Conflict of interest

The authors declare that they have no conflicts of interest.

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