Iraqi Journal of Science, 2025, Vol. 66, No. 9, pp: 3681-3694
DOI: 10.24996/ijs.2025.66.9.15

ISSN: 0067-2904

Curcumin as Efflux Pump Inhibitor in Regulation of *mexZ* Expression and Enhancing Tobramycin Susceptibility in Resistant *Pseudomonas aeruginosa* Isolates

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Received: 11/7/2024 Accepted: 30/8/2024 Published: 30/9/2025

Abstract

This research aims to explore the potential of curcumin as an efflux pump inhibitor to potentiate the antibacterial activity of tobramycin against *Pseudomonas aeruginosa*. From a total of 175 samples, 93 isolates of *P. aeruginosa* were successfully isolated and identified through 16S rRNA confirmation. Disc-diffusion method was used to choose the most-resisted antibiotic. The minimum inhibitory concentration of antibiotic tobramycin alone and in combination with curcumin were evaluated by agar dilution method. The expression of *mexZ* was also analyzed using quantitative real time polymerase chain reaction in the presence of antibiotic and antibiotic with curcumin. The minimum inhibitory concentration of tobramycin alone ranged from 2048 to 1024 µg/ml, with the highest percentage (55%). However, when curcumin was added at 70µg/ml, there was a significant enhancement in the sensitivity ($p \leq 0.05$) of *P. aeruginosa* isolates to tobramycin. The highest percentage of isolates (22.5%) showed two-fold change in MIC followed by four-fold (12.5%), seven-fold (10%), three-fold (7.5%). Smaller changes ranging from 2.5-5% were also observed. when treated isolates with 1/2 MIC of tobramycin, resulting in downregulation in *mexZ* in all isolates as well as a clear significant increase ($p \leq 0.001$) in the expression of the *mexZ* in most isolates after using of curcumin with tobramycin. In conclusion, curcumin can decrease the minimum inhibitory concentration value of tobramycin in *P. aeruginosa* and can be used as a native compound to enhance the treatment of resistant isolates to tobramycin.

Keywords: *Pseudomonas aeruginosa*, efflux pumps, curcumin, *mexZ,* Tobramycin.

الكركمين كمثبط لمضخة التدفق في تنظيم تعبير *mexZ* وتعزيز حساسية التوبراميسين في العزلات المقاومة من *Pseudomonas aeruginosa*

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الخلاصة

يهدف هذا البحث إلى استكشاف إمكانات الكركمين كمثبط لمضخة التدفق لتعزيز النشاط المضاد للبكتيريا للتوبراميسين ضد *Pseudomonas aeruginosa*. من إجمالي 175 عينة، تم بنجاح عزل وتحديد

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93 عزلة من *P. aeruginosa* من خلال تأكيد الرنا الريباسي S16. تم استخدام طريقة انتشار القرص لاختيار المضاد الحيوي الأكثر مقاومة. تم تقييم الحد الأدنى من التركيز المثبط للمضاد الحيوي توبراميسين وحده وبالاشتراك مع الكركمين بواسطة طريقة تخفيف الآجار. تم أيضًا تحليل التعبير عن *mexZ* باستخدام تفاعل البلمرة المتسلسل الكمي في الوقت الحقيقي في وجود المضادات الحيوية والمضادات الحيوية مع الكركمين. تراوح الحد الأدنى للتركيز المثبط للتوبراميسين لوحده من 2048 إلى 1024 ميكروجرام/مل، وكانت النسبة الأعلى (55%). ومع ذلك، عند إضافة الكركمين بتركيز 70 ميكروغرام/مل، كان هناك تحسن كبير في حساسية ($p\geq 0.05$) لعزلات *P. aeruginosa* للتوبراميسين. أظهرت أعلى نسبة من العزلات (22.5%) تغيراً مضاعفاً في MIC يليه أربعة أضعاف (12.5%)، سبعة أضعاف (10%)، ثلاثة أضعاف (7.5%). كما لوحظت تغيرات أصغر تتراوح بين 2.5−5%.عند معاملة العزلات بـ 2/1 التركيز المثبط الادنى من التوبراميسين، أدى ذلك إلى انخفاض تنظيم جين *mexZ* في جميع العزلات بالإضافة إلى زيادة معنوية واضحة ($p \geq 0.001$) في التعبير عن *mexZ* في معظم العزلات بعد استخدام الكركمين مع التوبراميسين. في الختام، الكركمين يمكن أن يقلل من قيمة التركيز المثبط الادنى للتوبراميسين في *P. aeruginosa* ويمكن استخدامه كمركب أصلي لتعزيز علاج العزلات المقاومة لمضاد التوبراميسين.

1. Introduction

Pseudomonas aeruginosa is a versatile Gram-negative bacterium that inhabits diverse environments [1, 2]. This microorganism appears as an elongated rod with a slight curve or straight shape. It lacks the ability to ferment glucose and form spores, and requires oxygen for growth [3]. The antimicrobials now in use are not effective against a variety of *P. aeruginosa* strains because innate, adaptive, and acquired resistance mechanisms [4]. Bacterial cells have changed in composition to become less susceptible to medications, or the drug's target within the cell may have altered [5]. Chromosome resistance is caused by chromosomal mutations, which can be caused by a variety of physically as well as chemically stimuli [6]. Cellular defense against a variety of substances is provided by the efflux system when paired with the outer membrane barrier [7]. The development of efflux pumps allowed bacteria to communicate with their surroundings [8]. Efflux pumps can export a variety of chemicals or can only be specialized for one substrate [9]. efflux pumps play a significant role in the development of MDR. *P. aeruginosa* has four multi-drug efflux pumps: *mexAB-OprM*, *mexXY-OprM* (OprA), *mexCD-OprJ* and *mexEF-OprN*, that have a role in the ejection hazardous compounds then lessen sensitivity to drugs [10]. The *mexXY* efflux pump in *P. aeruginosa* is the only multidrug efflux pump operon not having the coding to an outer membrane element [11]. Their production primarily regulated through suppressors *mexZ* as well considered inducible by antimicrobials [12], also through anti-repressor armZ that is induced by Aminoglycosides [13]. There are numerous species of Gram-negative bacteria that have developed resistance to nearly all effective antibiotics, especially in the case of Gram-negative pathogens. Therefore, controlling multidrug-resistant gram-negative bacteria necessitates the development of novel antimicrobial agents [14]. Certain bacterial efflux pumps are selective for a single substrate, while others are not. The non-selective efflux pumps are responsible for the transport of a diverse array of compounds and antibiotic classes, providing a phenotype of multiple drug resistance [14]. Bacterial efflux pumps are resistance-nodulation cell division (RND) family [15]. Active efflux is recognized as a significant factor in the development of antibiotic resistance in bacteria to the majority of antibiotics [14]. In the case of *MexXY* its expression is inducible and enhanced by the presence of certain antibiotics [16]. Specifically, the *MexXY* proteins may be over- produced constitutively due to mutations occurring inside or outside the putative repressor gene named *mexZ* [17]. The expression of the *mexXY* operon is negatively regulated by the *MexZ* repressor, and this repression is alleviated in response to antibiotic-induced ribosome stress,

which increases the synthesis of the antirepressor *ArmZ* (*ArmZ* acts as an anti-repressor that interacts with the *MexZ* protein, a transcriptional repressor of the *mexXY* operon.), interacting with *MexZ* [13].

In regards to its relationship with clinical status, *MexXY* is one of the notable *P. aeruginosa* efflux pumps in P. aeruginosa. Hydrophilic aminoglycoside is most common substrates for *MexXY* operon such as paromomycin, streptomycin, amikacin, gentamicin, and tobramycin [18]. Overexpression of efflux pumps can increase the minimum inhibitory concentration (MICs) of various antibiotics. many compounds have been recognized as efflux pump inhibitors (EPIs) because their capability to inhibit various known efflux pump in multidrug *P. aeruginosa* [19].

Another steroidal alkaloid compound, conessine, has the potential to act as an EPI to preserve the antibiotic activity against P. aeruginosa by inhibiting its efflux pump systems [20]. A numeral of natural products has been confirmed their role as synergists or enhancer of other antibacterial agents. One of them is Curcumin (phenolic compound) derived from the rhizomes of the plant *Curcuma longa* has been shown to possess direct broad spectrum antibacterial activities against Gram-negative and Gram-positive bacteria [21]. The synergy observed between plant extracts and antibiotics is likely due to plant extracts containing an assortment of structurally diverse compounds that can act through various biochemical pathways within microorganisms [22]. The function of curcumin as efflux pump inhibition is observed by decreasing in MIC values for numerous antimicrobial agents versus *P. aeruginosa* multidrug resistant strains [23]. Little is known about the effect of curcumin on efflux pump gene expression; therefore, the purpose of this study is to investigate the role of curcumin as an efflux pump inhibitor that increases the activity of tobramycin against the resistant isolates of *P. aeruginosa* as well as their effect on the expression of *mexZ*.

2.Material and methods

2.1. Identification of bacterial isolates

Pseudomonas aeruginosa isolates were collected from clinical samples by laboratories in Iraqi hospitals in Baghdad between September 2023 and December 2023. Ninety-three of the isolates were cultured in sterile conditions on MacConkey agar (Himedia, India) as a differential medium and cetrimide agar (Himedia, India) as a selective medium. In addition to biochemical testing using like catalase and oxidase. Finally, polymerase a chain reaction (PCR) method was used to amplify the *16S rRNA* gene using particular primers in order to confirm identification.

2.2.DNA extraction

Using the Genomic DNA Mini Kit (Presto™ Mini gDNA Bacteria Kit, Taiwan), the DNA of probable MDR isolates was extracted in accordance with the manufacturer's instructions.

2.3.16S rRNA Amplification

A conventional Polymerase chain reaction (PCR) technique was implemented to amplify the pass gene of *16S rRNA* [24]. Amplification of targeted DNA was carried out in 25 µL reaction volumes, each containing 12.5 µL of 2X PCR Taq Mixture (GoTaq® 1-Step RT-qPCR System, USA), 1 µl of forward primer (GGGGGATCTTCGGACCTCA), 1 µl of reverse primer (TCCTTAGAGTGCCCACCCG), and 2 µL of Template DNA were included in the reaction. D.W. subsequently determined that the volume is 25 µl. In an Eppendorf master cycler (Germany). the thermal program was optimized and executed at the following temperatures: 2 minutes at 94 ºC, 25 cycles of 25 seconds at 94 ºC, 25 seconds at 56.4 ºC, and 40 seconds at 72 ºC, and a final elongation step of 5 minutes at 72 ºC in the subsequent

phases. After gel electrophoresis, *P. aeruginosa* isolates were identified by the presence of 956 bp bands.

2.4. Disk Diffusion method

Within the guidelines of the Clinical Laboratory Standard Institute (CLSI) [25, 26], the disk diffusion method was employed on Muller-Hinton Agar medium using Amikacin (30 µg), Tobramycin (30 µg), Netilmicin (30 µg), Streptomycin (300 µg). The plates were aerobically was incubated for 18–24 hours at 37°C.

2.5. Curcumin extraction

Curcumin was extracted from turmeric rhizomes using a modified method previously described [27]. The rhizomes were first dried in an oven at 105°C for 3 hours, followed by extraction with Soxhlet apparatus. The desiccated rhizomes were quashed in a mortar and screened through an 80-mesh size sieve to produce a uniform powder with a particle size of 0.18 mm. The ground turmeric powder (25g) was weighed, embedded in a thimble, and inserted into the Soxhlet. The plant materials were placed in the Soxhlet extractor and acetone was used as the solvent, over 8 hours at 56°C. Once the extraction was completed, a rotary evaporator operating under vacuum at 35°C was employed to separate the extract from the acetone and concentrated the extract (100%). After evaporation, a first stock of extracted curcumin (150000 µg/ml) was prepared and stored at 4°C until use. The residue (oleoresin) was accurately measured and desiccated for subsequent applications [20].

2.6. Determination of Minimal Inhibitory Concentration (MIC)

Tobramycin was serially two-fold diluted (4-2048 µg/ml) from stock solution (80mg2/ml vial), and mixed with Muller Hinton aga. The bacterial inoculum (McFarland standard used to adjust the suspension's turbidity to 0.5 is equivalent to 1.5×10^6 cell/ml using Densi CHEK Plus Meter) was prepared and used within 30 minutes of preparation. The plates were labeled for inoculation, and 5µl of inoculums were applied to the surface. The plates were incubated at 37°C ± 1 for 18-24 hours. A second stock of curcumin (1000 µg/ml) was prepared from 150000 µg/ml with Dimethyl sulfoxide (DMSO) to prepare the concentrations used in this method. This above procedure was repeated when using tobramycin (4-2048 µg/ml) with curcumin (10,15,20,25,30,50,60,70,80,90,100 µg/ml). The minimal inhibitory concentration (MIC) breakpoint for tobramycin or for tobramycin and curcumin were determined based on the clinical and laboratory standards institute criteria [26].

2.7. Amplification of mexZ

Using conventional PCR, the target gene, *mexZ* 237 bp, was amplified. The 20 µl of mixture was composed of 10 µl premix ((GoTaq® 1-Step RT-qPCR System, USA) 1 µl of each primer (F 5′-AGGTCTGCCTGGCGATGTGC-3′and R 5′-AGCGTTGCCCCTGCTTCTCG-3′) [28], and 2 µl of DNA template, the rest volume was completed with DNase free water. The program began with an initial denaturation step of 4 min at 95 ˚C, followed by 35 cycles of denaturation at 30 seconds, annealing at 67.4°C for 30 seconds, extension at 72 C° (30 sec) and final extension at 72 C° (5min), and gel electrophoresis using agarose gel (1.5%) with SafeRed™ Loading Dye to confirm the presence and size of bands in the gel under a UV transilluminator.

2.8 RNA extraction and expression of mexZ gene

The transcription levels of *mexZ* were estimated by one step qRT-PCR in five isolates treated with sub-MIC (1/2 MIC) of tobramycin alone as well as in combination with curcumin and tobramycin. The RNA of treated and not treated bacterial cells were extracted

according to the manufacturer's directions using GENEzol TriRNA pure kit (Geneaid, Thailand) [29]. Each reaction consisted of 10 µl of prexix (Luna universal one-step RT-qPCR Kit, Biolabs, New England), Luna RT enzyme mix (1 µl), RNA (3 µl) 0.8 µl from each related primers and the volume was completed with DNase free water to reach 20 µl. The PCR reactions were performed using ABI PRISM 7500 sequence detection system (Applied Biosystems, USA). The protocol began with a reverse transcription step conducted at 55°C for 10 min. This was followed by an initial denaturation phase at 95°C lasting 1 min. Subsequently, the process involved 40 repetitive cycles, each consisting of an extension step at 60°C for 30 se. The primers used for *mexZ* amplification were the same used for conventional PCR. The housekeeping gene (*fbp*) was employed as an endogenous internal control, forward 5-CCTACCTGTTGGTCTTCGACCCG-3 and reverse 5-GCTGATGTTGTCGTGGGTGAGG-3 [30].The quantification of *mexX* gene expression was carried out using the ΔΔCT calculation and the fold of gene expression was given as 2-ΔΔCT.

3. Statistical analysis

All features were appeared as frequencies and percentages, and Pearson-Chi-square test was detected to reveal significant differences in percentages. $P \leq 0.05$ was measured significant. Our data were analyzed using SPSS v. 21.0 statistical software. Additionally use prism for drawing figures.

4. Results and discussion

4.1. Pseudomonas aeruginosa isolation and identification

Results of present study showed that 93 (53.14%) out of 175 sample of collected from many sources were positive for *Pseudomonas aeruginosa*. Additionally, current outcomes showed most of *P. aeruginosa* isolated from burn (30.1%) and urine (30.1%), and little of it from sputum (14.0%) and ear swab (7.5%) with significant different ($p<0.001$).

The ninety-three isolates were identified as *P. aeruginosa* through biochemical testing, such as catalase and oxidase, and cetrimide agar as well as *16S rRNA* gene in order to confirm identification. *P. aeruginosa* colonies, which exhibit an irregular growth pattern, are greenish-blue in color on cetrimide agar medium [31]. Our results agree with Conventional PCR *16S rRNA* was depended to complete the identification of *P. aeruginosa* [12], [2, 20] who The *16S rRNA* data analysis showed that *P. aeruginosa* sequences were present in all habitats. [32] also cultured *P. aeruginosa* on cetrimide agar. Another study reported results similar to those of study, which indicating that *P. aeruginosa* isolated from Burn (30%), urine (5.46%), wound (10%) and ear swab (2.73%) [33]. A recent study by Mohammad and Flayyih1 [34] isolated *Pseudomonas* spp. from wound, burn, and eye swab from hospitals in Baghdad.

The highest isolation percentage in burn may be due to the moist and warm environment that favors to growth of *P. aeruginosa*, which thrives in these circumstances. Geographic, climatic, and hygienic factors may be responsible for the variation in *P. aeruginosa* isolation percentages across various regions. The elevated number of burn patients in our community may be attributed to the high prevalence of *P. aeruginosa*. This increase may be the result of various factors, including increased kitchen errors, terrorist attacks, and fires caused by electrical shortages in our city. Further, *P. aeruginosa* is a multidrug-resistant pathogen causing numerous chronic infections including urinary tract disorders. Infection caused by this organism is difficult to treat because of the presence of its innate resistance to many

antibiotics and its ability to acquire further resistance mechanism to multiple classes of antibiotics [35].

Conventional PCR for *16S rRNA* was depended to complete the identification of *P. aeruginosa* as shown in (Figure 1). A clear single DNA band (956bp) identifies bacterial isolates as *P. aeruginosa* [24].

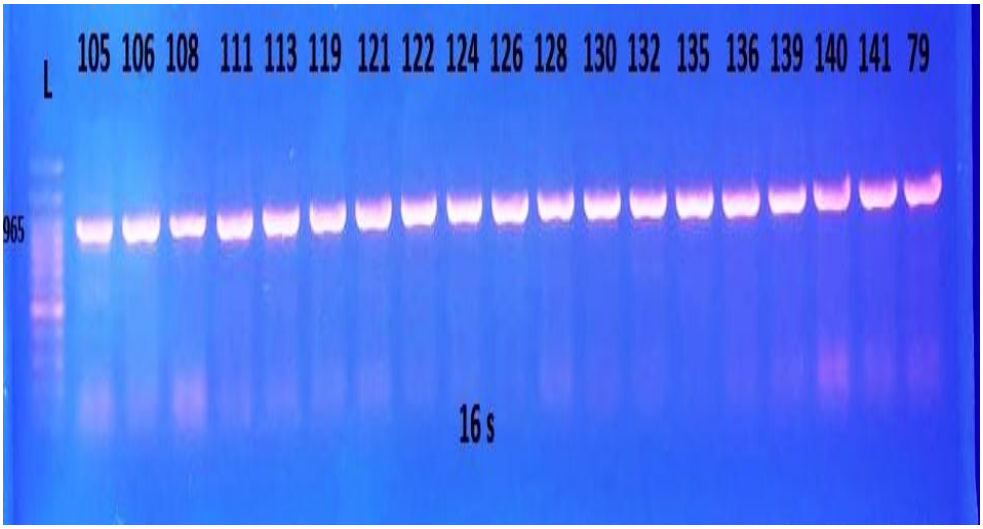

Figure 1: Agarose gel electrophoresis of PCR of the *16S RNA* (956 bp) of *P. aeruginosa* isolates. Agarose 1.5%, 100 V for 75 minutes and visualized on a UV transilluminator after being stained with a safe red dye. L: Leader 100-1200bp.

4.2. Disk Diffusion susceptibility

The current study demonstrated that the antibiotics play role in inhibiting growth of *P. aeruginosa* isolated from many sources, where it found the amikacin has highest inhibiting bacterial growth (53.8%), followed by tobramycin (50.5%), netilmicin (48.4%), and then streptomycin (46.2%). In another hand, present outcomes showed (50.5%) of isolates were resistance to netilmicin, (45.2%) to amikacin, and (43.0%) to tobramycin and streptomycin. The differences among percentages of antibiotic sensitivity for netilmicin, amikacin, tobramycin and streptomycin were significant ($p<0.001$) (Table 1).

Among of 68 *P. aeruginosa*, 47(69.11%) were susceptible to the tobramycin antibiotic [36] which detected the tobramycin MIC at which 50% of isolates are inhibited from 1,240 *P. aeruginosa* isolates. However, the results obtained by [37] showed 51% sensitivity to amikacin.

Table 1: Frequency and percentages of antibiotic sensitivity test of *P. aeruginosa* isolated from many sources.

Total number= 93		**N**	**%**	**P value**
Tobramycin	Sensitive	47	50.5%	P<0.001***
	Intermediate	6	6.5%	
	Resistance	40	43.0%	
Amikacin	Sensitive	50	53.8%	P<0.001***
	Intermediate	1	1.1%	
	Resistance	42	45.2%	
Netilmicin	Sensitive	45	48.4%	P<0.001***
	Intermediate	1	1.1%	
	Resistance	47	50.5%	
Streptomycin	Sensitive	43	46.2%	P<0.001***
	Intermediate	10	10.8%	
	Resistance	40	43.0%	

The present findings revealed that most of *P. aeruginosa* that resistance to tobramycin were obtained from burn (55.0%) and urine (20.0%), and little of it from sputum (10.0%) and ear swab (5.0%) with significant different ($p<0.001$) (Table 2).

Table 2: Distribution positivity of *P. aeruginosa* that resistance to tobramycin antibiotic according to sources of isolates.

Total number=40		Count	Percent	P value
Sources of isolates	Burn	22	55.0%	P<0.001***
	Wound	4	10.0%	
	Urine	8	20.0%	
	Sputum	4	10.0%	
	Ear swab	2	5.0%	

Mutations that modify the expression and/or function of chromosomally encoded mechanisms or the acquisition of resistance genes on mobile genetic elements, such as plasmids, are mechanisms by which *P. aeruginosa* can develop resistance to antibacterial agents. Plasmids are essential for the acquisition and dissemination of antibiotic resistance genes among bacteria [38]. Both approaches have the potential to significantly restrict the therapeutic options available for the treatment of severe infections by fostering drug resistance [39].

After conducting the MIC test on 40 tobramycin-resistant isolates using the agar dilution method recommended by the clinical and laboratory standards institute guidelines [40], the results showed the MIC values ranged between 32-2048 µg/ml (Table 3). The MIC values 2048 and 1024 µg/ml scored highest percentage (55%) were significant ($p<0.05$) compared to others concentrations (27.5% for 512 µg/ml, 10% for 256 and 128 µg/ml, 5% for 64 µg/ml and 2.5% for 32 µg/ml).

The findings of this investigation demonstrated that curcumin was capable of decreasing the MIC value of tobramycin in isolates of *P. aeruginosa* that were obtained from a diverse array of hospitals. Additionally, curcumin can be employed as a native compound to improve the effective treatment of tobramycin-resistant isolates. The MIC of tobramycin was determined after adding of different concentrations of curcumin (10,15,20,25,30,50,60,70,80,90,100 µg/ml)**.**

The MIC of tobramycin combination with curcumin was determined (Table 3). There was a significant enhancement in the sensitivity ($p\leq 0.05$) of *P. aeruginosa* isolates to tobramycin upon the use of 70µg/ml of curcumin. While other concentrations caused lower reduction in MIC or inhibit the bacteria without antibiotic. Other studies indicated that the optimal concentration was 50 µg/ml of curcumin when used with ciprofloxacin [21].

The highest percentage of isolates (22.5%) observed two-fold change in MIC followed by four-fold (12.5%), seven-fold (10%), three-fold (7.5%) while other folds ranged between 2.5-5% (Figure 2).

At the 70 µg/ml of curcumin, none of the isolates were susceptible to curcumin alone, indicating its inhibitor activity of efflux pump. Additionally, the concentration 1024 and 2048 scored highest percentage (97.5%), followed by 512 (65.0%), 256 (60.0%), then 128 (50.0%).

Table 3: Effect of many concentrations of tobramycin antibiotic and tobramycin antibiotic with curcumin extracts on *P. aeruginosa* that isolated from many sources.

Antibiotic concentration	Bacterial growth	tobramycin		P value	tobramycin with curcumin		P value
		N	%		N	%	
2048	No	22	55.0%	P>0.05	39	97.5%	P<0.001***
	Yes	18	45.0%		1	2.5%	
1024	No	22	55.0%	P>0.05	39	97.5%	P<0.001***
	Yes	18	45.0%		1	2.5%	
512	No	11	27.5%	P<0.001***	26	65.0%	P>0.05
	Yes	29	72.5%		14	35.0%	
256	No	4	10.0%	P<0.001***	24	60.0%	P>0.05
	Yes	36	90.0%		16	40.0%	
128	No	4	10.0%	P<0.001***	20	50.0%	1.00
	Yes	36	90.0%		20	50.0%	
64	No	2	5.0%	P<0.001***	13	32.5%	P<0.05*
	Yes	38	95.0%		27	67.5%	
32	No	1	2.5%	P<0.001***	9	22.5%	P<0.001***
	Yes	39	97.5%		31	77.5%	
16	No	0	0.0%	1.00	5	12.5%	P<0.001***
	Yes	40	100.0%		35	87.5%	
8	No	0	0.0%	1.00	4	10.0%	P<0.001***
	Yes	40	100.0%		36	90.0%	
4	No	0	0.0%	1.00	4	10.0%	P<0.001***
	Yes	40	100.0%		36	90.0%	
P value		P<0.01**			P<0.001***		

These results are consistent with other studies that observed significant differences between the effects of gentamicin and ciprofloxacin when combined with curcumin on *P. aeruginosa* isolates [20, 23]. The combination of antibiotic with an efflux pump inhibitor can be used as an effective strategy the resistance in bacterial isolates and restore the infectivity of antibiotic to increase bacterial cell death [41], Ballard E and Coote PJ. demonstrated that the MICs of antibiotics can be reduced by combining natural curcumin with them [42].

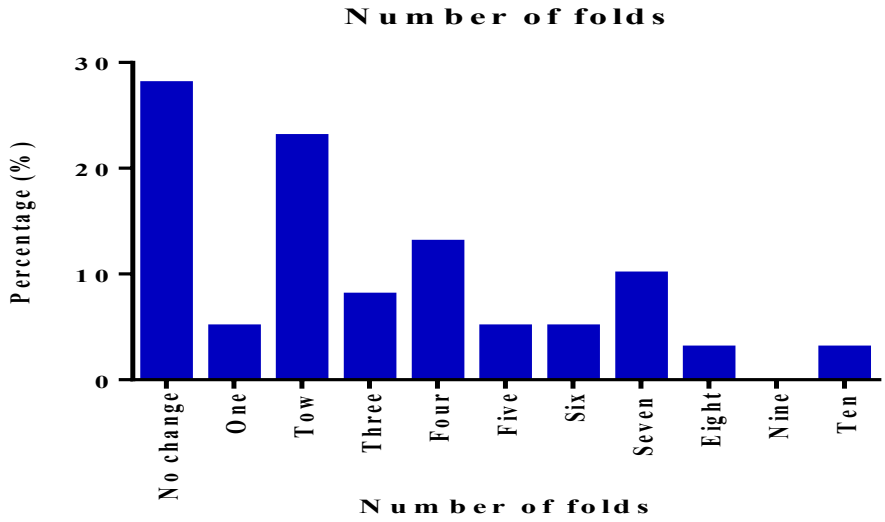

Figure 2: Fold change of tobramycin MIC after adding of 70 µg/ml Curcumin.

4.3. *mexZ Amplification and Expression*

PCR was performed on 40 tobramycin-resistant isolates to detect the *mexZ* (237 bp). The presence of gene (*mexZ*) has confirmed in all isolates (Figure 3). [38] found that 100 % positive result *mexZ* genes. *mexXY* under negative control by *mexZ* protein at ordinary state but when exposed to induced antibiotics the *MexXY* proteins may be overproduced constitutively as a result of mutations occurring inside or outside the repressor gene *mexZ*. Thus, allow *P. aeruginosa* to adapt rapidly to inhibitory concentrations of aminoglycosides, tetracyclines, and macrolides [17].

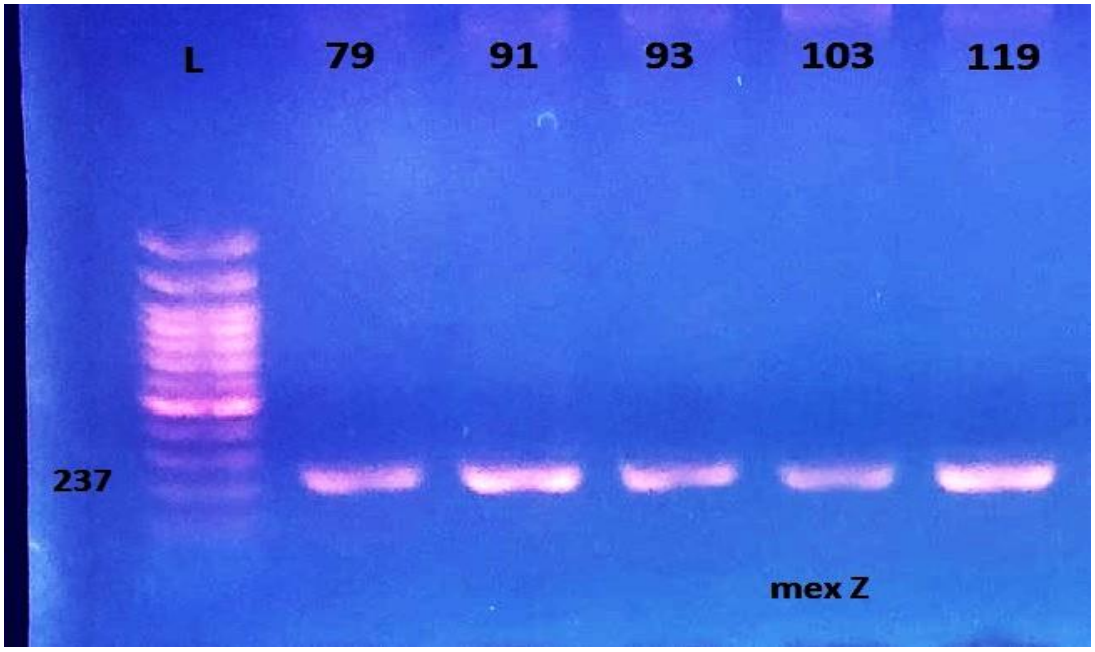

Figure 3: Agarose gel electrophoresis of PCR of the *mexZ* gene (237 bp) of *P. aeruginosa* isolates. Agarose 1.5%, 100 V for 75 minutes and stained with safe red dye and visualized on a UV transilluminator. L: Lader 100-1200bp.

The *MexXY-OprM* efflux pump is an important contributor to aminoglycoside tolerance in *P. aeruginosa* reference strains and expression of the *mexXY* genes is repressed by the *MexZ* repressor protein [43].

Expression of *mexZ* was assessed in five isolates of *P. aeruginosa* which had four-fold change in MIC after treatment with tobramycin and curcumin. The MIC of tobramycin for four isolates was 2048 µg/ml and 512 µg/ml for the rest isolate. when treated the bacterial cell with 1/2 MIC of tobramycin, resulting in downregulation in *mexZ* in all isolates about 0.49-0.001-fold. Although there was a decrease in *mexZ* expression after treatment of the same isolates with 1/2 MIC tobramycin and curcumin but this decrease was less than saw with treatment with tobramycin only.

This indicated that presence of curcumin may be restores the activity of the regulatory gene (*mexZ*) and this result was reinforced by decreased gene expression of *mexX* (data not shown) after add curcumin. When comparing the gene expression of a gene in the presence of an antibiotic and an antibiotic with curcumin, it was found that there was a clear significant increase ($p \leq 0.001$) in the expression of the *mexZ* in three isolates of about 52-206-fold with slight decrease in other isolates (0.05-0.6-fold) (Table 4).

Table 4: Effect of tobramycin antibiotic and tobramycin antibiotic with curcumin on gene expression of *mexZ.*

Isolate code	bacteria+antibiotic	bacteria and antibiotic +curccumin	P value
79	0.31208	0.01746	p<0.001***
91	0.01060	2.18859	p<0.001***
93	0.00102	0.06839	p<0.001***
103	0.01010	0.53219	p<0.001***
119	0.49655	0.00008	p<0.001***
control	1.00000	1.00000	1.00
P value	p<0.001***	p<0.001***	

One of the key mechanisms underlying tobramycin resistance is the upregulation of genes responsible for the MexXY-oprM efflux pump system, which is often triggered by mutations in the mexZ regulatory gene [18]. Little known about the expression of this system in the presence of curcumin, although curcumin has been shown to be an efflux pump inhibitor [38]. MDR isolates showed significant reduction in MIC after adding curcumin (50µg/ml) with selected antibiotics, while no change in MIC was observed when were used alone, indicating their efflux pump inhibitor [23].

The *mexZ* binds to intergenic region located between *mexZ* and *mexX*, Mutations in or modulation of *mexZ* activity by inducible antibiotic leads to overexpression of the *MexXY-OprM* system in *P. aeruginosa* [44].

PAβN is another efflux pump inhibitor when investigated for their role to reduce of ciprofloxacin MIC through *mexY* expression in clinical *P. aeruginosa* isolates found no statistically significant correlations between gene expression and the MIC reduction detected. For explain these results mentioned that PAβN not just close efflux pump system but also permeabilizes the outer membrane facilitating antibiotic entry [45].

Conclusion

In conclusion, curcumin can decrease the MIC value of tobramycin in efflux pump resistant isolates of *P. aeruginosa* and can be used as a native compound to enhance the treatment of resistant isolates with tobramycin. Curcumin has synergistic effect with tobramycin that leads to increase the expression of *mexZ* gene, this can help in restore the effectiveness of tobramycin against resistant isolates.

Acknowledgements

We thank to Mohammed Fahmi and Nada Sabah Oudah for their assistance.

Disclosure and Conflict of Interest:

The ethics committee of the College of Science, University of Baghdad, approved this work (Ref.CSEC/1123/0107). All participants agreed to provide the investigator with the

specimens. Informed consent according to the Declaration of Helsinki was obtained from all participants. The authors declare that they had no conflicts of interest.

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