ISSN: 0067-2904

Detection of *mex*B Multidrug Efflux Gene in Some Local Isolates of *Pseudomonas aeruginosa*

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Received: 11/4/2022 Accepted: 28/6/2022 Published: 30/1/2023

Abstract

According to the prevalence of multidrug resistance bacteria, especially *Pseudomonas aeruginosa*, in which the essential mechanism of drug resistance is the ability to possess an efflux pump by which extrusion of antimicrobial agents usually occurs, this study aims to detect the presence of *mex*B multidrug efflux gene in some local isolates of this bacteria that show resistance towards three antibiotics, out of five. Sensitivity test to antibiotics was performed on all isolates by using meropenem (10µg/disc), imipenem (10µg/disc), amikacin (30 µg/disc), ciprofloxacin (5µg/disc) and ceftazidime (30 µg/disc). Conventional PCR results showed the presence of *mex*B gene (244bp) in four isolates out of ten (40%). In addition,25, 50µg/ml of curcumin was used to detect its efficacy with the antibiotics that the bacteria showed resistance towards. Results showed the highest resistance for ciprofloxacin (80%), while all of them were sensitive to imipenem. In addition, the present results show that both concentrations of curcumin (25, 50µg/ml) were effective in increasing the zone of inhibition from zero to 10 mm for isolates towards amikacin. Same result was obtained towards ciprofloxacin, except for an increase of inhibition zone from zero to 7 mm to one isolate (38T) when treated with 50 µg/ml, and finally an increase in sensitivity to ceftazidime was found and inhibition zone was increased from 8 to 11 for the second isolate (42E), which revealed that curcumin potentiates antibiotics activity by inhibition of efflux pump mechanisms that can be related to the synergetic activity between antibiotics and curcumin.

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Keywords: *Pseudomonas aeruginosa*, *mex*B efflux gene, Antibiotic resistance, Inhibition of efflux pump, Curcumin.

التحري عن جين مضخة الادوية المتعددة ***mexB*** في بعض العزلات المحلية لبكتريا الزائفة الزنجارية

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الخلاصه

نظرا لانتشار مقاومة البكتريا للعديد من الادوية وخاصة بكتريا الزائفة الزنجارية والتي تمتلك الية اساسية لمقاومة الادوية وهي مضخة التدفق والتي عن طريقها يتم قذف المضادات الحيوية، لذا فان الهدف من هذه الدراسة هوالتحري عن وجود جين مضخة الادوية المتعددة *mex*Bفي بعض العزلات المحلية لبكتريا الزائفة الزنجارية والتي اظهرت مقاومة تجاه ثلاثة مضادات من اصل خمسة تم استخدامها في الدراسة الحالية. تم اجراء اختبار الحساسية للمضادات الحيوية وذلك باستخدام الميروبينيم والاميبينيم (10مايكروغرام/ قرص)، الاميكاسين والسيفتازيديم (30 مايكروغرام/ قرص) ومضاد السيبروفلوكساسين (5مايكروغرام/ قرص). أظهرت نتائج اختبار تفاعل البلمرة التسلسلي التقليدي وجود جين *mex*B(حجم 244bp) في اربع عزلات من اصل عشرة (40%) اضافة لاستخدام تراكيز (25 و50) مايكروغرام/ مل من الكركم للتحري عن فاعليته مع المضادات تجاه البكتريا التي اظهرت مقاومة تجاه تلك المضادات. أظهرت النتائج ان اعلى نسبة مقاومة من البكتريا كانت تجاه السيبروفلوكساسين (80%)، ولكن كانت جميع العزلات حساسة للاميبينيم. اضافة لذلك اظهرت النتائج ان تركيزي الكركم (25و50) مايكروغرام/مل كانت لها فعالية كبيرة حيث ادت الى زيادة قطر التثبيط من 0 الى 10 مليمتر للعزلات المقاومة تماما للاميكاسين وكذلك زيادة قطر التثبيط من صفر الى 7 مليمتر للعزلة (T 38) لمضاد السيبروفلوكساسين عند معاملته بتركيز 50 (مايكروغرام/مل)، واخيرا زيادة الحساسية تجاه السيفتازيديم للعزلة (E 42)حيث زاد قطر التثبيط من 8 الى 10 (مليمتر).وهذا يوضح بان الكركم له القابلية على تقوية فعالية المضادات وذلك عن طريق تثبيط الية مضخة التدفق.

Introduction

Pseudomonas aeruginosa is considered as an important pathogen because of the wide range of resistance mechanisms. *P. aeruginosa* is known to have the ability to thrive in a wide spectrum of environments, which is responsible to make main problems for doctors and nurses [1].

Efflux impact became an important strategy in the clinics due to its ability to confer a moderate resistance level that makes antibiotics to be inefficient in infected sites when less than optimal antibiotic concentrations are used [2], thus leading tocross-resistance towards unrelated classes of antibiotics [3]. A specific interest in the Mex efflux pumps of this bacteria occurred due to its wide substrate. 12 potential efflux systems of this family have been identified in this bacterial genome [4].4 of them (MexAB-OprM, MexCD-OprJ, MexEF-OprNand MexXY-O) are best characterized as antibiotic transporters [5]. Resistance nodulation division (RND) family is known to be responsible for the antibiotic expulsion through multidrug resistance (MDR) efflux systems, which is considered to be the key mechanism of *P. aeruginosa* antibiotic resistance [6].

Significant drug resistance is known to be contributed with MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY, MexJK and MexVW [7]. Resistance of *P. aeruginosa* to many antibiotics is largely attributed to the expression of the MexAB-OprM efflux pump [8] which was found to target many antibiotic classes [9, 10].

Many natural compounds have shown the ability to inhibit efflux pump. Curcumin, which is a phenolic compound, has been studied and has showed enhancement of the antibiotics effect and susceptibility alteration that can be attributed to efflux pumps inhibition [11, 12]. The present study aim is the detection of *mex*B gene in the local isolates and to study the effect of curcumin on bacteria that showed antibiotic resistance.

Materials and Methods

Bacterial Isolates

In the current study ten bacterial isolates of *P. aeruginosa*were obtained from the Department of Biology/ College of Science / University of Baghdad after their diagnosis, phenotypically and genotypically, which in turn were isolated from patients suffering from different infections such as wounds infections, urinary tract infections and pulmonary tract infections.

Disk Diffusion

A sensitivity test to antibiotics was performed by using Kirby–Bauer disk diffusion method according to the guidelines of CLSI (2018)[13]. The disks used are meropenem (10µg/disc), imipenem (10µg/disc), amikacin (30µg/ disc), ciprofloxacin (5µg/disc), ceftazidime (30µg/disc) and imipenem (10µg/disc). These disks were prepared by Mast Company (Turkey). Plates were aerobically incubated for 24 hrs. at 37°C.

DNA Extraction

The DNA of the isolates that gave high antibiotic resistance, were extracted according to the protocol of ABIOpure Extraction Genomic DNA Mini Kit (ABIOpure, USA). Extracted DNA was stored at-20ºC until its use.

Detection of *mex*B Gene

Amplification of the tested gene was performed by conventional PCR and the primer sequence was taken from Pourakbari *et al*.[14]. The final optimized PCR reaction consisted of 1µl of forward primer (GTGTTCGGCTCGCAGTACTC) and 1 µl of reverse primer(AACCGTCGGGATTGACCTTG) (10pmol /µl) (Macrogen, Korea),10 µl (2X) Green master mix, 2 µl DNA , 6 µl nuclease free water polymerase (Promega, USA), to give a final volume of 20 µl. Adjustment of the cycling program was: initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 30 sec for 61°C, as well as 30 sec for 72°C and 7 mins for the final extension at 72°C.

Curcumin Extraction

Two concentrations of aqueous extract of curcumin,50, 25 µg/ml (that were studied in other previous studies), were prepared by dissolving curcumin in boiled water and were then left for several minutes until cooled.

To investigate the capacity of curcumin in efflux inhibition, which is a certain mechanism that *P. aeruginosa* usually utilizes to resist a broad spectrum of antibiotics as a defense strategy [15], antibiotic discs that were not affected on the tested isolates were chosen to be exposed to the effect of curcumin after they were soaked for some seconds in the previously prepared concentrations of curcumin [16].

Results

Results of the current study appeared that the local isolates resistance was of about 70% for amikacin, 80% for ciprofloxacin, 40% for ceftazidime and 10% for meropenem, while all isolates were 100% sensitive for imipenem. 40% of the local isolates were resistant to three kinds of antibiotics out of five.

While using PCR overall isolates that had shown to have the highest resistance towards antibiotics, *mex*B efflux pumps gene was detected in all isolates under study that showed a band of 244 bp (Figure 1).

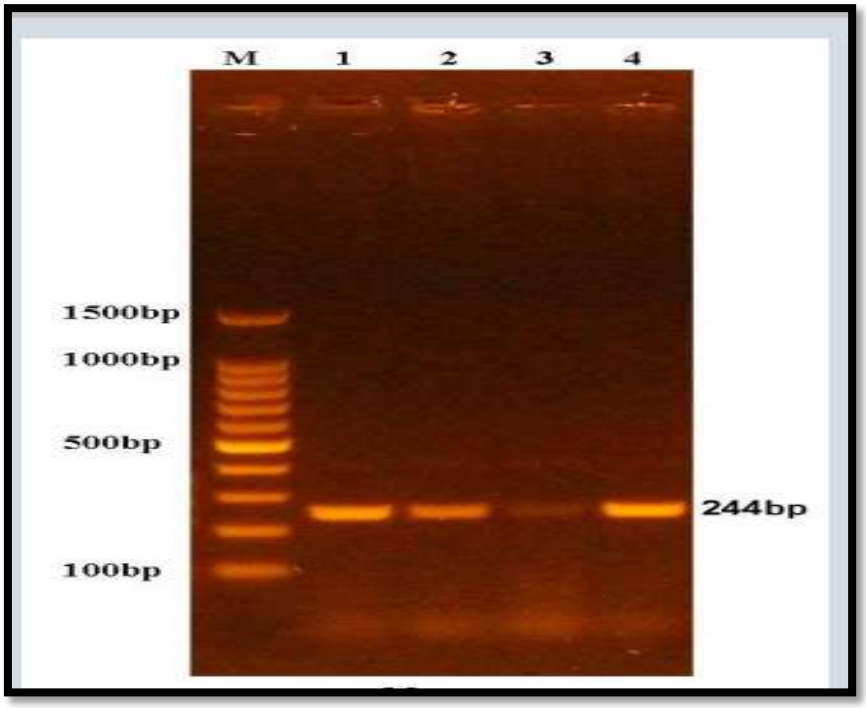

Figure 1: Amplification of *mex*B gene of *P. aeruginosa* samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100 bp ladder marker. Lanes 1-4 resemble 244bp PCR products.

Effect of Curcumin on Efflux Pump

Two isolates of the tested bacteria were chosen according to their full resistance to amikacin, meropenem, ciprofloxacin and ceftazidime, to estimate the effect of curcumin. Present results show that both concentrations of curcumin,25 and 50µg/ml, were effective in increasing the zone of inhibition from zero to 10 mm for both isolates towards amikacin antibiotic. Sensitivity towards meropenem did not increase. Same result was obtained towards ciprofloxacin, except an increase of inhibition zone from zero to 7 mm to one isolate after treatment with 50 µg/ml. Finally, an increase in sensitivity to ceftazidime was found and inhibition zone increased from 8 to 11 for the second isolate. Isolates, whose resistance dropped after the addition of curcumin, were thought to have a mechanism of efflux pump [12].

These results can be agreed with another study which revealed that up to 15 µg/ml, no curcumin with antibiotics impact can be gained.However,by increasing the concentration from 20-50 µg/ml, the strains sensitivity elevation was noted [11], in addition to another study which mentioned that favorable results were detected at 50µg/ml [12]. The results of the current study are shown in Figures2&3.

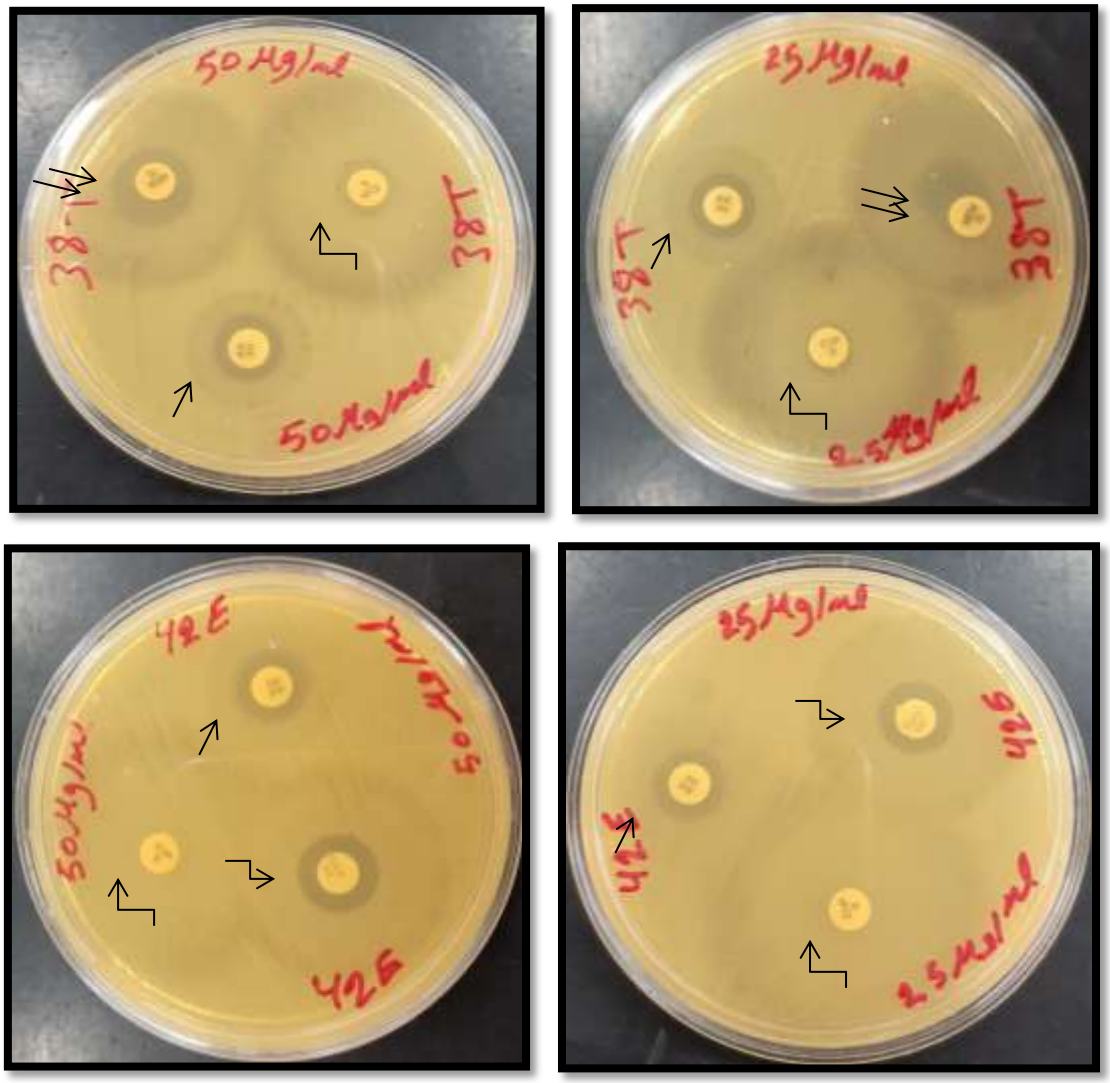

Figure 2: Effect of curcumin concentrations 25, 50 µg/ml on increasing of inhibition zone of some antibiotics. ( ) shows a sensitivity for amikacin, ( ) shows a sensitivity for ciprofloxacin, ( ) shows a sensitivity of 42 E for ceftazidime, ( ) shows a sensitivity of 38 T for meropenem.

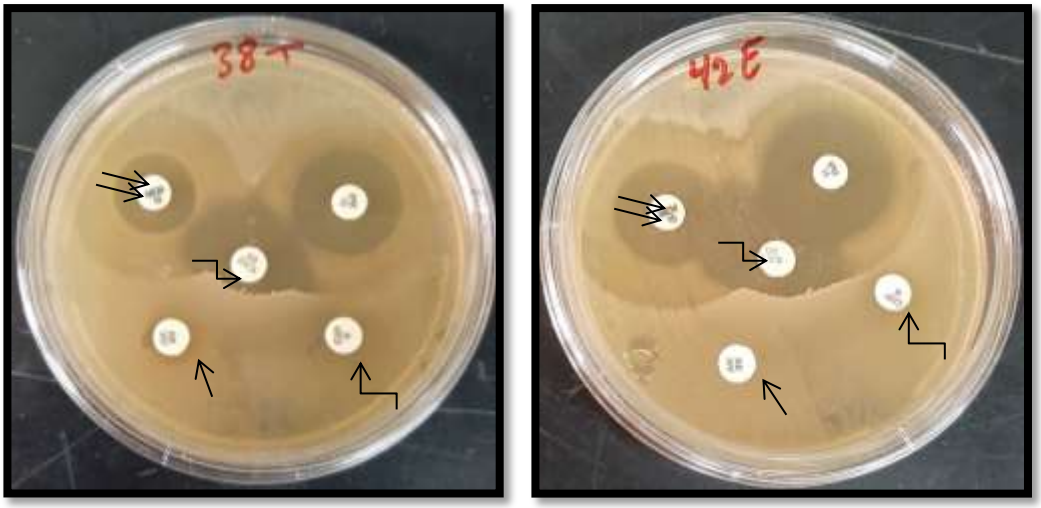

Figure 3: Bacterial antibiotic resistance without the addition of curcumin.

Discussion

The emergence of multidrug resistant *P. aeruginosa* during last decades has been observed worldwide [17].

The highest resistance rate was found in ciprofloxacin which disagrees with another local study that showed 40% and 12.5% of resistance to this antibiotic [17, 1]. This result can be close the result that was obtained by another local study carried by Al-Zaidi [18], besides its disagreement with Babak Pourakbari*et al.* 2016 [14]. On the other hand, resistance for amikacin showed a high percentage that reached 70% which, in turn is considered incompatible with another local study carried out by Sulaiman and Abdulhasan who obtained only 12% of local isolates of this particular bacteria for this antibiotic [17].It is also incompatible with other local study which showed that all local isolates were sensitive to amikacin and no resistance was shown [1]. The lowest resistance percentage in the current study appeared towards meropenem which showed only 10% which is close to the result obtained by Babak Pourakbari *et al.* 2016 [14] but differs from another study carried out by Negi *et al*., 2014 which showed 50% resistance [11].

Efflux systems expression in combination with low permeability of the outer membrane is responsible for *P. aeruginosa* multidrug resistance [1]. Substance transporting out of the cell explains the mean of efflux [18]. Extrusion of multi types of drugs, antimicrobials and signaling molecules, can be a result of efflux pumps [19]. The highest resistance of this bacteria to some antibiotics used in the current study, may also be referred to the abuse of these antibiotics by people.

Potentiation of the antimicrobial agent's activity can be achieved by using inhibitors of efflux pump (EPIs) due to the homology in a structure that efflux pumps possess with them [14]. The mechanism of EPIs action mechanism is by the action of these inhibitors that act as a substrate for efflux pumps competitively instead of the target antibiotics. As a result, cell death eventually occurs when an increase in the concentration of antibiotic intracellularly happens as an extrusion of these inhibitors is achieved outside the cells by the efflux pumps [11].

In other words, attainment of the inhibition of these mechanisms can be through expression interference of those changing the chemical structure of antibiotics or antibiotics efflux can be caused by blocking of outer pores. Disturbance of assembly of their components or energy interference that is necessary for the activity of the pump is considered another efflux pump inhibition mechanism [20, 21]. The importance of these inhibitors is attributed to successful therapy, decreasing the resistance level and increasing the drugs concentration intracellularly. Toxicity is the principal challenge in EPIs production [22].

An effective strategy for combating bacteria that are multidrug resistant, can be attained by a combination of antibiotics and which can be used as a remedy for these types of bacteria, which in turn can result in inhibition of the antibiotics that are extruded, and efficacy of the antibiotics can be restored to elevate bacterial cell death [23].Due to its properties, besides its safety at high doses which can be reached 12 g/day in humans [24], curcumin is considered as an effective new complementary drug for the treatment of various diseases. *In vitro*, it has been revealed that curcumin acts as an EPI against MDR and that the MIC reduction was caused by the inhibition of the efflux-pump of many antibiotics that are used against these isolates [25]. It was found that the antibacterial mechanism of curcumin was by the

polymerization inhibition of the protein FtsZ, which is considered to be important in cell division of prokaryotic cells by which cytokinesis can be prevented [26].
Current results can be considered to be compatible with other studies that illustrated that decreasing of efflux pump and potentiation of the effect of antimicrobial compounds was obtained by using curcumin [17, 11].

Conclusion

As a result of the current study, 40% of the local isolates showed resistance to three antibiotics out of five and 100% of these isolates contained *mex*B efflux pump gene in their genome. In addition, potentiation of the effect of antibiotics by using curcumin and by changing their susceptibility pattern in current results are because of the inhibition of efflux pump which can be related to the synergetic activity between antibiotics and curcumin that requires several genotypic studies.

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