Detection of the Antiseptic Resistance Gene among *Pseudomonas aeruginosa* Isolates

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Abstract

   Pseudomonas aeruginosa is an opportunistic pathogen that causes a number of infections in immunocompromised patients. This organism appears to improve resistance to many antimicrobial agents and a high percentage of clinical isolates of *P. aeruginosa* exhibit multidrug resistance (MDR) phenotype . The purpose of this study is to screen the antibiotic susceptibility patterns and the prevalence of *qacE delta1* gene among bacterial isolates. Accordingly, 145 samples were collected from different clinical sources from patients who admitted to different hospitals in Baghdad city in a period ranged 23/8/2018-1/1/2019. The isolates were diagnosed as *P. aeruginosa* based on routine bacteriological methods and confirmed by a molecular method using *16SrRNA* gene. The antibiotic susceptibility test was performed to all identified isolates by Kirby-Bauer Disk Diffusion method using ten types of antibiotics. The results of antibiotics susceptibility test revealed high levels of resistance toward Piperacillin (72.22%), Trimethoprim (68%), Ceftazidime (68%), Colistin (40.28%), and Levofloxacin (33.33%). And , the minimum inhibitory concentration (MIC) of Cetrimide was tested using different concentrations (2.048 to 0.004μg/100μl) and the results showed that MIC values ranged between 2.048 and 0.016) μg/100μL, and the concentration of 0.256 μg/100μl was more frequent . Finally, the prevalence of *qacE delta1* gene among bacterial isolates was detected in percentage 63.88% among bacterial isolates .

Keywords: *pseudomonas aeruginosa*, *qacEdelta 1* gene ,Antiseptic .

الكشف عن جين مقاومة المطهرات بين عزلات الزائفة الزنجارية

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الخلاصة

   تعتبر بكتريا *Pseudomonas aeruginosa* من الممرضات الانتهازية التي تسبب عدد من الاصابات للأشخاص ذو المناعة المنخفضة . يبدو أن هذا الكائن الحي يحسن المقاومة للعديد من العوامل المضادة للميكروبات ونسبة عالية من العزلات السريرية لل *P. aeruginosa* تظهر المقاومة المتعددة للأدوية (MDR). الغرض من هذه الدراسة هو فحص أنماط الحساسية للمضادات الحيوية وانتشار الجين *qacE delta1* بين العزلات البكتيرية. تم في هذه الدراسة جمع 145 عينة من مصادر سريرية مختلفة من مرضى دخلوا مستشفيات مختلفة في مدينة بغداد في الفترة من 23/8 / 2018−1 / 2019/1. تم تشخيص العزلات

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على أنها *P. aeruginosa* على أساس الطرق البكتريولوجية الروتينية وتم تأكيدها بالطريقة الجزيئية باستخدام

جين *16srRNA*. تم إجراء اختبار الحساسية للمضادات الحيوية على جميع العزلات التي تم تحديدها بطريقة

Kirby–Bauer Disk Diffusion باستخدام عشرة أنواع من المضادات الحيوية. كشفت نتائج اختبار

الحساسية للمضادات الحيوية عن مستوى عالٍ من المقاومة تجاه piperacillin   (72.22٪) ،

trimethoprim (68٪) Ceftazidime ، (68٪) colistin ، (40.28٪) ، levofloxacin (33.33٪).

تم اختبار MIC لـ cetrimide باستخدام تراكيز مختلفة (2.048 إلى 0.004 ميكروغرام / 100 ميكرو

لتر) وأظهرت النتائج أن قيم MIC تراوحت بين (2.048–0.016) ميكروغرام / 100 ميكرو لتر وكان

التخفيف بتركيز 0.256 ميكروغرام / 100 ميكرو لتر أكثر تكرارًا . وأخيرًا ، تم اكتشاف انتشار جين *qacE*

delta1 بين العزلات البكتيرية بنسبة 63.88٪.

Introduction

P. aeruginosa is an ubiquitous non-fermentative bacterium [1]. It is one of the most common causative agents of nosocomial infections, with a high mortality rate that could reach 60% [2,3]. In addition,it is responsible for several inflammations of burns, wounds, urinary tract infections, otitis media, eye infection, endocarditis,brain membrane inflammation (Meningitis),and respiratory infections such as pneumonia,especially in patients with cystic fibrosis [4].

P. aeruginosa infections is a growing global public health concern due to the ability of the bacteria to produce a variety of virulence factors as well as their resistance to the bulk of current antibiotic regimes used in hospitals [5]. In hospitals, biocides (disinfectants and antiseptics like Cetrimide) are used extensively and their effectiveness is very important in controlling microbial populations and preventing the transmission of infections [6,7]. Acquisition of biocide resistant genes can occur through mobile genetic elements (integrons, plasmids, transposons and prophages); bacteria can acquire these genes via horizontal gene transfer from the same or different bacterial species [8].

Biocide resistant genes, which confer resistance to a wide range of antibiotics and antiseptic agents, are located within a gene cassette in the variable region of integron (*intI*) [9].
These genes result in increasing the resistance ability of *P. aeruginosa* strains to antibiotics and biocides. This study aimed to detect the antibiotic susceptibility patterns and determine the prevalence of *qacE delta1* gene among *P. aeruginosa* bacterial isolates.

Sample collection

Totally, 145 samples were obtained from different clinical sources (burns, wounds, respiratory system infections, eye, ear infections, urinary tract infections, cerebral spinal fluid, sputum pus, joints and catheter). The samples were collected from patients who admitted to Baghdad hospitals (The Burn Centre, Baghdad Teaching Hospital, Medical City-Ghazi Hariri for Specialized Surgery, Educational Laboratory, Al-Sadder Hospital, Al-Karamu Hospital, Al-Yarmouk Educational Hospital, and the Burn Centre in Al-Yarmouk Hospital) during the period between 23/8/2018 and 1/1/2019. The clinical samples were identified by routine biochemical tests such as Gram stain, oxidation and fermentation test (O/F), oxidase test, inability to fermentate lactose, and growth on Cetrimide agar medium (Himedia, India).

Antibiotic susceptibility test

All identified isolates (n=72) were subjected to antibiotic susceptibility test that was carried out by Kirby-Bauer diffusion method using different types of antibiotics, including Imipenem (10 μg), Meropenem (10 μg), Trimethoprim (5μg), Aztreonam (30 μg), Tobramycin (10 μg), Gentamycin (10 μg), Levofloxacin (5μg), Ceftazidime (30 μg), Piperacillin (100 μg) and Colistin (10 μg) (Himedia, India). The results of susceptibility were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute [10].

Minimum inhibitory concentration of cetrimide

The MIC test was performed according to a previously published method [10,11].

Cetrimide was dissolved in sterile distilled water to prepare a stock solution of 8,192 μg/ mL, and then it was filtered via filter paper (size 0.22 μm). Later, it was diluted in Mueller Hinton Broth (MHB) medium (Himedia, India) to obtain two fold serial dilutions that ranged from 2.048 to 0.004μg/100μl. Bacterial inoculum was prepared according to the colony suspension method using MHB, and any resulting turbid solutions were visually compared with the McFarland standard for turbidity *vs.* cell concentration. The result was verified by measuring the absorbance of the suspension

spectrophotometrically on 600 nm. The reading between 0.08 and 0.10 was accepted as equal as the turbidity adjusted to $1*10^8$ cfu/ml and equal to 0.5 McFarland.

Then, 100 μl was taken from stock (bacterial growth) and added into 9.900μl normal saline to obtain an inoculum with a concentration of $1.5*10^6$ cfu/ml. A 96 well microtiter plate was filled with 100μl of MHB, then serial concentrations of Cetrimide (100μL) were added to each column, with the corresponding concentration of the target disinfectant. For each test plate, two biocide-free controls were used, the first one was sterility control (200 μl medium only) and the second was growth control (100 μl of medium- inoculum suspension). After overnight incubation, resazurin sodium stain was added to each well in plates and incubated for 2-4 hours. The reduction of resazurin stain (purple-blue*)* to resorufin (pink-colorless) was observed in each well containing the active living cells, while the stain remained as purple-blue in wells that contained dead cells.

Molecular study
DNA extraction

Chromosomal DNA was extracted according to the manufacturer's procedure of Reagent Genomic DNA Kit, Geneaid (Taiwan).

Amplification of *P. aeruginosa* 16s *rRNA* and *qacE delta1* genes

PCR method was performed to amplify *16srRNA* and *qacE delta1* genes, and specific primers were used (Table-1). These primers were utilized in a 25 μl reaction solution containing 12.5 μl of Go Taq®Green Master Mix (Promega, USA), 1 μl of each primer (10 pmol), 7.5 μl of DNase RNase free water, and 3 μl of the purified DNA template. The PCR cycle conditions were: Initial denaturation at 95°C for 5 min, thirty cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 1min, extension at 72°C for 30 sec, and final extension at 72°C for 5 min. The PCR products were separated by gel electrophoresis using 1.5% agarose gel (Thermo Fisher Scientific, USA) and visualized by gel documentation system using ethidium bromide. A 1500 bp DNA Ladder (Bioland -USA) was used to detect the correct amplicon size.

Table 1- Primers sequences of *16srRNA* and *qacE delta1* genes

Name	Sequence	Product size
16SrRNA	**F5´CGGACCTCACGCTATCAGAT3´**	**249 bp**
	R5´CTGCCCTTCCTCCCAACTTA3´	
qacE delta1	**F-5´TAGCGAGGGCTTTACTAA GC3´**	**207bp**
	R5´CCCATACCTACAAAGCCCCA3´	

Result and discussion

72 clinical isolates, out of 145 clinical samples, were identified as *P. aeruginosa* by routine bacteriological methods. In addition, the isolates were subjected to molecular detection by *16srRNA* gene to confirm bacterial genus and species as shown in (Figure-3).

P. aeruginosa can cause various clinical infections and it is considered one of the most important problems confronting the world, especially the developing countries. In addition, it is considered as an important cause of nosocomial infections due to its ability to persist in unfavourable environmental conditions by developing biofilms and tolerating the antibiotics, antiseptics, and disinfectants [12].

Susceptibility of *P. aeruginosa* for different antibiotics

Figure-1 demonstrates an obvious resistance among ***P. aeruginosa*** isolates to Piperacillin (72.22%), Trimethoprim (68%), and Ceftazidime (68%). Moreover, all the isolates showed resistance to Aztronem in a percentage of 47.22%. Also, the resistance percentages to Tobramycin and Gentamycin were 45.83% and 51.39%, respectively. On the other hand, the isolates showed a moderate–high resistance to Meropenem and Imipenem (44.44% and 40.28%, respectively). For the remaining antibiotics (Colistin and Levofloxacin), the resistance values were 40.28% and 33.33%, respectively.

Our study is consistent with a previous report [13] which demonstrated that *P. aeruginosa* isolates were highly resistant to Aztreonam (86.7%), but inconsistent with the same study in relation to Piperacillin resistance rate (93.3%). Another study [14] reported that the isolates of *P. aeruginosa* collected from burn patients who referred to Mottahari hospital in Tehran province, Iran, were resistant to the antibiotics of Aztreonam (80.2%) and Ceftazidime (74.8%). Another study recorded a

different rate of Piperacillin resistance (75%) [4]. Also, in agreement with our results, resistance rates to Piperacillin and ceftazidum were recorded to be 69.9% and 68.8%, respectively [15].   According to the present study, resistance to Imipenem was high, in comparison with previous studies which reported values of 20% [16] and 38.6% [17]. In addition, an earlier work [18] recorded a high rate of resistance to Meropenem (30.6%) among *P. aeruginosa* isolates, which is comparable with recent reports that recorded values of 38.3% [17] and 36.4% [19]. The reason for the high resistance to Meropenem may be related to the excessive use of this drug [18].

The isolates showed highest resistance values to aminoglycosides (Gentamycin and Tobramycin), whereas an earlier study showed a significantly lower resistance to Gentamycin [20].

This study showed a high percentage of Colistin resistance among the tested isolates, while many other studies recorded a high sensitivity that ranged 96%-100% [21, 22]; however, increasing the administration of Colistin to treat the infections caused by the MDR organisms may have resulted in the emergence of Colistin-resistant strains [5].
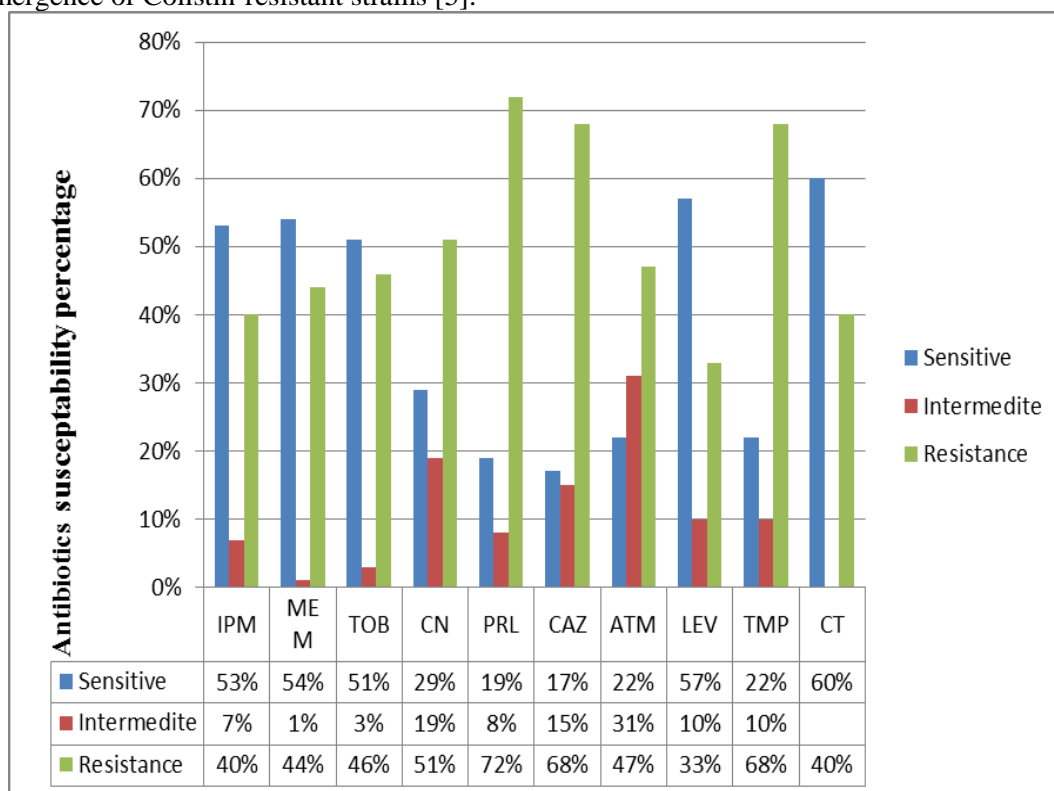

	IPM	MEM	TOB	CN	PRL	CAZ	ATM	LEV	TMP	CT
Sensitive	53%	54%	51%	29%	19%	17%	22%	57%	22%	60%
Intermedite	7%	1%	3%	19%	8%	15%	31%	10%	10%	
Resistance	40%	44%	46%	51%	72%	68%	47%	33%	68%	40%

Figure 1-The percentage of antibiotic susceptibility for *P. aeruginosa* isolates. Aztreonam(ATM), Ceftazidime(CAZ),   Colistin(CT),   Gentamycin(CN),   Imipenem(IPM),   Levofloxacin(LEV), Meropenem (MEM), Piperacillin(PRL), Tobramycin (TOB), Trimethoprim(TMP).

Minimum inhibitory concentration of Cetrimide
The results of MIC of Cetrimide against *P. aeruginosa* isolates showed that the MIC values ranged between 2.048 and 0.016 μg/100 μl, and the concentration of 0.256 μg/100 μl was more frequent (Figure- 2).
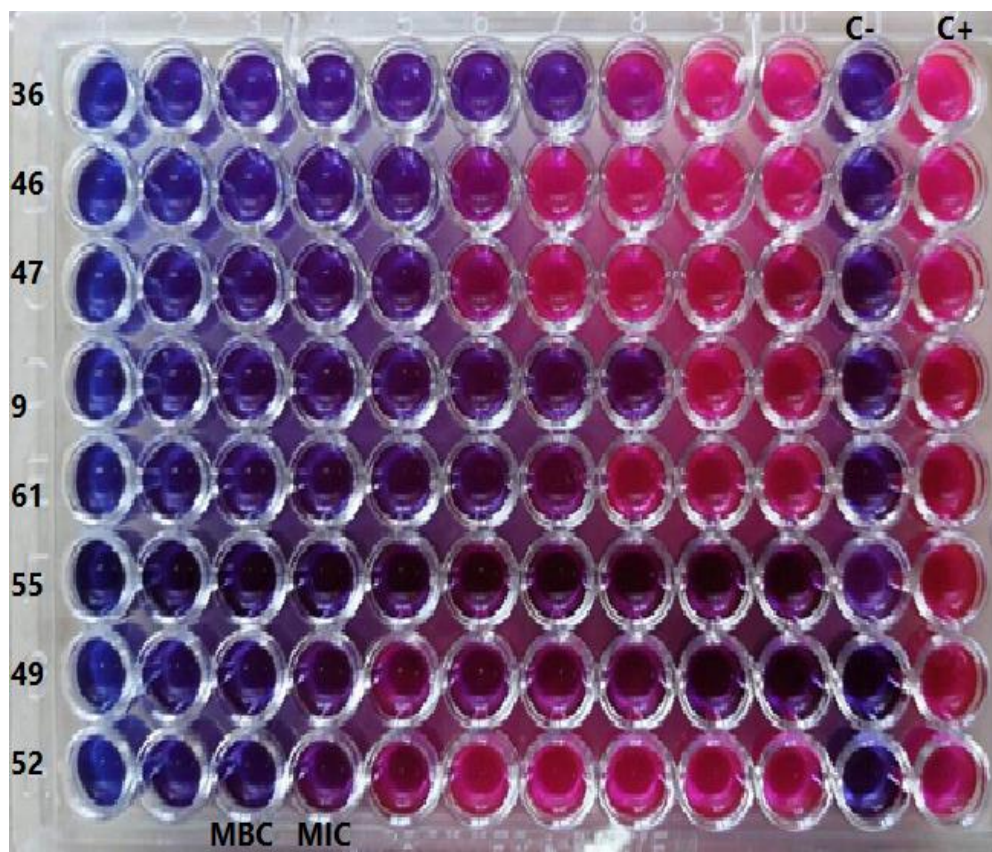
Figure 2-Determination of the minimum inhibitory concentration of cetrimide among *P. aeruginosa* isolates by using Resazurin sodium stain (60 µl per well), with incubation for 2-4 h for the observation of colour change. C-: Column 11 refers to the negative control (blue/purple), C+: Column12 refers to the positive control, MBC: minimum bactericidal concentration.

Molecular detection of *16SrRNA* and biocide resistance gene *in P. aeruginosa* isolates

　　All the 100 DNA samples of *Pseudomonas spp.* were subjected to molecular identification by PCR amplification technique. The results revealed that 72 isolates (100%) gave positive and specific band size of 249bp. To confirm our results, the products were sent for sequencing and the results were aligned with the NCBI blast tool for *P. aeruginosa*, showing similarity and identity of 100%.
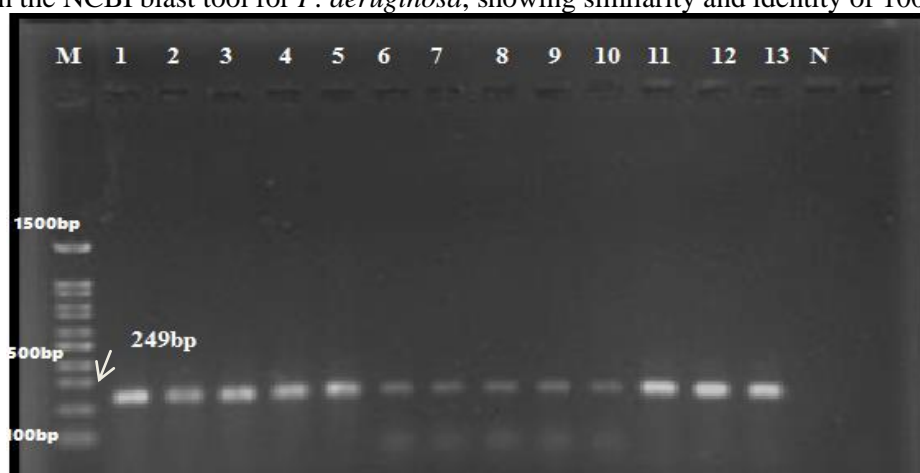

Figure 3-PCR product visualised by 1.5% agarose gel electrophoresis (1hr:30mins) showing the amplification of a 249bp fragment of *16S rRNA* gene of *P. aeruginosa* isolates. M: 1500bp represents ladder, lane 1 represents positive control, lanes 2,-13 represent positive isolates, and lane 14 represents negative control using water instead of gDNA.

The results of molecular detection of *qacE delta 1* , which is responsible for biocides resistance, showed that from a total of 72 extracted DNA samples of *P. aeruginosa* ,only 46 isolates (63.88%). were provided a specific band with a molecular size of 207bp, following electrophoresis on 2% agarose gel (Figure-4). A proportion of A study on the Egyptian population [23] indicated that 57.8% of *P. aeruginosa* isolates have this gene. In contrast to our findings, another study reported that 30.5% of the isolates possessed this gene [6], while other studies [24, 25] detected *qacEDelta 1* gene in *P. aeruginosa* isolates with a high incidence rate of 100%.
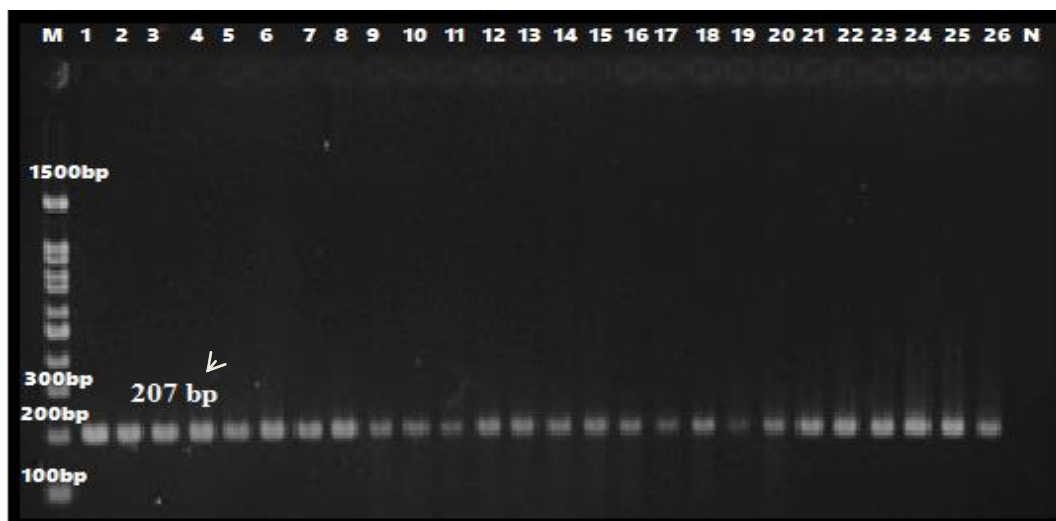

Figure 4-PCR product on 2% agarose gel electrophoresis (1hr:30mins), representing an amplification profile of *qacEdelta1* gene of *P. aeruginosa* isolates with a specific band size of 207 bp. M:1500bp represents ladder; lanes 2-26 represent positive isolates, lane 1 represents positive control, and lane 27 represents negative control which contains water instead of gDNA.

According to our results, 63.88% of the biocides resistance isolates possessed this gene. As mentioned above, this gene confers the ability to resist both disinfection and sterilization compounds, especially quaternary ammonium compounds (QACs). Out of 26 isolates, 8 isolates did not have this gene but showed resistance to Cetrimide. This might be attributed to membrane impermeability that is resulted from the repeated exposure to chemical disinfectants or excessive use of antibiotics. Moreover, it might be resulted from mutations that lead to a change in the cell wall of bacteria, or proteins known as porins which cause the channels on the wall to narrow, reducing the entry of antimicrobial agents, including antiseptics. In addition, the acquisition of resistance genes from plasmid or transposons leads to failure of disinfection in hospitals and health centers, which renders them sites for spreading the infection [26].

Conclusions

In conclusion, our findings indicate that *P. aeruginosa* isolates showed a high resistance to antibiotics and biocides. Hence, the optimization of antimicrobials use and the control of infection are recommended measures to prevent the increase in the population of drug resistant organisms in the hospitals of Baghdad city.

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