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JOURNAL OF UNIVERSITY OF BABYLON

For Pure and Applied Sciences (JUBPAS)

Vol.31; No.2.| 2023



ISSN: 2312-8135 | Print ISSN: 1992-0652 | www.journalofbabylon.com | jub@itnet.uobabylon.edu.iq | info@journalofbabylon.com

# Genetic Detection of *IMP-1* Gene and its Relationship with Biofilm Formation in *Klebsiella pneumonia*

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# الكشف الجيني لجين *IMP-1* وعلاقته بتكوين الغشاء الحيوي في *Klebsiella pneumoniae*

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| Received: | 23 /3/2023 | Accepted: | 10/5/ 2023 | Published: | 30 /6/2023 |
|---|---|---|---|---|---|

## ABSTRACT

**Background:** *Klebsiella pneumoniae* were considered as normal flora of skin, and intestine. It can cause damage to human lungs; the danger of this bacterium is related to exposure to the hospital surroundings.
**materials and methods:** the detection of *Klebsiella pneumoniae* on morphological and biochemical tests and then assured with VITEK 2 system. Resistance to antibiotics was determined by Kirby-Baeur method. And genotyping of *IMP-1* in isolates was done by PCR technique, then biofilm formation was identified by Micro titer plate method.
**Results:** The present study included a collecting of 50 specimens from different clinical specimens, (blood 40%, urine 30%, sputum 20%, wound infection 10%); 10 isolates were identified as *Klebsiella pneumoniae* .
All isolates, under study, developed high resistance toward Cefitrixon, Ampicillin, Amoxicillin, Ticarcillin, Ticarcillin+Clavulanic acid, and Ceftazidim estimated by disc diffusion method. All isolates characterized by harboring the highest resistant in a percentage reached 100% against antibiotics, under study. This study determined the Minimal Inhibitory Concentration were detected by eight E-test strips for isolates. As well as the isolates were strong biofilm production for three isolates, while three were moderate of biofilm formation and other isolates were weak former; at the value of *($P \leq 0.05$)* was considered as a significant. Genotype detection of Metalo-beta lactamase (*IMP-1)* by PCR technique in *Klebsiella pneumoniae* . Upon using PCR technique exposed only three isolates;30% of isolates (two from urine, one from blood) samples harbored *IMP-1* gene. The study was also found relationship between *IMP-1* and biofilm formation in isolates which were harboring these genes, when ($P \leq 0.05$).
**Conclusions:** *K. pneumoniae* were isolated from different sources. All isolates were resistant to most antibiotics used in this study. The isolates have Metallo-beta lactamse. PCR was showed *K. pneumoniae* have *IMP-1 gene*,.This study also found there was relationship between biofilm formation and *IMP-1* gene in *K. pneumoniae* ($P \leq 0.05$).

**Key words:** *Klebsiella pneumoniae*, *IMP-1*, genotype, Metalo-beta lactamases, biofilm.

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JOURNAL OF UNIVERSITY OF BABYLON
For Pure and Applied Sciences (JUBPAS)
Vol.31; No.2.| 2023

## الخلاصة:

**المقدمة:** *Klebsiella pneumoniae*, تعتبر بكتريا طبيعية موجودة على الجلد وفي الامعاء .لكن تسبب تلف الرئة و خطر هذه البكتريا هو التعرض لها اثناء التواجد في المستشفى.

**المواد وطرق العمل:** تم تشخيص البكتريا بواسطة الطرق المظهرية و البايو كيميائية وتم التاكيد على التشخيص بنظام الفايتك .مقاومة البكتريا للمضادات الحياتية تم باستخدام طريقة كيربي–باير.وبالطريقة الجينية تم الكشف عن وجود جينات الميتالوبيتالاكتاميز باستخدام تفاعل البلمرة و بعدها تحديد تكوين البايوفيلم بالعزلات بطريقة الاطباق الدقيقة .

**النتائج والمناقشة:** تضمنت الدراسة الحالية جمع 50 عينة من عينات سريرية مختلفة (40٪ من الدم ، 30٪ من البول ، 20٪ من البلغم، 10٪ من الجروح). 10 *Klebsiella pneumoniae*

واظهرت جميع العزلات قيد الدراسة مقاومة عالية تجاه السيفيتريكسون ، الأمبيسلين ، الأموكسيسيلين ، التيكارسيلين ، التيكارسيلين + حامض كلافولانيك ،و السيفتازيديم باختبار الانتشار بالاقراص. تميزت جميع العزلات باحتوائها على أعلى مقاومة بنسبة 100٪ ضد المضادات الحيوية قيد الدراسة. حددت هذه الدراسة قيمة التركيزالمثبط الأدنى لثمانية شرائط باستخدام اختبار E للعزلات. وكذلك كانت العزلات ذات إنتاج بيوفيلم قوي لثلاثة عزلات ، بينما كانت ثلاثة عزلات متوسطة تكوين الأغشية الحيوية والعزلات الأخرى كانت ضعيفة الانتاج, عندما كانت قيمة ($P \leq 0.05$) تعتبروجود فروق معنوية. والكشف عن النمط الجيني لجينات Metalo–beta βlactamase ، (*IMP–1*), بواسطة تقني PCR تفاعل البلمرة المتسلسل في *Klebsiella pneumoniae* ، تم الكشف عن ثلاث عزلات فقط ؛ 30٪ من العزلات (اثنتان من البول وواحدة من الدم) تحتوي على الجين (*IMP–1*) .كما وجدت الدراسة علاقة بين جين .وتكوين الأغشية الحيوية في العزلات التي كانت تحتوي الجين عندما كانت ($P \leq 0.05$).

**الاستنتاجات:** تم عزل البكتريا من مصادر مختلفة. كانت جميع العزلات مقاومة للمضادات الحياتية قيد الدراسة . العزلات كانت حاملة لجينات المقاومة IMP–1, كما وجدت الدراسة لن هناك علاقة بين وجود جين IMP– 1. وتكوين البايوفيلم في هذه البكتريا .

**الكلمات المفتاحية:**

**التشخيص الجيني , الميتالو بيتالاكتاميز ,البايوفيلم *IMP-1*) ,** *Klebsiella pneumoniae,* **)**



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## INTRODUCTION

*Klebsiella pneumoniae* were considered as normal flora of skin, and intestine. It can cause damage to human lungs; resulting in hematic spit, the danger of this bacterium is related to exposure to the hospital surroundings **[1 and 2].** Klebsiella species are resistant to numerous antibiotics used in treatment **[3].** Klebsiella infections are seen mostly in illness affects older people with other diseases. *K. pneumoniae* which are carried the carbapenemase genes may be giving also resistance to aminoglycosides, beta lactams, but also to other groups of antibiotics **[4].** On the other hand, Klebsiella can also cause urinary tract and wound infections, alsocan produce extended-spectrum beta-lactamases (ESBLs), which are resistant to all beta-lactam antibiotics, except carbapenems. It possesses resistance also to aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol are created by bacteria that have resistance to penicillins, cephalosporins, and carbapenems **[5 and 6]. .**The metalo-beta lactamase *K. pneumoniae* was one of the most important infection in patients with covid-19 in Portugal and other countries in the period of pandemic **[7 ].**All (metallo-β-lactamases B) are antibacterial agents with broad spectrum that are used the last treatment for multi-drug resistant *K. pneumoniae* infections **[8].** (*IMP-1*) is one member of a large gene family that encodes beta lactamase enzymes called carbapenemases. Spread of *Metallobeta lactamase* in different region of world is very dangerous to human beings. The prevalence of its must be controlled by using newly antibiotics **[9].**

## MATERIALS AND METHODS:

### Isolation of bacteria:

During the period from September to December 2019, fifty specimens from different clinical specimens (blood, urine, wound infection, sputum) from hospitalized patients in Baghdad, ten isolates were identified as *Klebsiella pneumoniae*. Then they were cultured onto MaCconkey agar, blood agar, and incubated at 37°C for 24hrs. **[10].**

### Identification of bacteria:

*Klebsiella pneumoniae* were identified depending on the morphological and microscope features. The plates of MaCconkey agar were streaked with a pure colony of tested bacteria and then incubated at 37°C for 24 hrs. Then confirmed identification with VITEK 2 system **[10].**

### The sensitivity test with antibiotics discs:

The plates of Mueller-Hinton agar were inoculated by dipping a sterile swab into the inoculums culture with $1.5 \times 10^8$ CFU/ml by adjusting to McFarland standard tube (No. 0.5) by **[10].**

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Sensitivity test against some antibiotics was done and the results were compared with CLSI data according to **[11].**

**MIC determining by E-test method:**

Eight antibiotics strips were used: Cefoperazone, Cefoxitin, Cefotaxime, Azithromycin, Cefotaxime/ Cefotaxime +, Ampicillin, Imipenem + EDTA and Imipenem (Bioanalyse). the sensitivity test of isolates against these antibiotics was done according to **[10].**

**Detection of *IMP-1*gene with PCR:**

PCR amplifications were performed with 100 ng of DNA bacteria plus 12.5 µl of Master Mix (Bioneer/ Korea), 1.5 µl of primer (the primers described in **[**table **1]** were prepared to 10 pmol/µl concentration as work primer), and distal water to reach the final volume to 25 µl. The PCR program with an initial denaturation was done for 2 min at 95°C. then for 30 s in 90°C with 30 cycles, 52 °C for 1 min and 72 °C for 1 min, with a final extension for 8 min at 65°C. Then all PCR amplified products were planned with 2% (w/v) agarose gel in Tris-acetate-EDTA buffer. The products were run for 120 min at 90 V. The bands were observed after staining with ethidium bromide by using an ultraviolet-light **[12].**

**Table (1): The specific primer of *IMP-1, NDM-1* genes:**

| Primer | Sequence | GC (%) | roduct size | eferences |
|---|---|---|---|---|
| orward *IMP-1* | (F-5/ CCTCATGTTTGAATTCGCC-/ | 50.0 | 198 bp. | 12] |
| Reverse *IMP-1*; | / CTCTGTCACATCGAAATCG ( | 50.0 | | |

**Biofilm Formation Assay of bacteria:**

The method identified in **[13]** was followed as the standard test for biofilm formation detection. Briefly, only the bacterial cultures of the diluents (200 µL), and another with 50 µL, it was then a negative control which added 200 µL of Brain heart infusion broth eight wells with no further additions. For 18-24hrs, the micro titer plate was incubated at 37℃, then, was washed five times with distilled water, and was left in dry air for 15 min. The plate was stained with 0.1% Crystal Violet 200 µL for 15 min, and was washed with distilled water. So 200µl of 95 % methanol was added for 10 min. to each well. The amount of crystal violet collected by the ethanol in each well was quantified using an ELISA reader to calculate the OD 580nm.Statistical analysis was expressed as Mean ±SD between the control and each of bacteria with Excel software. Cut off value (ODc) can provide isolates as shows in **[**table **2].**

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**Table (2): The Cut off value of biofilm production:**

| | |
|---|---|
| ) ≤ ODc | biofilm formation |
| )c<OD ≤ 2 × ODc | ak biofilm formation |
| ODc< OD | ong biofilm formation |

## Statistical Analysis

Data presented as a frequency and percentage, while Mean ±SD was used to analyze the data in this study. ($P \leq 0.05$) considered statistically by using program SPSS Statistics (2012) **[14].**

## RESULTS AND DISSCUTIONS:

**Identification of bacteria by biochemical tests:**

Ten isolates of *K. pneumoniae* were isolated from different clinical specimens (40% of blood,30% of urine, 20% of sputum, 10% of wound infection) as shows in **[**fig.**1].**

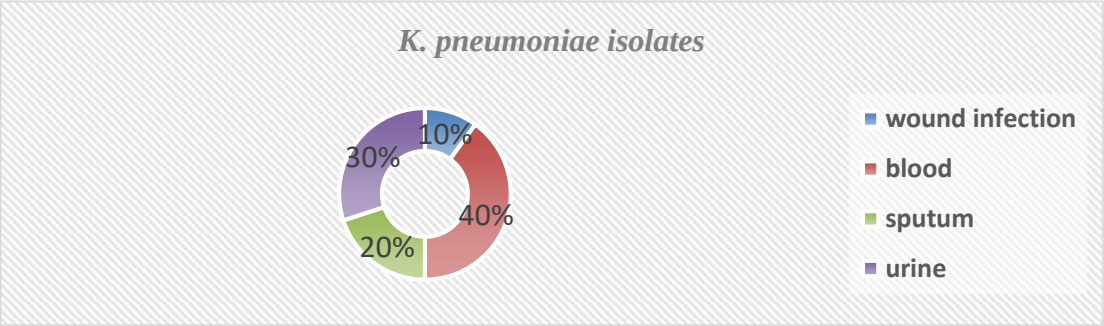


**Figure (1): Distribution of *K. pneumoniae* in clinical specimens**

*K. pneumoniae* were cultured on MacConkey agar, the bacteria were lactose fermenter so that appeared pink colonies. And also were cultured on Simmon Citrate agar the bacteria can utilize the citrate; therefore, the media color was changed to blue. Indole test was negative for *K. pneumoniae.* On blood agar bacteria gave gamma hemolysis **[10].** The confirmation of identification was done by VITEK 2 system.

**Sensitivity test for antibiotics:**

The sensitivity of *K. pneumoniae* isolates were tested to a number of antibiotics used to treat some of the infections caused by this species in humans. **[**fig.**2]** shows that *K. pneumoniae* used in test were resisted (100%) to Amoxicillin, Cefitrixon, Ticarcillin, Ticarcillin +Clavulanic acid (TCC), Ceftazidim and Kanamycin. While Ticarcillin +Cilactin (IC), Sparfloxcin (SPX) were sensitive in 75% and 25% intermediate in sensitivity. And Ciprofloxacin, Nitrofuranton were given 66.6%, 25%, 8.4% respectively. In the other hand, all isolated bacteria were sensitive to

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Imipenem 90 %, and only one isolate was resisted to it. But Amikacin was showed 41.7% of sensitive isolates, and 25% intermediate and the rest were sensitive in 33.3%.

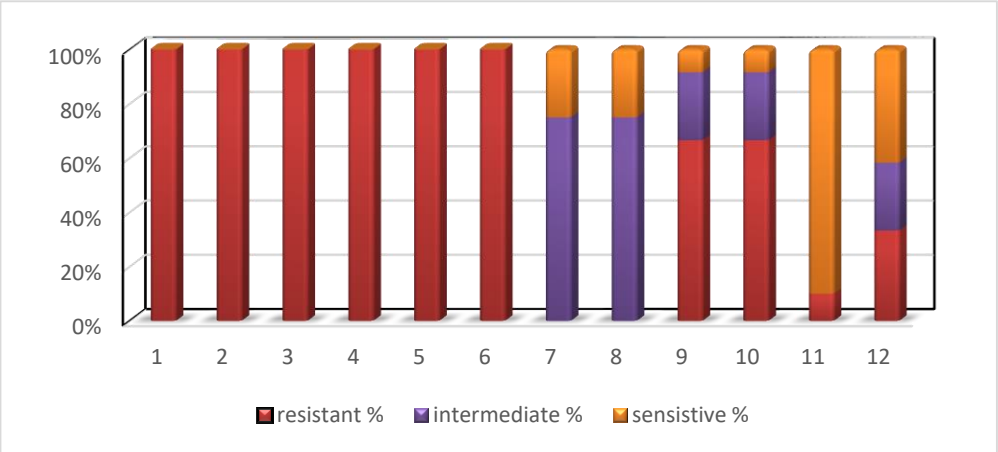


**Figure (2): The results of antibiotic sensitivity test for isolates**
**1: Amoxicillin, 2: Cefitrixon, 3: Ticarcillin, 4: Ticarcillin/Clavulanic acid, 5: Ceftazidim, 6: Kanamycin, 7: Ticarcillin+Cilactin, 8: Sparfloxcin, 9: Nitrofuranton, 10: Ciprofloxacin ,11: Imipenem, 12: Amikacin**

another study from Taiwan about *K. pneumoniae* , their data revealed decreased susceptibilities to most β-lactam antibiotics (all generation of Cephalosporins) and fluoroquinolones **[15].** In the other hand, other study was showed ESBL producing *K. pneumoniae* isolates were resisted to ampicillin, and third-generation cephalosporins. And these isolates were sensitive to meropenem, amikacin, and ciprofloxacin **[16].** A study with disc diffusion method was found, that *K. pneumoniae* were sensitive to, Cefotaxime, Imipenem /Cilactin, Sparfloxacin, and Norfloxacin antibiotics **[17 and 18];** these results agree with result of this study, *K. pneumoniae* were resisted to most antibiotics used in this study.

**MIC of E-test strips against *K. pneumoniae:***

The E-test was used in this study to determine the Minimum Inhibitory Concentration (MIC) for antibiotics by using diffusion methods. The result showed as elliptical inhibition zone around the strips. In this study used Ampicillin, Cefoxitin, Cefperazone strips for isolates of *K. pneumoniae*, and they were not giving MIC value for isolates because they resistant to them. While Azithromycin was giving MIC value (8 -16**)** µg/ml**.** Ceftriaxone strip was showed MIC value (19- 20) µg/ml, and Imipenem was 1 µg/ml for sensitive isolates with Imipenem + EDTA was 4 µg/ml as shows in **[**table **3].**

**Table (3): Results of E-test antibiotic strips against *K. pneumoniae*:**

| ID | est antibiotic strips | C of *K. pneumoniae* (µg/ml) |
|---|---|---|
| | **ıpicillin** | |
| | **ithromycin** | **6** |
| | **ftriaxone** | **20** |
| | **foxitin** | |
| | **foperazone** | |
| | **fotaxime/ Cefotaxime +** | **2/2-1.5/0.16** |
| | **ipenem + EDTA** | **R** |
| | **ipenem** | **R** |

In another article, the MBL by E-test was interpreted as a positive. The elliptical for each of the bla IMP- positive *K. pneumoniae* isolates were cut off the lowest value of (Imipenem), 4< µg/ml and Imipenem + EDTA ,1<µg/ml) of inhibition zone, according the method of CLSI by **[11].**

**Identification of Metallo-beta lactamase genes by PCR:**

From ten isolates of *K. pneumoniae*, results of PCR were showed only three isolates possess *IMP-1* gene (No.5,9,10), while the other isolates (1,2,3,4,6,7,8) don't have this gene**.** The isolates (No.5, 9, 10) were clinical isolates (two from urine, one from blood), on the other hand, all isolates were not possessed *NDM-1* gene; the results showed in **[**fig. **3]** about 30% of isolates were carried the gene of *IMP-1*. In the United States, a study found that PCR was performed for genes of MBLs, including VIM, IMP, molecular result showed that *VIM-1* and *IMP-1* genes are 15.6 and 6.4%, respectively. But in this study all three *IMP-1* producing *K. pneumoniae* isolates and this agrees with this study result of found *IMP-1* gene in *K. pneumoniae* **[19].** Another study in Japan found that a KPC-producing organism to become endemic in Japan is currently of great interest in *Klebsiella pneumoniae* ST258 isolated from a Japanese patient **[20].** And this study does not agree with Egyptian study which mentioned that positive for *bla KPC, bla VIM, bla NDM, blaOXA-48*- and none was *bla IMP*-positive **[21].**

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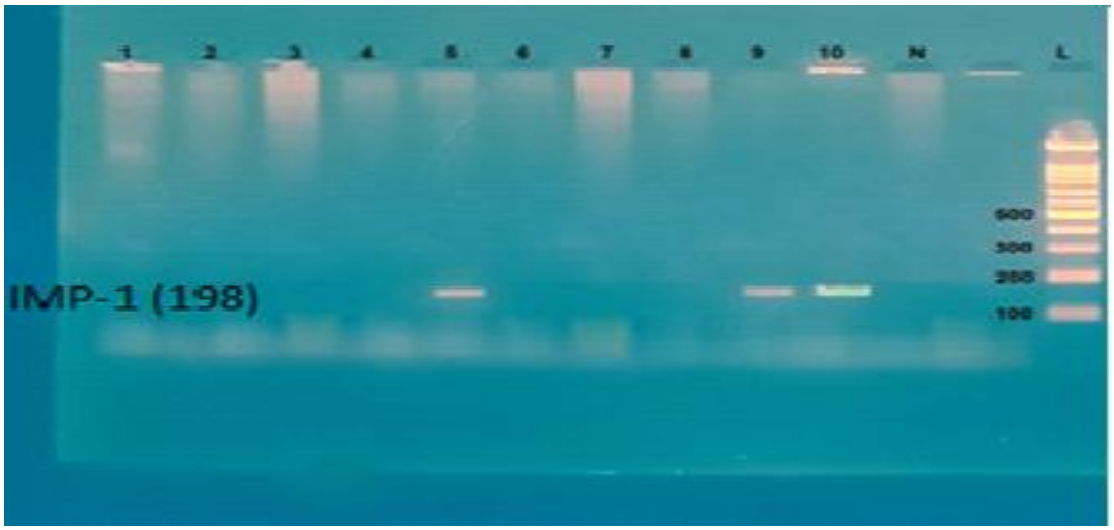


**Figure (3): Agarose gel electrophoresis of *IMP-1* gene PCR product (198 bp) , only (5, 9, 10) lanes were positive for *IMP-1* gene, on 1.5% agarose gel electrophoresis stained with Ethidium Bromide at 100 volts/Amp for 75 min, L: 100bp ladder marker**

**Biofilm formation relationship with *IMP-1, NDM-1* genes in *K. pneumoniae:***

The three isolates contained the *IMP-1* gene, and they also formed a strong biofilm, and these isolates were more resistant to antibiotics. The results showed that the bacteria used in this study were strong biofilm production for three isolates, while three were moderate of biofilm formation; while other isolates were weak producer. The value of (*P≤0.05)* was considered as a significant. The results as show in **[**table 4 and 5].

**Table (4): Results of biofilm formation in *K. pneumoniae*:**

| Isolates of bacteria | Specimens | Mean ±SD | *P* value |
|---|---|---|---|
| Kp1 | blood | 0.127±0.046* | 0.042 |
| Kp2 | urine | 0.345±0.087 | 0.076 |
| Kp3 | urine | 0.308±0.13 | 0.153 |
| Kp4 | urine | 0.338±0.16 | 0.186 |
| Kp5 | blood | 0.532±0.07 | 0.243 |
| Kp6 | sputum | 0. 251±0.056 | 0.121 |
| Kp7 | blood | 0.183±0.11 * | 0.05 |
| Kp8 | Wound infection | 0.05±0.021* | 0.014 |
| Kp9 | blood | 0.06±0.034* | 0.016 |
| Kp10 | sputum | 0.02±0.012* | 0.012 |

***P≤0.05* was considered significant**

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Acquisition of unique antibacterial resistance can undermine or enhance the formation of biofilms among the bacterial population. And this study supporting the relationship regarding biofilm formation and the development of antibiotic resistance. Therefore; there was relationship between the biofilm formation and metalo -β lactamase found in *K. pneumoniae*, results were showed in **[**table 5**].**

**Table (5): Relationships between biofilm formation and Metalo-β lactamase genes in *K. pneumoniae*:**

| Biofilm formation in *K. pneumoniae* | No. of isolates with biofil | No. of isolates with *IMI* gene |
|---|---|---|
| strong | 3* | 3* |
| moderate | 3* | - |
| weak | 4 | - |
| tal no. of isolates | 10* | 3* |

***P≤0.05* was considered significant**

The results of this study agreed with another study which found a significant correlation between the ability to form biofilms and the isolates of *K. pneumoniae* **[19].** It concluded that raising the carbapenem resistance of strains with biofilm producing *K. pneumoniae* and this agrees with the results of this study **[22].**

## CONCLUSION:

*K. pneumoniae* were isolated from different sources. All isolates were resistant to most antibiotics used in this study. The isolates have Metallo-beta lactamse ;(class B) carbpemenase. PCR showed that *K. pneumoniae* have *IMP-1 gene* in a clinical isolate. This study also found that there was relationship between biofilm formation and *IMP-1* gene in *K. pneumoniae* ($P \leq 0.05$).

## Conflict of interests.

There are non-conflicts of interest.

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