Biofilm Formation of KPC-producing and non KPC-Producing *Klebsiella pneumoniae* ssp. *pneumoniae* and Inhibitory Effect of some Watery Plant Extracts on Biofilm Formation

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Abstract

During a period of two months ((March and April, 2014), 16 clinical strains of *Klebsiella pneumoniae* ssp. *pneumoniae* were isolated from urine samples of inpatients in the Martyr Gazi Al-Hariry hospital in Baghdad. Bacterial strains showed different degrees of sensitivity towards antibiotics, they were resist 100% to Ampicillin and 68.75% to Cefazolin. On the other hand 87.5% of those strains were sensitive to Imipenem, Ertapenem and Levofloxacin, 81.25% to Piperacillin/ Tazobbactam, 75% to Aoxicillin/ Clavulanic acid and Ampicillin/ Sulbactam, 68.75% to Nitrofurantoin, Ceftriaxon, Ceftazidime and Cefepime, 56.25% to Gentamicin, Tobramycin and Trimethoprim/Sulfamethoxazole and 50% to Ciprofloxacin. Two strains were KPC-producing by phenotypic detection and genotyping detection was confirmed by PCR detection of $bla_{KP\ C-1}$ gene in these two strains. Out of 16 strains 10 (62.5%) strains were positive (+) for slime production while 6(37.5%) strains were negative (-) for slime production. The *K. pneumoniae* ssp. *pneumoniae* strains showed different degrees of biofilm formation by tube method while results by microtitre plate method showed all strains were biofilm forming when compared with the OD of the control, strains number 12 and 14 achieved the highest biofilm thickness (OD: 1.134 and 1.019, respectively) followed by strains number 11, 4 and 16 gave moderate biofilm thickness (OD: 0.805, 0.791 and 0.773, respectively), and other strains showed weak biofilm thickness in comparison to control.

The inhibitory effect of four hot and cold watery plant extracts (*Allium sativum, Adi- antum capillus-veneri* ,*Elettaria cardamomum* and *Zingiber officinale*) was investigated and the results revealed that the OD of the *K. pneumoniae* ssp. *pneumoniae* biofilm was de- clined in comparison with the data of biofilm pretreatment with extracts ($P<0.05$). All four extracts had a significant effect ($P<0.05$) on biofilm of both *K. pneumoniae*ssp. *pneumonia*e (KPC-producing strains). Hot watery extract of*Allium sativum*was the most effectivewatery plant extract on biofilm in comparison with other watery plant extracts used in this study ($P<0.05$).

Keywords: Klebsiella pneumoniae ssp. pneumoniae, blaKPC-1 gene, Biofilm formation, Inhibitory effect

1. Introduction

Klebsiella pneumoniae ssp. *pneumoniae* (*K. pneumoniae* ssp. *pneumonia*e) is a Gram- negative bacterium, belongs to the family *Enterobacteriaceae* [1]. It is a non-motile, encap- sulated, facultative anaerobic and rod-shape bacterium [1] [2]. The opportunistic pathogen, *K. pneumoniae* ssp. *pneumoniae* is responsible for causing a wide range of nosocomial and community-acquired infections [3], such as bacteremia, pneumonia and urinary tract infections [2] [3]. It is usually infects patients with indwelling medical devices, especially urinary catheters, on which this microorganism is able to grow as a biofilm [3] . Strains of *K. pneumoniae* having plasmids that code for extended spectrum beta lactamase enzymes (ESBL), and that promote resistance to beta-lactam antibiotics which led to the failure of treatments and high morbidity and mortality rates among patients. [1]. KPC- producing *K. pneumoniae* ssp. *pneumoniae* strains are susceptible to fewer antibiotics. Many of these strains are susceptible to colistin, tigecycline and one aminoglycosides, but some of strains are resistant even to these drugs [3]. One major factor contributing to antibiotics resis- tance is the ability of the pathogen to form biofilms on biotic and abiotic surfaces including catheters and other [2] [4] . The biofilm is often involved in hospital infections, making it highly important to consider the biofilm mode of growth in antibiotic treatment resolutions, clinically this issue is very importance due to the association between biofilm formation and the resistance to antibiotic treatment. [5] .

Biofilm is a complex community of microorganisms attached to a surface inside the body and on invasive devices enclosed in an exopolysaccharide matrix of microbial and host origin to produce a spatially organized three dimensional structure. Biofilm is occurring in aquatic systems as well as in a large number of environments and medical devices relevant for public health. Biofilm-associated bacteria have been shown to be associated with colonize a wide variety of medical devices and several human diseases. The role of biofilm formation by microorganism has been suggested to be an important stage in the pathogenesis of *K. pneumonia*e .The formation of biofilm within the urinary tract is one of the best explanation for the recurrent and chronic infections [6].

2. Aim of this study

The aim of this study was to characterize the ability of KPC-producing and non KPC- producing *Klebsiella pneumoniae* ssp. *pneumoniae* to form biofilm and to examine the effect of some plant extracts on biofilm formation

3. Materials and methods

Bacterial strain

Sixteen clinical strains of *Klebsiella pneumoniae* ssp. *pneumoniae* were isolated from urine samples of inpatients in the Martyr Gazi Al-Hariry hospital in Baghdad. These strains were isolated during a period of two months (March and April, 2014). Bacterial strains were identified depending on morphological characteristics and Vitek-2 system (Bio-Merieux , France) using ID-GNB cards according to the manufacturer's instructions.

Antibiotic susceptibility test and phenotypic detection of KPC-producing strains

Each strain was reported to be resistant or not to Imipenem and Ertapenem and multiple other antibiotics including: Ampicillin, Aoxicillin/Clavulanic acid, Cefazolin, Piperacillin/ Tazobbactam, Ceftriaxon, Ceftazidime, Cefepime, Ciprofloxacin, Gentamicin, Nitrofuran- toin, Tobramycin, Levofloxacin, Ampicillin/Sulbactam and Trimethoprim/Sulfamethoxazole by Vitek-2 system (Bio-Merieux , France) using AST-GN69 cards according to the manu- facturer's instructions. phenotypic detection of KPC-producing strains was observed from the resistant of those strains to Imipenem and Ertapenem antibiotics.

Genotyping detection of bla_{KPC-1} gene

PCR assay was performed in a monoplex pattern in order to amplify the ragments of bla_{KPC-1} gene for each Imipenem and Ertapenem resistant strain.

DNA was extracted by suspending 2-3 colonies of each test strain grown on MacConkey agar plates in 500 μl of nuclease-free water (Promega, USA) and heating at 90˚C for 10 min using a water bath . Samples were spun at 10000 rpm for 10 min. These samples were used as the bacterial DNA template for PCR assay [7]. Amplification reactions (25μl for each sample) were performed using the Master mix 2X (Kapa , India) (12.5μl) , nuclease-free water (3.5μl) , DNA sample (5μl) , bla_{KPC-1} Forwared primer:5'- TGT CAC TGT ATC GCC GTC -3' (2μl) and Reverse primer:5'- CTC AGT GCT CTA CAG AAA ACC -3' (2μl) (Alpha DNA , Canada). Temperature cycling conditions included an initial denaturation at 95˚C for 5 min followed by 35 cycles of 95˚C for 1 min, annealing at 58˚C for 30 sec. and extension at 72˚C for 1 min. Cycling was followed by a final extension at 72˚C for 10 min [8].

Bioftlm formation assay

All *Klebsiella pneumoniae* ssp. *pneumoniae* strains were subjected to biofilm production.

1- Congo red agar method

Qualitative evaluation of biofilm producers using the Congo red agar method to detect slime production was performed as follows: The medium is comprised of brain heart infusion broth (Hi media /India) 37 g/L, sucrose (BDH /England) 50 g/L, Congo red (Fluka) 0.8 g/L and agar (Biolife/Italy) 10g/L. Inoculated plates were incubated at 37 ˚C for 24 hrs. Slime producing strains presented black colonies while non producing strains developed red colonies [9].

2- Tube method

The quantitative assay for biofilm formation was performed as follows: Two sets of glass tubes filled with 3 mL of Brain Heart Infusion (BHI) broth (Hi media /India) were inoculated with a loopful of a pure culture of a strain of *K. pneumoniae* ssp. *pneumonia*e grown overnight from agar plate. After 24 and 48 hr. incubation at 37˚C, the content of each tube was decanted. The tubes were then stained with 1% crystal violet for 7 min. Then the tubes are washed with distilled water for 5 min. A positive result was indicated by the presence of an adherent film of stained material on the inner surface of the tube [10].

3- Microtitre plate method

Studied *K. pneumoniae* ssp. *pneumonia*e strains cultured in Brain Heart Infusion (BHI) broth (Hi media /India) incubated at 37ºC for 18 hrs., after that bacterial culture was diluted in BHI broth and adjusted in comparison to MacFarland tube no. 0.5. Two hundred microliters of this bacterial culture were used to inoculate pre-sterilized 96-well polystyrene microtiter plates and later incubated for 48 hrs. at 37ºC. After incubation, all wells were washed with sterile physiological saline for the elimination of unattached cells. Afterward, 200 μl of 1% crystal violet was added to each well. After 15 min. at room temperature, each well was washed with 200 μl sterile physiological saline. This process was repeated three times.

The crystal violet bound to the biofilm was extracted later with 200μl of ethyl alcohol, and then absorbance was determined at 540 nm in an ELISA reader (Human/German). Controls were performed with crystal violet binding to the wells exposed only to the culture medium without bacteria. All the assays were performed in triplicates [6]. The cut-off optical density (ODc) for the microtitre-plate is defined as three standard deviations above the mean OD of the negative control. Strains were classified as follows: OD ≤ ODc non- adherent, ODc < OD ≤ 2 x ODc weakly adherent, 2 x ODc < OD ≤ 4 x ODc moderately adherent, 4 x ODc < OD strongly adherent [10].

Plant materials

Four plant species were collected from local markets including:*Allium sativum, Adiantum capillus-veneris, Elettaria cardamomum* and *Zingiber officinale* were selected for inhibitory activity of biofilm formation by *K. pneumoniae* ssp. *pneumonia*.

Preparation of hot watery extracts

Purchased dry each plant species were ground into powder in an electric blender. 20 g of each plant powder was dissolved in 180 mL of distilled water in a glass bottles, heated to 85 ºC in a water bath and kept at this temperature with shaking for 8 hrs. after cooling, the liquid was filtered through the Whatman No. 1 filter paper. The filtrates were then condensed and dried in smaller glass bottles at 37 ºC for 48 hrs. Then, 1 gram of dried extracts was dissolved in 5mL of distilled water to obtain concentrations 200 mg/ml [11].

Preparation of cold watery extracts

Fifteen grams of powdered sample were added to 180 ml of cold water in flask for 18-24 hrs. in room temperature then filtered through a Whatman No. 1 filter paper. The filtrates were then condensed and dried at 37 ºC for 48 hrs. [12].

In vitroinhibitory effect of plant extraction on bioftlm

Biofilm inhibition carried out in 96 wall plates adopting modified method of biofilm inhibition spectrophotometric assay [13]: Studied *K. pneumoniae* ssp. *pneumoniae* strains cultured in Brain Heart Infusion (BHI) broth (Hi media /India) incubated at 37ºC for 18 hrs., after that bacterial culture was diluted in BHI broth and adjusted in comparison to MacFarland tube no. 0.5. Two hundred microliters

of this bacterial culture were used to inoculate pre-sterilized 96-well polystyrene microtiter plates and later incubated for 48 hrs. at 37ºC. After incubation, all wells were washed with sterile physiological saline for the elimination of unattached cells. Then, before the staining step, the previously prepared plant extracts were added to the biofilm containing wells: Subsequently, the tray was incubated for another 24 hrs. at 37ºC, after incubation period all wells were washed and stained as the same procedure described above.

4. Statistical analysis

The Chi-square (χ^2) test was used for comparison among groups. A p-value of < 0.05 was considered to be statistically significant.

5. Results

Antibiotic susceptibility test and phenotypic detection of KPC-producing strains

Antibiotic susceptibility of 16 clinical *Klebsiella pneumoniae* ssp. *pneumoniae* strains was detected by Vitek-2 system (Bio-Merieux, France) using AST-GN69 cards. The result showed different degrees of sensitivity towards antibiotics under test as shown in table-1. the rate of sensitivity to Ampicillin was very low 0(0%) followed by Cefazolin 5(31.25%). On the other hand 14(87.5%) of those strains were sensitive to Imipenem, Ertapenem and Levofloxacin, 13(81.25%) to Piperacillin/ Tazobbactam, 12(75%) to both Aoxicillin/ Clavulanic acid and Ampicillin/ Sulbactam, 11(68.75%) to Nitrofurantoin, Ceftriaxon, Ceftazidime and Cefepime, 9(56.25%) to Gentamicin, Tobramycin and Trimethoprim/Sulfamethoxazole,8(50%) to Ciprofloxacin.

Table-1: Susceptibility and resistance rates of *Klebsiella pneumoniae* ssp. *pneumoniae* strains towards different antibiotics under study.

Antibiotics	N (S%)	N (R%)
Ampicillin	**0(0%)**	**16(100%)**
Aoxicillin/Clavulanic acid	**12(75%)**	**4(25%)**
Ampicillin/Sulbactam	**12(75%)**	**4(25%)**
Cefazolin	**5(31.25%)**	**11(68.75%)**
Ceftriaxon	**11(68.75%)**	**5(31.25%)**
Ceftazidime	**11(68.75%)**	**5(31.25%)**
Cefepime	**11(68.75%)**	**5(31.25%)**
Ciprofloxacin	**8(50%)**	**8(50%)**
Gentamicin	**9(56.25%)**	**7(43.75%)**
Ertapenem	**14(87.5%)**	**2(12.5%)**
Imipenem	**14(87.5%)**	**2(12.5%)**
Nitrofurantoin	**11(68.75%)**	**5(31.25%)**
Tobramycin	**9(56.25%)**	**7 (43.75%)**
Levofloxacin	**14(87.5%)**	**2(12.5%)**
Piperacillin/ Tazobbactam	**13(81.25%)**	**3(18.75%)**
Trimethoprim/Sulfamethoxazole	**9(56.25%)**	**7 (43.75%)**

Phenotypic detection of KPC-producing strain (resistant to Imipenem and Ertapenem) showed that two *Klebsiella pneumoniae* ssp. *pneumoniae* strains were multi-drug resistant and KPC-producing (No. 12 and No.14). The isolate No. 12 was resist to all the antibiotics under the study but it sensitive only to Trimethoprim/ Sulfamethoxazole and the isolate No. 14 was also resist to all the antibiotics under the study but it is sensitive only to Levofloxacin and Trimethoprim/ Sulfamethoxazole.

Genotyping detection of $bla_{KP\ C-1}$ gene

PCR assay was done to detect the presence of $bla_{KP\ C-1}$ gene in *K. pneumoniae* ssp. *pneumonia* strains. Three strains of *K. pneumoniae* ssp. *pneumonia* were subjected to PCR assay, two strains of these (No. 12 and No. 14) were KPC-producing byphenotypic detection while the third strain (No. 5) was non KPC-producing byphenotypic detection as negative. The results presented in figure-1 showed the presence of $bla_{KP\ C-1}$ gene (its amplicon size was 1010 bp) in the strains number 12 and 14 while its negative PCR product for the third strain (No. 5).

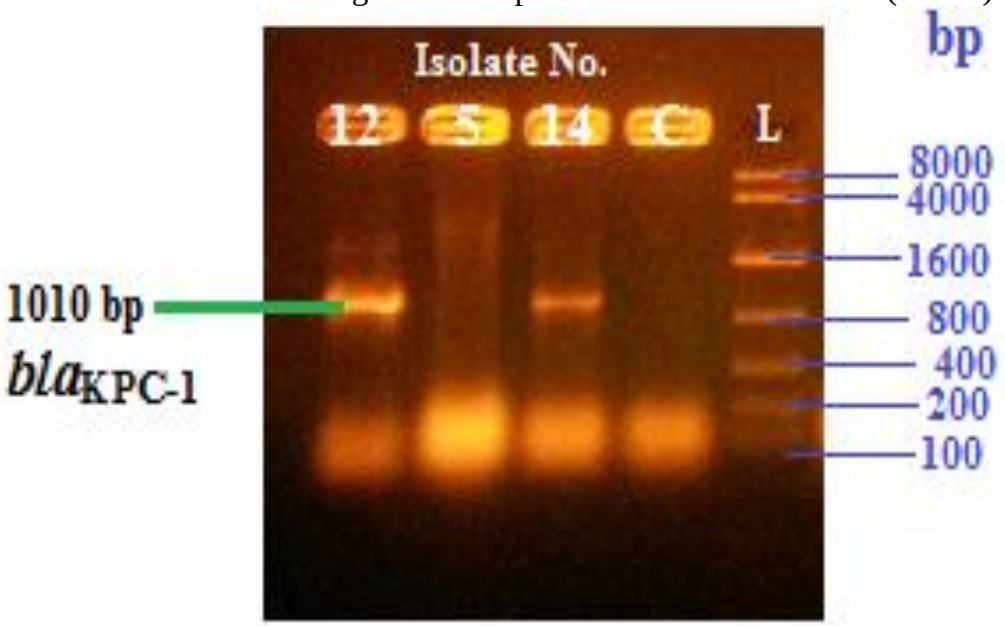

Figure-1: Agarose gel electrophoresis (1%) of bla_{KPC-1} gene PCR product at 5 V/cm for 1 hr. stained with ethidium bromide and visualized on a UV transiluminator documentation system. Lanes 12 and 14: 1010bp of bla_{KPC-1} gene PCR product; Lane 5: negative PCR product; lane C: Negative control (had all PCR mixture including water instead of DNA template); lane L: DNA ladder (Kapa

Bioftlm formation by *K. pneumoniae* ssp. *pneumoniae* strains

All 16 strains of *K. pneumoniae* ssp. *pneumoniae* were assayed for the production of biofilm: the results of slime production which detected by congo red agar method was presented in table -2. From which out of 16 strains 10(62.5%) strains were positive (+) for slime production while 6(37.5%) strains were negative (-) for slime production (figure-2).

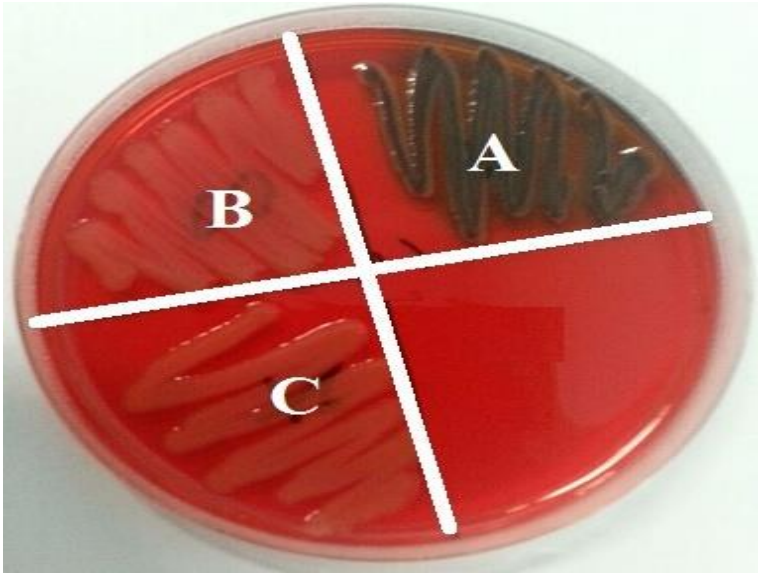

Figure-2: The congo red agar after 24 hrs. of incubation , in which : A= slime produced strain ;B and C= non slime produced strains.

The results of biofilm formation by tube method were also presented in table-2. From which after 24 hr. 14(87.5%) strains were positive (+) for biofilm formation, 6 from those 14 strains were strongly adherent while 8 from those 14 strains were weakly adherent and only 2(12.5%) strains were negative (-) for biofilm formation. On the other hand all *K. pneumoniae* ssp. *pneumonia*e strains 16(100%) were positive (+) for biofilm formation, from which 7 strains were strongly adherent and 9 strains were weakly adherent (figure-3).

Figure-3: Formation of biofilm on glass tube after 24 hr. in which; Tube 1: non adherent , Tube 2: weakly adherent and Tube 3: strongly adherent.

Table-2: The results of biofilm formation by Congo red and tube method.

Isolate No.	Congo red method	Tube method (24 hr.)	Tube method (48 hr.)
1	-	-	+
2	-	+	+
3	-	+	+
4	+	++	++
5	-	+	+
6	+	+	++
7	-	-	+
8	-	+	+
9	+	+	+
10	+	+	+
11	+	++	++
12	+	++	++
13	+	+	+
14	+	++	++
15	+	++	++
16	+	++	++

For tube method; ++ = Strongly adherent, + = weakly adherent and - = Negative. For congo red method; + = positive for slime production and - = Negative for slime production.

All strains tested for production of biofilm by microtitre plate method and all the results presented in table -3. From this table we notice that all *K. pneumoniae* ssp. *pneumonia*e strains were biofilm forming when compared with the OD of the control (OD: 0.184) and each strain shows a different potential in biofilm production, strains No. 12 and 14 which achieved the highest biofilm thickness (the absorbance (OD): 1.134 and 1.019, respectively) followed by strains No. 11, 4 and 16 which gave moderate biofilm thickness (OD: 0.805, 0.791 and 0.773, respectively), while other strains showed weak biofilm thickness in comparison to control.

Table-3: The absorbance (OD) at 540 nm for biofilm of *K. pneumoniae* ssp. *pneumoniae* strains.

Isolate No.	Absorbance at 540 nm	Isolate No.	Absorbance at 540 nm
1	0.451	9	0.496
2	0.397	10	0.583
3	0.352	11	0.805
4	0.791	12	1.134
5	0.420	13	0.489
6	0.488	14	1.019
7	0.434	15	0.504
8	0.531	16	0.773

In vitro inhibitory effect of plant extraction on bioftlm

In microtitre plate, the inhibitory effect of four hot and cold watery plant extracts (*Al- lium sativum, Adiantum capillus-veneri* ,*Elettaria cardamomum* and *Zingiber officinale*) was investigated against two *K. pneumoniae* ssp. *pneumoniae* strains which formed the thickest biofilm after 48 hr. of incubation. The results of*in vitro* inhibitory effect of hot and cold watery plant extracts on biofilm were summarized in table-4. From this table, the results revealed that the absorbance (OD) of the *K. pneumoniae* ssp. *pneumoniae* biofilm was declined in comparison with the data of biofilm before treatment with watery plant extracts (significant differences at $P<0.05$). All four hot and cold watery plant extracts had a significant effect ($P<0.05$) on biofilm of both *K. pneumoniae*ssp. *pneumonia*e strains (No. 12 and No. 14). Hot watery extract of*Allium sativum*was the most effectivewatery plant extract on biofilm in comparison with other watery plant extracts used in this study (significant differences at $P<0.05$).

Table-4: The inhibitory effect of watery plant extracts on *K. pneumoniae* ssp. *pneumonia*e biofilm.

Isolate	Watery plant extracts	Absorbance (OD)		
		Before treatment	After treatment	
			Cold extract	Hot extract
Isolate No. 12	***Allium sativum***	1.134	0.308	**0.174**
	Elettaria cardamomum		0.468	0.344
	Zingiber officinale		0.357	0.347
	Adiantum capillus-veneris		0.482	0.407
Isolate No. 14	***Allium sativum***	1.019	0.327	**0.188**
	Elettaria cardamomum		0.351	0327
	Zingiber officinale		0.333	0.329
	Adiantum capillus-veneris		0.473	0.370

6. Discussion

Due to highly resistance to antibiotics, biofilm-associated infections are very difficult to treat [14], also formation of biofilm by *K. pneumoniae* ssp. *pneumoniae*on the tissues of the hosts is thinking to protect these bacteria against host defense mechanisms [13].

Based on our results, multi-drug resistance and KPC-producing *K. pneumoniae* ssp. *pneumonia*e strains were produced the highest biofilm thickness in comparison with other bacterial strains under study and this may be due to the presence of bla_{KPC-1} gene in these strains or due to resistant to most antibiotics under test (multi-drug

resistance) or due to the both. *Carattoli et al.* reported that with the increasing worldwide occurrence of multi drug resistance KPC-producing *K. pneumoniae*, the data on their ability to product biofilms and their response to the antimicrobial agents in the biofilm state is crucial [15].

All watery plant extracts used in our study showed reduction of the *K. pneumoniae* ssp. *pneumonia*e biofilm but hot watery *Allium sativum* extract showed the most effective extract on biofilm which highly reduced *K. pneumoniae* ssp. *pneumonia* biofilm in comparison with other extracts used in this study ($P<0.05$). A study was done by Nidadavolu *et al*. reported that *Allium sativum* prevented biofilm formation by *Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, Staphylococcus epidermidis* and *Staphylococcus aureus* [16]

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الخلاصة

خلال فترة شهرين (اذار و نيسان ، 2014) ، تم عزل 16 سلالة سريرية من بكتريا الكليبسيلا الرئوية *Klebsiella pneumoniae* ssp. *pneumoniae* من عينات الادرار لمرضى راقدين في مستشفى الشهيد غازي الحريري في بغداد. أظهرت السلالات البكتيرية درجات مختلفة من الحساسية تجاه المضادات الحيوية ، كانت مقاومة 100 ٪ لمضاد Ampicillin و 68.75 ٪ لمضاد Cefazolin بينما من ناحية أخرى كانت 87.5 ٪ من تلك السلالات حساسة للمضادات Ertapenem, Imipenem, Levofloxacin و 81.25٪ لمضاد Piperacillin/ Tazobbactam و 75٪ للمضادين Aoxicillin/ Clavulanic acid و Ampicillin/ Sulbactam، 68.75٪ للمضادات Nitrofurantoin, Ceftriaxon, Ceftazidime و Cefepime ، 56.25٪ للمضادات Gentamicin, Tobramycin و Trimethoprim/ Sulfamethoxazole و50٪ لمضاد Ciprofloxacin. كانت سلالتين من هذه البكتريا منتجة لانزيمات ال KPC من خلال الكشف المظهري و التنميط الجيني وهذا ما تم تاكيدة من خلال تقنية تفاعل تضاغف سلسلة البلمرة (PCR) للكشف عن جين *bla*KPC−1 في هاتين السلالتين. كانت 10 سلالات من بين ال 16 سلالة وبنسبة (62.5٪) موجبة (+) لإنتاج الطبقة اللزجة بينما كانت 6 (37.5٪) سلالات سالبة (−) لإنتاج الطبقة اللزجة. أظهرت السلالات الرئوية درجات مختلفة من انتاج الغشاء الحيوي بطريقة ألانبوبة بينما أظهرت النتائج وفقا لطريقة لوحة التلازن ان جميع السلالات منتجة للغشاء الحيوي بالمقارنة مع الكثافة الضوئية (OD) السيطرة, السلالات رقم 12 و 14 حقق أعلى سمكاً للغشاء الحيوي (OD : 1.134 و 1.019 ، على التوالي)، تليها سلالات رقم 11 و 4 و 16 للغشاء الحيوي (OD: 0.805, 0.791 و 0.773 على التوالي) ، فيما انتجت بقية العزلات غشاء حيوي ضعيف مقارنة مع السيطرة .

تم التحري عن التأثير التثبيطي للمستخلصات المائية الحارة والباردة للنباتات الاربعة (الثوم , كرفس البئر, الهيل و الزنجبيل) وقد اظهرت النتائج ان الغشاء الحيوي قد تأثر مقارنة مع قراءات الغشاء الحيوي قبل المعاملة بالمستخلص ($P<0.05$) . كان للمستخلصات الاربعة تأثير معنوي ($P<0.05$) تجاة بكتريا *K. pneumoniae* ssp. *pneumoniae* المنتجة لأنزيم KPC. اظهر المستخلص المائي الحار لنبات (الثوم) تأثيرا اعلى مقارنة مع المستخلصات المائية للنباتات قيد الدراسة .

الكلمات المفتاحية: الكلبسيلا الرئوية ,الجين , الغشاء الحيوي ,المثبطات , المستخلصات النباتية .