The Inhibitory effect of pyocyanin pigment produced by*Pseudomnas aeruginosa* on biofilm formation of *Klebseila pneumoniae*

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Abstract:

The Pyocyanin was extracted from clinical isolate of *Pseudomonas aeruginosa* that isolated from patient suffering from cystic fibrosis ; and the *Klebsiella pneumoniae and Klebsiella oxytoca* isolates werecollectedfrom Catheter Associated Infections (CAI) and Urinary tract infection (UTI) patients with 25 (20.49%) and 3 (2.54%)of total 122 urine specimens respectively; represented the degree of the biofilm thickness among 25 isolates of *K. pneumoniae*, 3(12%) isolates (K21, K42 and K129) were considered as good producers of biofilm after incubation for 24 h at 37°C and evaluated by using crystal violet staining solution technique and their optical density (O.D) was determined at 540 nm. The susceptibility pattern ofantibiotics *against K. pneumoniae* isolates (K21 and K42) were showed resistant to Ceftriaxon, Cefotaxime, Piperacillin, Metronindazole, Cephalothin and Ceftazidime whereas sensitive to Imipenem, Gentamicin, Tobramycin, Amikacinand Ciprofloxacin; However, K129 was shown different behavior against same antibiotics. The biofilm of K21, K42 and K129 were reduced significant differences ($P<0.05$) after treatment with (2048µg/ml) of the pyocyanin solution comparatively with differences MIC values of sensitive antibiotics for these isolates after incubated for 24 h at 37 °C; the viablecells count of these isolates were shown insignificant differences ($P<0.05$). The pyocyanin solution has an inhibitory effect on planktonic isolates K21, K42 and K129 were shown increased diameter of inhibition zones with increased concentration after incubated for 24 h at 37 °C.

Keywords: Inhibitory effect, catheter, *Pseudomonas aeruginosa*, pyocyanin, *K. pneumoniae* and biofilm.

Introduction

Klebsiella pneumoniae is an opportunistic pathogen which frequently causes hospital-acquired urinary and respiratory tract infections (1). The urinary tract consider isthe most regular area for this bacterium colonization, it cause from 2 -15% of cystitis cases. Moreover, the incidence of *K. pneumoniae* increases in the nosocomial infections (2). The microbial biofilms defined as adherent microorganisms to a surface within a polymeric matrix, comprising exopolysaccharide of microbial and host origin that develops into a complex community; it is share about 80% of the microbial infections in the body (3). Bacteria in biofilms are innately more resistant to antimicrobial agents by one or more mechanisms compared with cultures grown in suspension (planktonic) in conventional liquid media, with up to 1000-fold decreases in susceptibility (4), and easily to evade the immune defense (5). Biofilm-associated *K. pneumoniae* have been shown to be associated with several human diseases (6), and to colonize a wide variety of medical devices (7). *P.aeruginosa* is theonly known organism of the pseudomonads and other glucose–nonfermenting gram-negative bacilli capable of producing the very distinctive water-soluble pigment pyocyanin (8). It is considered as a product of secondary metabolites, pyocyanin has phenazine nucleus that means it belongs to the phenazine family (9).Pyocyanin is a blue-green, phenazine pigment, it is a redox compound (electron acceptor), which stimulates redox cycling in bacteria, and human epithelial and liver cells (10).Pyocyanin has a variety of pharmacological effects on eukaryotic and prokaryotic cells and has bactericidal activity against many bacteria such as *Escherichia coli, Staphylococcus aureus* and *Mycobacterium smegmatis*, which may allow *P. aeruginosa* an advantage over competing bacteria occupying the same niche (11).

Hence, the present work aimed to investigate the inhibitory role of the pyocyanin pigment solution on the biofilms formation and viability cells of *K. pneumoniae* that isolated from human with catheter associated infections and urinary tract infections.

Materials and Methods

Bacterial strains

P. aeruginosa was obtained from postgraduate students Laboratories of Biology Department / College of Science/ University of Baghdad. That previously isolated from patient suffering from Cystic Fibrosis. API 20 NE system was employed for the identification of *P. aeruginosa.*

A total of 122 urine specimens were collected in the period between of mid of July until to the mid of September of 2010 from AL-Kadhmiya Teaching Hospital in Baghdad City. Specimens were collected from out-patients in sterile screw cupped containers. Regarding inpatients, catheter was withdrawn and cut

into pieces of 1 cm in length, rinsed in phosphate buffered saline (PBS) and transported to 5 ml of Brain Heart Infusion broth (BHIB) and incubated at 37°C for 24 h.

The urine specimenswere streaked directly on blood agar and incubated for 24 h at37ºC; the non hemolytic grayish-white colonies were sub-cultured on MacConkey agar and incubated for 24h at37ºC. Pink mucoid colonies and biochemical tests were carried out depending on (12, 13).

Extraction and purification of pyocyanin

P. aerugiosa isolate was produced dark blue color on King A medium were cultured on glycerol-alanine minimal media (6gm of L-alanine,10ml glycerol, 2gm $MgSO_4$,0.1gm K_2HPO_4, 0.01gm $FeSO_4$ and 20gm agar in 1Liter distilled water). The pH was adjusted to 7.2 and sterilized for 15 min by autoclave (14). All plates were exposed incubation over night at 37 ºC and then to light source at 25 ºC for 24h after (15). Extraction of pyocyanin was followed of Ingledew and Campbell procedure (16) with some modifications of Al-Azawi procedure (17) it is briefly: 2 volume of chloroform was added on 1 volume of bacterial culture on glycerol-alanine minimal medium that pyocyanin production and left for 2 hr at 25 ºC in order to pyocyanin dissolving. The blue chloroform was washed with acidified water (0.1M HCL) that lead to convert the pigment to red color with continuous mixing repeated 2-3 times, the acidified layer (red layer) was collected by pipette aspiration and the pH was neutralized to 7 by 1M trisbase (hydroxymethyl amino methane, pH=11.0). Then re-extraction of pyocyanin was accomplished by chloroform 4-5 times to extract the most pyocyanin from the mixture. The acidifiedwater layer was discarded and pyocyanin was removed from chloroform by adding 0.05M HCL. pH was adjusted to 7.5 by 0.1M of NaOH, Needlelike crystals were formed in the chilled solution trapped on a 0.45-µm filter, washed with water, dried and weighed (18).

Screening of Biofilm production (Microtitration plate Method)

The method described by Maldonado *et al* (2) was followed to achieve biofilm formation, bacterial isolates was cultured in (BHI) broth incubated at 37ºC for 18 h diluted by same medium to obtained 1×10^8 cell/ml and adjusted by depending on comparison to MacFarland tube 0.5. 200µl of bacterial culture were transposed to sterilized 96-well polystyrene microtiter plates and incubated for 24 h at 37ºC. Then, all wells were washed with (PBS) for the elimination of unattached cells. Afterward, 200µl of 1% crystal violet solution was added to wells with shaking for three times to help the colorant to get the bottom of the well and left for 10 minutes at 25 ºC , each well was washed with 200 µl sterile (PBS) to remove the planktonic cells and stained not adhered cells to the well. Above process were repeated three times. The adhered bacteria that formed

biofilm were remained on the surface of the well. The crystal violet that bounded to the biofilm was extracted with 200µl of ethyl alcohol, and the degree of biofilm formation was determined at 540 nm in an ELISA reader (Beckman coulter, Austria) Controls were performed with crystal violet solution binding to the wells that exposed to the BHIB medium .

The biofilm degree was calculated as follows

$$OD_{\text{at 540 nm}} = OD_{\text{sample}} - OD_{\text{control}}$$

To determine the viability of bacterial cells within the biofilm was performing according the procedure that described by(12).

Antibiotic susceptibility

The antibiogrm of the highest biofilm producing of *K. pneumoniae* was determine by single disk method against: Ciprofloxacin (CIP) 5 µg, Metronindazole (MET) 5 µg, Tobramycin (TOB) 10 µg, Imipenem (IMP) 10 µg, Amikacin (AK) 10 µg, Ceftriaxon (COR) 30 µg, Cefotaxime (CTX) 30 µg, Gentamicin (CN) 30 µg, Ceftazidime (CAZ) 30 µg, Cephalothin (KF) 30 µg and Piperacillin (PRL) 100 µg, The diameter of inhibition zone for identified antibiotics were measured in millimeters and values were interpreted as resistant and sensitive categories according to NCCLS (19, 20).

A standard *E. coli* strain (*E. coli* ATCC 25922) was used as the quality control strain.

Minimum Inhibitory Concentration (MIC) of pyocyanin

This test was performed according to the method of (21) as follows: Double dilutions of pyocyanin, IMP, CIP, TOB, AK and CN were freshly prepared using Mueller-Hinton broth as a diluents 1ml was distributed in sterile test tubes, Pure isolated colonies of the highest biofilm producing of *K. pneumoniae* isolates were grown on MacConkey agar then suspended in 5 millilitres of normal saline (NS), mixed well and balanced with Macfarland 0.5 turbidity standard. The standardized inoculum was diluted in Muller Hinton Broth (1:100). 1ml of the adjusted inoculums was added to each tube containing the given pyocyanin and antimicrobial agent. The final concentration in tube will contain 5×10^5 cfu/ml (13). For each batch of MIC determinations, a negative control tube was performed by mixing 2 ml of Mueller Hinton broth to a sterile empty tube. In addition, a positive control was also prepared by adding 1 ml of test isolate broth to1 ml of Mueller Hinton broth; all tubes were incubated at 37°C for 24 h and the growth was determined visually among the different tubes of the serial dilution by comparing with the positive and negative control. The lowest concentration of antibiotic or pyocyanin pigment that inhibited the growth of bacteria was termed the MIC.

Inhibitory effect of pyocyanin and antibiotics on biofilm formation and cells viability

For the inhibition of biofilm assay, the highest biofilm producing isolates of *K .pneumoniae* isolateswere elected to be assayed. Subsequently, same protocol previously described was followed to produce a biofilm. Then, just before the staining step, two hundred microliters of BHIB containing a pyocyanin or antibiotic at a final concentration equals to MIC were added to *K. pneumoniae* biofilm wells. All plates were incubated for 24 h at 37ºC.

The bacterial biofilm was evaluated using crystal violet solution. The OD reading designated as OD1of K. *pneumoniae* biofilmwas included as a positive control 1; its OD represented the reading before treatment and OD2 of *K. pneumoniae* biofilmwas included as a positive control 2; its O.D represented the reading after treatment K. *pneumoniae* biofilm with pyocyanin solution and antibiotics. Negative controls were performed with crystal violet solution binding to the wells that exposed to the BHIB medium. All the assays were performed in triplicates. The biofilm degree was calculated as follows:

$OD1_{\text{at 540 nm}} = OD_{\text{before treatment (positive control)}} - ODc_{\text{negative control}}$

$OD2_{\text{at 540 nm}} = OD_{\text{after treatment (positive control)}} - ODc_{\text{negative control}}$

At the same time viable count depending on the procedure described earlier by (15) was perfor- med to determine the viability of bacterial cells within the biofilm.

Statistical analysis

Data was presented as mean ± standard deviation. ANOVA and $LSD_{0.05}$ were employed for data analysis using Statistical Analysis System- SAS (2004)(22).

Results and discussion:

P. aeruginosa was isolated from cystic fibrosis patient; it was produce pyocyanin on king A agar and liquid media kept production in large amounts.The glycerol-alanine minimal medium was used to pyocyanin production, it was large amount than king B agar, this may attributed to stimulate the responsible gene of pyocyanin and suppress production of fluorescein (pyoverdin) by K^{+} and Mg^{+2} salts that essential ingredient of medium, while phosphate that found in king B agar contains that inhibit pyocyanin expression (11). This results agreement with report that observed *P. aeruginosa* were isolated from organs associated with air completely and the aerobic conditions are the essential factor required to produce pyocyanin, and this may attributed to O_2 role that induces genes responsible for encoding pyocyanin or aerobically metabolism that needs O_2 for growth and different activities especially pyocyanin production (23). The pyocyanin was extracted by chlorofom was shown as needle like cluster (Fig. 1).

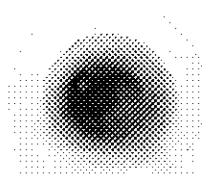

Figure 1: Needlelike crystals of pyocyanin produced by *P.aeruginosa*

The clinical isolates of *Klebseilla* were isolated from 122 urine specimens of out-patients and inpatients with catheter, the results were shown 28 (28.95%) isolated belong to *Klebsiella*, 25 (20.49%) of than belong to *K. pneumoniae* and 3 (2.54%) were identified as *K. oxytoca* (table 1).

Table 1: Phenotypic identification and origin of *Klebseila* strain isolated from clinical sources

Clinical source	Phenotypic identification
Catheter	*K. pneumoniae* (21) *K. oxytoca* (2)
Mid-stream urine	*K. pneumoniae* (4) *K. oxytoca* (1)
Total	28(28.95%)

Our results were incompatible with other study it was shown 41% of total isolates belong to *K. pneumoniae* were obtained from urine (25). Many studies were referred to *Klebsiella* spp. were consider biggest and important ratio among many clinically isolated pathogens that prevailing in hospitals (Nosocomial pathogens) that causes UTI (26). Implanted foreign polymer bodies became a common practice of modern medical care and used of foreign material may led to many complications due to the insertion or implantation of medical devices that related with microbial infections, The morbidity and mortality of device-associated infections may increased problem of nosocomial infections, these results may indicate that the most of the strains that isolated from patients with urethral catheterization belong to *K. pneumoniae* due to role of type 1 and type 3 pili in mediating *K. pneumoniae* colonization (27).

The degree of the biofilm thickness of *K. pneumoniae* isolates were showed 3 (12 %) isolates were considered as good producers, while 9 (36%) were moderate and 13(48%) of the isolates were weak producers. The strains K21, K129and K42 belong to*K. pnumoniae* were gives thickest biofilm that O.D was 6.112, 4.872 and 4.102, respectively at 540 nm (table 2). *K. pneumoniae* isolated from CAI showed a remarkable high capability to form biofilm than isolated of UTI.

Table 2: Determination of biofilm production from *K. pneumoniae* at 540 nmby microtiter plate assay and statistical analysis.

	Strain no.	Mean O.D ± SD
1	K21	6.112 ± 0.112 a
2	K129	4.872 ± 0.436 a
3	K42	4.102 ± 0.102 a
4	K25	3.252 ± 0.252 b
5	K24	2.541 ± 0.389 b
6	K59	2.235 ± 0.651 b
7	K25	1.574 ± 0.301 b
8	K27	1.462 ± 0.352 b
9	K14	1.444 ± 0.112 b
10	K1	1.412 ± 0.294 b
11	K19	1.199 ± 0.460 b
12	K28	1.139 ± 0.268 b
13	K24	0.707 ± 0.132 c
14	K2	0.555 ± 0.506 c
15	K5	0.518 ± 0.106 c
16	K15	0.484 ± 0.129 c
17	K16	0.397 ± 0.058 c
18	K17	0.336 ± 0.074 c
19	K11	0.327 ± 0.053 c
20	K20	0.302 ± 0.032 c
21	K6	0.278 ± 0.073 c
22	K22	0.267 ± 0.075 c
23	K9	0.260 ± 0.051 c
24	K23	0.238 ± 0.038 c
25	K65	0.217 ± 0.038 c

Each datum is a mean of triplicate. SD= standard deviation. LSD= 0.44. P= 5.41E-35 a= Good biofilm producer (O.D 6-4), b= Moderate biofilm producer (O.D 3.9-1) and c= Weak biofilm producer (O.D lower than 0.9)

The variant in biofilm thickness may attributed to many reasons belong to isolates capacity, the primary number of cells, ability to cells adherence or quality and quantity of autoinducers (quorum sensing signaling molecules) that produced from isolates that play an important role. (28). One of the more important variables influencing biofilm susceptibility is the age of the biofilm (29).

The susceptibility testing of K21 and K42 isolates to antibiotics were determinate by antibiogram and shown resistant to (CRO), (CTX), (PRL), (MET), (KF) and (CAZ)whereas (IMP), (CN), (TOB), (AK)and (CIP); were sensitive. While K129 was shown resistance to (CRO), (CTX), (PRL), (MET), (KF), (CAZ),(CN) and (TOB) whereas sensitive to (IMP), (AK)and (CIP).

Among many problems associated with infection that related with medical devices is the low sensitivity of the bacteria to the antibiotics by different mechanism such as resistance genes by horizontal gene transfer (HGT) and β-lactmase (30). Three hundred clinical isolates of *K. pneumoniae* were collected from urine; blood and sputum in Nigeria were appeared resistant to cefotaxime (74%), ceftriaxone (79.6%) and ceftazidime (69%) (31). The hospital-acquired strains were significantly more resistant to all tested antimicrobial agents than the community-acquired organisms However, plasmid-mediated resistance to these cephalosporins has been reported in many countries in the last few years and several nosocomial outbreaks have occurred due to these strains, recently study described the capacity of ceftazidime-resistant *K. pneumoniae* to become established and cause serious infections within the hospital under the selective pressure of ceftazidime use despite an active antibiotic control program (32).

MICs are a minimal concentration of antibiotic in this paper MIC of pyocyanin pigment was detected that is necessary for inhibition of visible bacterial growth after incubation period at 37°C for 18-24 hour. The lowest MIC was shown CIP (2µg/ml) and highest MIC was AK (32 µg/ml) for K21 and K42respectively; while (4µg/ml) of IMP was the lowest MIC and CN (2048 µg/ml) was highest MIC for K129, MIC of pyocyanin pigment was (2048 µg/ml) for all isolates. Result of MICs shown in (table 3). In other study they reported that MIC of CIP was (0.125µg/ml) while MIC of AK was (16µg/ml) for *K. pneumoniae* (33).

Table 3: Values of MICs of elected antibiotics for K2, K42 and K129isolates

Antibiotic and pyocyanin concentration µg/ml						
	IMP	CN	TOB	AK	CIP	pyocyanin
No. of Isolates						
K21	16	8	8	32	2	2048
K42	8	8	8	32	2	2048
K129	4	-	-	256	8	2048

The effect of pyocyanin on biofilm and viable cells count of *K. pneumoniae* comparatively with different antibiotics shown in (table 4), the biofilm of K21, K42 and K129 were reduced significant differences ($P<0.05$) after treatment with pyocyanin solution for 24 h at 37ºC, while the viablecells count of these isolates were shown insignificant differences ($P<0.05$).

Table 4: O.D. and viability count (cfu/ml) for *K. pneumoniae* biofilm after treatment with IMP, CIP, AK, CN, TOB and pyocyanin and statistical analysis.

No. of isolates	Parameter	Befor treatment	After treatment						LSD[3] value
			IMP	CIP	AK	CN	TOB	PYO	
K21	O.D[1].	0.449±0.02	0.316 ± 0.02	0.278 ± 0.01	0.331 ± 0.03	0.299 ± 0.01	0.308 ± 0.02	0.249±0.01	0.0545*
	P- value		0.002	0.001	0.045	0.003	0.006	0.0008	
	LSD value		0.056*	0.056*	0.113*	0.065*	0.076*	0.059*	
	V.C[2].	17.76±5.78	15.91 ± 1.62	14.61 ± 1.73	14.91 ± 1.76	13.31 ± 1.14	12.99 ± 1.72	6.48±1.93	5.159 *
	P-value		0.773	0.629	0.661	0.492	0.473	0.138	
	LSD value		16.69 INS[4]	16.77 INS	16.80 INS	16.38 INS	16.77 INS	16.94 INS	
K42	O.D.	0.446±0.006	0.359±0.001	0.348±0.009	0.294±0.007	0.299±0.029	0.316±0.026	0.253±0.013	0.054 *
	P- value		0.0002	0.0009	0.0001	0.008	0.008	0.0002	
	LSD value		0.017*	0.031*	0.026*	0.084*	0.074*	0.042*	
	V.C	15.06 ± 4.67	17.05 ± 2.39	11.12 ± 0.54	15.53 ± 1.61	13.53 ± 2.17	10.95 ± 2.16	6.78 ± 2.02	5.923 *
	P-value		0.724	0.448	0.929	0.781	0.469	0.179	
	LSD value		14.59 INS	13.07 INS	13.73 INS	1432 INS	14.31 INS	14.14 INS	
K129	O.D	0.428±0.002	0.262±0.006	0.363±0.020	0.270±0.012	0.328±0.012	0.265±0.013	0.248±0.009	0.034 *
	P- value		0.0001	0.022	0.0001	0.001	0.0001	0.0001	
	LSD value		0.018*	0.049*	0.033*	0.033*	0.027*	0.032*	
	V.C	7.705 ± 7.33	12.41 ± 2.71	8.32 ± 3.12	14.99 ± 2.27	16.01 ± 1.61	12.33 ± 1.64	3.51 ± 1.85	7.009 *
	P-value		0.580	0.942	0.396	0.331	0.572	0.572	
	LSD value		21.71 INS	22.14 INS	21.33 INS	20.86 INS	20.88 INS	21.02INS	

O.D. = optical density, [2]VC= viable count, [3]LSD= least significant difference. Each datum is the mean of triplicate, INS= insignificant differences in the same raw, *=this sign in the same raw refer to significant differences.

Dependently, these finding strongly suggest that most efficient pyocyanin and antibiotics lead to destroy exopolysaccharide layers of biofilm and decrease the viability cells of *K. pneumonia*e while failed to effectively kill all bacterial cells within able to establish a new biofilm. This result agreement with other studies that shown the pyocyanin extraction from agar medium by using chloroform; it has high titers of antibacterial activity, that refer to pyocyanin has a variety of pharmacological effects on eukaryotic and prokaryotic cells (23, 34).

In other study *P. aeruginosa* was isolated from cystic fibrosis patient was showed inhibitory effect to the biofilm formation of environmental strains of the *Burkholderia cepacia* when both isolates mixed together, authors suggested pyocyanin as the active compound of the soluble inhibitor (35).The biofilm formation proceeds in two stages; a rapid attachment of the bacteria to the polymeric surface is followed by a more prolonged accumulation phase that involves cell proliferation and intercellular adhesion (36). The biofilm is formed at the interphase between the inert support of the microplate and the liquid media (37). Our results agreed with many reports that refer to the bacterial biofilm were significantly less responsive to antibiotics and antimicrobial stressors than viable cells of the same species (38). The failure may attribute to ability of antibiotics to penetrate a biofilm (39).

The pyocyanin solution developed an inhibitory effect on planktonic *K. pneumoniae* isolates K21, K42 and K129 were investigated on Muller Hinton Agar and distilled water was employed as control, the diameter of inhibition zones around pyocyanin solution containing wells were measured in millimeter and compare with control. The best results were show for K129, it showed increased of inhibition zones with increased concentration of pyocyanin solution, such as: 10, 12, 16 and 24 mm at 6.25, 12.5, 25 and 50 mg/ml, respectively (Figure 2).

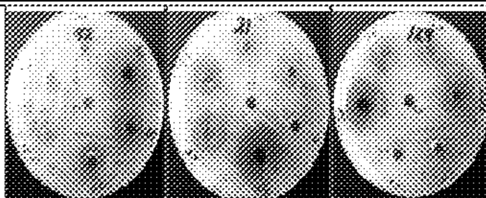

Figure 2: Inhibitory effect of pyocyanin on planktonicagainst K21, K42 and K129

The direct mechanism of pyocyanin toxicity remains unclear, the wide range of biological activity is thought to be due to its ability to catalyze the formation of toxic radicals such as superoxide (O^{2-}) and hydrogen peroxide (H_2O_2) (40). Our results were compatible with other study it was shown that pyocyanin has antibiotic activity against other bacteria and fungi, it is bactericidal for such bacteria: *Escherichia coli, Staphylococcus aureus* and *Mycobacterium smegmatis,* this killing is observed on agar plates as clear zones on lawns of sensitive bacteria (23).The present work can conclude that although *K. pneumoniae* biolim is hardly to be eradicated by bacterial antibiotics, the pyocyanin extracted from a *P. aeruginosa* has reduction *K.pneumoniae* biofilm and decrease viability cells, results showed the pyocyanin developed an inhibitory effect on planktonic cells, also it can be used as a therapeutic agent after adequate in vivo experimentation or as a disinfectant agent on the a biotics surfaces.

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التأثير التثبيطي لصبغة البايوسيانين المنتجة من بكتريا *Pseudomnas aeruginosa*على تكوين الغشاء الحياتي لبكتريا *Klebsiella pneumoniae*

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الخلاصة:

استخلصت صبغة البايوسيانين من عزلة سريرية تابعة لبكتريا *Pseudomonas aeruginosa* والمعزولة من مريض مصاب بداء التليف الرئوي، وعزلتبكتريا *Klebsiella pneumoniae* و*Klebsiella oxytoca*من بعض المرضى المستخدمين للقنطرة البولية والمرضى المصابين بالتهاب المسالك البولية بنسبة عزل 25 (20.49%) و3 (2.54%)على التوالي من مجموع 122 عينة ادرار. قدرت قابلية 25 عزلة من بكتريا *K. pneumoniae*على انتاج الغشاء الحياتي واظهرت العزلات K21 وK42 وK129 بواقع3 (2.54%) من العزلات ذات انتاجية جيدةمن الغشاء الحياتي بعد فترة حضن 24 ساعه وبدرجة حرارة 37 م°وقدر باستعمال تقنية التصبيغ بمحلول البنفسج البلوري وحددت قيمة الكثافة الضوئية عند طول موجي 540 نانومتر. اظهرت العزلات K21 و K42 مقاومة ضد السيفترياكسون و السيفوتاكسيم والبيبيراسيلين والميترونيدازول وسيفالوثين والسيفتازايدام وحساسة للأيميبينيم والجنتامايسين والتوبرامايسين والاميكاسين والسيبروفلوكساسين،اما العزلة K129 فاظهرت سلوكا مغايرا ضد هذه المضادات . اظهر الغشاء الحياتي للعزلات K21 و K129 و K42 فروقا معنوية ($P<0.05$) بعد معاملتها بـــ 2048 مايكروغرام/ مل من محلول صبغة البايوسيانين بالمقارنه مع قيمة التركيز المثبط الادنى للمضادات الحساسة لهذه العزلات بعد فترة حضن 24 ساعه وبدرجة حرارة 37 م° ، اما العدد الحي لذات العزلات فقد اظهر فروقا غير معنوية($P<0.05$). واظهر محلول صبغة البايوسيانين تاثيرا مثبطا على الخلايا الهائمة للعزلات K21 وK42 وK129 وزيادة قطر التثبيط بزيادة التركيز بعد فترة حضن 24 ساعه وبدرجة حرارة 37 م.