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Study The Relationship Between The Ability of Biofilms Formation and Antibiotic Sensitivity for *Klebsiella pneumonia* Isolated From Different Clinical Sources

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Abstract:

Klebsiella pneumoniae is a dangerous pathogens that can cause severe diseases. The aim of this study is to examine the ability of *K.pneumoniae* to produce biofilms and the relationships between biofilms formation and antibiotics resistance.

This study included isolation of 50 isolates of *K.pneumoniae* from different clinical sources from different hospitals in Baghdad city, the number and percentage of isolates according to the sources (urine, blood, sputum, burns, ear swabs, pus, wounds and stool) were 22(44%), 11(22%), 4(8%), 4(8%), 3(6%), 3(6%), 2(4%) and 1(2%) respectively. Antibiotic sensitivity of the isolates was done by vitek 2 compact system using antibitics (amikicin, azteronam, cefepime, ceftazidime, ciprofloxacin, gentamycin, impenem, meropenem, minocycline, piperacillin, piperacillin/ tazobactam, ticracillin, tobaromycin and trimethoprim/ sulfamethoxazole).

The obtained results showed that the antibiotics amikicin, impenem and meropenem were more effective against the isolates. On other hand, the isolates showed different ability to produce biofilms according to the clincal sources was test by using two methods (Congo-red agar methods-CRA- and Tissue culture plate methods-TCP-), the results showed that the percentages of isolates formed biofilms in (CRA) 72% produce biofilms and 20% non productive and 8% non-specific. The percentages by TCP methods were 80% produce biofilms and 20% was not able to form biofilms. Higher production of biofilms isolates were exposed to Ciprofloxacin and Meropenem to make a comparison the antibiotic resistance between planktonic and biofilms producers isolates, the results showed that the resistance to antibiotics became 10 times higher than planktonic isolates**.**

Form this study we can conclude that *K.pneumoniae* could be isolated from differents sources (that were multi-drug resistant) had the ability to produce biofilm in different methods.

Key words: *Klebsiella pneumoniae, Biofilm formation, Multi-drug resistant.*

دراسة تأثير الأغشية الحيوية بواسطة *Klebsiella pneumonia* المعزولة من مناطق سريرية مختلفة على بعض دفاعات الجهار المناعي

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الخلاصة:

تعتبربكتريا *Klebsiella pneumonia* من الممرضات الخطرة التي من الممكن أن تسبب اصابات حادة.شملت الدراسة عزل 50 عزلة من البكتريا من مصادر سريرية مختلفة من مستشفيات مختلفة في مدينة بغداد لغرض دراستها من محاور عدة بعد أن شخصت البكتريا بالإعتماد على الطرق التقليدية بالتشخيص من حيث الإعتماد على الصفات الزرعية والكيموحيوية ومن ثم أستعمل جهاز vitek2 compact system لتأكيد التشخيص.

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أظهرت النتائج أن عدد ونسبة عزل وفقا للمصادرالسريرية (البول ،الدم ،القشع، حروق، مسحة الأذن، القيح، الجروح والخروج) كانت 22 (44٪)، 11 (22٪)، 4 (8٪) (3) (6٪)، 3 (6٪)، 2 (4٪) و 1 (2٪) على التوالي.
تم دراسة المقاومة للمضادات المختلفة وتحديد التركيز الادنى للمضادات الحيوية لتثبيط نمو البكتريا، ولوحظ أن المضادات amikicin, impenem and meropenem أكثر فعالية ضد هذه البكتريا، ومن جهة أخرى أظهرت العزلات قدرة مختلفة على إنتاج الأغشية الحيوية، حيث أستخدمت في هذه الدراسة طريقتان لمعرفة قدرة العزلات لتشكيل الأغشية الحيوية، كانت النسبة في طريقة Congo-Red Agar (CRA) 72 % مكونة للغشاء و 20 % غير منتجة و 8 % غير محددة ، في حين طريقة أطباق المعايرة الدقيقة 80 % مكونة و20 % غير قادرة على تكوين الغشاء. العزلات الأقوى لإنتاج الأغشية الحيوية اختيرت لمعرفة مقاومتها للمضادين Ciprofloxacin and Meropenem لأجراء مقارنة المضادات بين الخلايا المفردة والخلايا المكونة للاغشية الحيوية، وأظهرت النتائج أن المقاومة للمضادات تصبح أكثر بحوالي 10 مرات بالنسبة للخلايا المكونة للاغشية الحيوية مقارنة مع الخلايا المفردة.

Introduction:

Klebsiella pneumoniae is an opportunistic pathogen responsible for causing a spectrum of hospital community-acquired and nosocomial infection and especially infect patients with indwelling medical devices such as urinary catheters[1].

K. pneumonia is member of Entero-bactericeae, Gram-negative, rod shape non-motile, facultative anaerobic, lactose fermenter with a prominent capsule, which is ubiquitously present in the environment such as soil, vegetation, water and readily isolated from mammalian mucosal surfaces[2].

Biofilms are defined as structured bacterial communities enclosed in a self-produced exopolysccharide matrix and adherent to abiotic or biological surfaces[3]. It is characterized by the cells that are irreversibly attached to a substratum or to each other bacteria seem to initiate biofilm formation in response to specific environmental conditions such as nutrient and oxygen availability[4]. The importance of biofilm is to protect and safe the bacteria from host immune system and antibiotic treatment, creating a source of toxic metabolites and persistent infection, biofilm not only provide a physical barrier to antimicrobial agent and host antibodies, but as well as facilitate the exchange of antibiotic-resistant genetic material[5].

The presence of other bacteria in the hospital environment that have a high antibiotic resistance properties may facilitate the transfer of resistance genes between them, in addition to these, isolates may be able to produced an enzymes (e.g.: β-lactamases) which cause degradation of antibiotic[6].

Biofilm producing bacteria exhibit resistance to antibiotics by various methods like restricted penetration of antibiotic in to biofilm, decreased growth rate and expression of resistance genes[7].

The aim of this is to study the ability of bacteria to form biofilms and select the strains that are more resistant to antibiotic and have the ability to form strong biofilm.

Materials and Methods:

Bacterial isolates:

Fifty *Klebsiella pneumoniae* isolates were isolated from hospitals in Baghdad city included Ibn-El Balady hospital, Al-Kendy teaching hospital and Teaching laboratories in medical city, during July 2014 to December 2014. They were isolated from different clinical sources including urine, blood, sputum, ear swab, burn, stool, wounds and pus.

Diagnostic kits:

The VITEK® 2 Compact system is dedicated to the identification and susceptibility testing of clinically signifi-cant bacteria. The system includes the VITEK®2 Compact instrument, computer, and printer. The software provided with the VITEK® 2 Compact system includes analysis and data-management programs according to the leaflet of company.

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Determination of Minimum inhibitory concentration (MIC) via VITEK® 2 compact:

The MIC for some antimicrobial agents were determined using VITEK® 2 Compact system.

The break point for each antimicrobial used was according to Clinical and Laboratory Standards Institue (CLSI, 2012, table-1)[8].

Table-1: Minimal inhibitory concentration (MIC) interpretive standards for isolates (CLSI), 2012.

Antibiotics	Code	MIC interpretive criteria µg/ml		
		S	I	R
Amikicin	AK	<=16	32	>=64
Azteronam	ATM	<=4	8	>=16
Cefepime	FEP	<=8	16	>=32
Ceftazidime	CAZ	<=4	8	>=16
Ciprofloxacin	CIP	<=1	2	>=4
Gentamicin	GM	<=4	8	>=16
Impenem	IPM	<=1	2	>=4
Meropenem	MEM	<=1	2	>=4
Minocycline	MNO	<=4	8	>=16
Piperacillin	PIP	<=16	32-64	>=128
Piperacillin/Tazobactam	TZP	<=16/4	32/4-64/4	>=128/4
Ticracillin	TIC	<=16/2	32/2-64/2	>=128/2
Tobaromycin	TM	<=4	8	>=16
Trimethoprim/ sulfamethoxazole	SXT	<=2/38		>=4/76

S: Sensitive, I: Intermediate, R: Resistance

Congo red test:

Plates were inoculated by pure single isolated colony and incubated aerobically for 24-48 hr at 37°C, positive result was indicated by black colonies with a dry crystalline consistency. The weak slime producers usually remained pink, though an occasional darkening at the centers of the colonies was observed . A darkening of the colonies, with the absence of a dry crystalline colonial morphology, indicated an indeterminate result[9].

Tissue Culture Plate Method:

The assay was performed in triplicate using 96-well flat-bottomed cell culture plates (Nunc, New York, NY, USA) as described previously[10]. Ten ml of Trypticase soy broth with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar. The broth was incubated at 37°C for 24 hours. The culture was further diluted 1:100 with fresh medium. Ninety-sixt flat bottom wells tissue culture plates were filled with 0.2 ml of diluted cultures individually. Only sterile broth was served as blank. Similarly control organisms were also diluted and incubated. All three controls and blanks were put in the tissue culture plates. The culture plates were incubated at 37°C for 24 hours. After incubation, gentle tapping of the plates was done. The wells were washed with 0.2 ml of phosphate buffer saline (pH 7.2) four times to remove free floating bacteria. Biofilms which remained adherent to the walls and the bottoms of the wells were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was

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washed with deionized water and plates were dried properly. Optical densities (OD) of stained adherent biofilms were obtained with a micro ELISA auto reader at wave length 570 nm. Experiment was performed in triplicate and repeated thrice. Average of OD values of sterile medium were calculated and subtracted from all test values.

Antibiotic susceptibility assay on abiotic surfaces:

Biofilms allowed to form as above, after incubation 24hr, two antibiotics were prepared in different concentration from stock using this formula:

C1V1 =C2V2

This experiment were done as described as[3].These antibiotic were dissolved in brain heart infusion broth and then 180 µl from different concentration of antibiotics were added to 20 µl of bacteria in microtiter plate and incubated 24hr at 37°C, and thus as described above.

Results and Discussion:

K.pneumoniae are known to be an opportunistic pathogen causing serious infections such as pneumonia, urinary tract infection and septicemia. The wildly spread of bacteria in hospitals environment in some Baghdad hospitals is the main reasons made this pathogen to multidrug resistant and cause nosocomial infections. The ability of this pathogens to cause diseases and resist antibiotics related to biofilms formation was tested.

The number and percentage of isolates according to sources were as follow: 22(44%) isolates from urine, 11(22%) blood, 4(8%) sputum, 4(8%) burn patients, 3(6%) ear swab, 3(6%) pus, 2(4%) wounds infection, 1(2%) and stool (table-2).

Identification of the isolate have been done with VITEK® 2 Compact system..

Table-2: Prevalence of *K. pneumoniae* on different clinical sources.

Type of specimen	Number of isolates	*K.pneumoniae isolates*, (%)
Urine	22	44
Blood	11	22
Sputum	4	8
Burn	4	8
Ear swab	3	6
Pus	3	6
Wounds	2	4
Stool	1	2
Total	50	100

Antimicrobials Susceptibility:

The isolates were evaluated for antimicrobial susceptibility with Vitek 2 compact system. Susceptibility was tested to 14 antimicrobials: Amikicin, azteronam, cefepime, ceftazidime, ciprofloxacin, gentamicin, impenem, meropenem, minocycline, piperacillin, piperacillin/ tazobactam, ticracillin, tobaromycin and trimethoprim/sulfamethoxazole.

The results of antimicrobial resistance for isolates under study were as follows: 98% for ticracillin, 94% for piperacillin, 70% for each of azteronam, ceftazidime and cefepime, 60% for trimethoprim/sulfamethoxazole, 28% for tobaromycin, 22% for gentamicin, 12% for piperacillin/tazobactam, 8% for impenem, meropenem, 2% for ciprofloxacin. In addition, some isolates had been show intermediate resistance against some antimicrobials such as minocycline 50%, ciprofloxacin 14%, tobaromicin and piperacillin/tazobactam 2%, while amikicin revealed 100% sensitivity of isolates (Table-3).

These results shown that the local isolates of *K. pneumoniae* were highly resistant in term of multi-drug resistant pathogen, especially against penecillins and cephalosporins, also increase of percentage resistance to trimethoprim/ sulfamethoxazole and gentamicin was found. On another hand, the most effective antibiotics were amikicin, impenem, meropenem.

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Table-3: Antibiotics resistant patterns for isolates.

No.	AM	ATM	FEP	CAZ	CIP	GM	IPM	MEM	MNO	PIP	TZP	TIC	TM	SXT
1	S	S	S	S	S	S	S	S	S	S	S	R	S	S
2	S	S	S	S	S	S	S	S	S	R	S	R	S	S
3	S	R	R	R	S	S	S	S	S	R	S	R	S	S
4	S	S	S	S	S	S	S	S	S	R	S	R	S	S
5	S	R	R	R	S	R	S	S	S	R	S	R	R	R
6	S	S	S	S	S	S	S	S	S	R	S	S	S	S
7	S	S	S	S	S	S	S	S	S	R	S	R	S	S
8	S	S	S	S	S	S	S	S	S	S	S	S	S	S
9	S	S	S	S	S	S	S	S	S	R	S	R	S	S
10	S	S	S	S	S	S	S	S	S	R	S	R	S	S
11	S	R	R	R	S	S	R	R	I	R	R	R	S	R
12	S	S	S	S	S	S	S	S	S	R	S	R	S	S
13	S	S	S	S	S	S	S	S	S	S	S	R	S	S
14	S	R	R	R	S	S	S	S	I	R	S	R	S	R
15	S	S	S	S	S	S	S	S	S	R	S	R	S	S
16	S	R	R	R	S	S	S	S	I	R	S	R	R	R
17	S	S	S	S	S	S	S	S	S	R	S	R	S	S
18	S	R	R	R	S	S	S	S	I	R	S	R	S	R
19	S	R	R	R	S	S	S	S	S	R	S	R	S	R
20	S	R	R	R	S	S	S	S	I	R	S	R	S	S
21	S	R	R	R	I	R	R	R	I	R	R	R	R	R
22	S	R	R	R	S	S	S	S	I	R	S	R	S	R
23	S	R	R	R	S	S	S	S	I	R	S	R	S	R
24	S	S	S	S	S	S	S	S	S	R	S	R	S	S
25	S	R	R	R	S	S	S	S	S	R	S	R	S	S
26	S	R	R	R	S	S	S	S	I	R	S	R	S	R
27	S	R	R	R	S	S	S	S	I	R	S	R	S	R
28	S	R	R	R	S	S	S	S	S	R	S	R	S	R
29	S	R	R	R	I	R	S	S	I	R	S	R	R	R
30	S	R	R	R	S	S	S	S	I	R	S	R	S	R
31	S	R	R	R	S	S	S	S	I	R	S	R	S	R
32	S	R	R	R	S	R	S	S	S	R	S	R	R	R
33	S	S	S	S	S	S	S	S	S	R	S	R	S	S
34	S	R	R	R	I	R	S	S	I	R	I	R	R	R
35	S	R	R	R	I	R	S	S	I	R	S	R	R	R
36	S	R	R	R	S	S	S	S	S	R	S	R	R	R
37	S	R	R	R	S	R	S	S	I	R	S	R	R	R
38	S	R	R	R	S	S	S	S	S	R	S	R	S	S
39	S	R	R	R	S	S	S	S	I	R	S	R	R	R
40	S	R	R	R	S	R	S	S	S	R	R	R	R	R
41	S	S	S	S	S	S	S	S	S	R	S	R	S	S
42	S	R	R	R	S	S	S	S	I	R	S	R	S	R
43	S	R	R	R	S	S	S	S	I	R	S	R	S	R
44	S	R	R	R	S	S	S	S	S	R	S	R	S	R
45	S	R	R	R	I	R	R	R	I	R	R	R	R	R
46	S	R	R	R	I	S	S	S	I	R	R	R	S	R
47	S	R	R	R	I	S	S	S	I	R	S	R	R	R
48	S	R	R	R	S	R	S	S	I	R	S	R	I	R
49	S	R	R	R	S	S	S	S	I	R	S	R	S	R
50	S	R	R	R	R	R	R	R	I	R	R	R	R	S

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Asati[11] have been reported that *K. pneumonia* was found to be most sensitive to amikacin, gatifloxacin, gentamicin and chloramphenicol. Consequently, considering the antimicrobial susceptibility, cost, side effects and many other factors, amikacin, gatifloxacin, gentamicin and chloramphenicol should be preferred drugs for *K. Pneumoniae* infection isolated from pus. This resistance was due to the production of β-lactamase enzymes which cause the hydrolysis of β- lactam ring resulting in inactivation of β-lactam antibiotics.

Overall resistance to third generation cephalosporins was high on account of the production of extended spectrum β-lactamases (ESBLs) by the *K. pneumoniae*, the resistance may also be due to the production of metallo-β-lactamases (MBL), which can be chromosomally encoded or plasmid mediated, the dose as well as the incidence of toxicity subsequently reduced if beta lactamase inhibitors are used with β-lactam antibiotics[12].

Detection of biofilm formation:

K.pneumoniae has a tendency to form biofilms on biotic and abiotic surfaces, including catheters and other medical devices, which is a contributing factor to their antibiotic resistance[13].

Several factors required for biofilms formation have been identified in *K.pneumoniae* clinical isolates from the gastrointestinal tract and in strains that are associated with pneumoniae and urinary tract infection[14].

The results showed that 72% (36/50) of isolates produced strong slime layer indicated by formation black colonies, while 20% (10/50) of isolates did not produce slime layer indicated by formation of pink colonies and 8% (4/50) of isolates produced a darkening of the colonies with the absence of a dry crystalline colonial form indicated an indeterminate result, while from the total of 50 clinical isolates (figure-1), TCP method detected 60% (30/50) as highest value of biofilm formation due to strong adherence, 26% (13/50) as moderate or weak biofilm former and adherence and 14% (7/50) as non biofilm producers (figure-2, table-4)

The Congo Red agar method also required the use of a highly nutritious medium- in this case, the brain heart infusion broth with a 5% sucrose supplementation. The Congo Red method was rapid, more sensitive, and reproducible and it had the advantage of the colonies remaining viable on the medium[15].

Acidity, temperature, and ion concentration have all been shown to influence biofilm formation by microorganisms in different conditions[16]. Slime production also varies among different species of isolates[17].

This result partially agree with Ariadnna *et al*., revealed 64% of isolates were high biofilm former, while 26% were medium biofilm former and 10% were low biofilm formet[18]. On the other hand Samia *et al.*, their results showed that 52% of isolates are high producer while 40% are moderate producers and 8% are non producers[3].

Carlos *et al*., revealed that 76% of isolates were determined to be positive for biofilm formation while 24% of isolates were to be negative for biofilm formation[19]. Tissue culture plate method is more quantitative, most reliable and easy method for the detection of biofilm forming microorganisms as compared with CRA method, and can be considered as a general screening method for detection of biofilm producing bacteria in laboratories[20,21]. Biofilm formation provides bacteria with a mean of persistently colonizing either living or inert surfaces within a human host[22].

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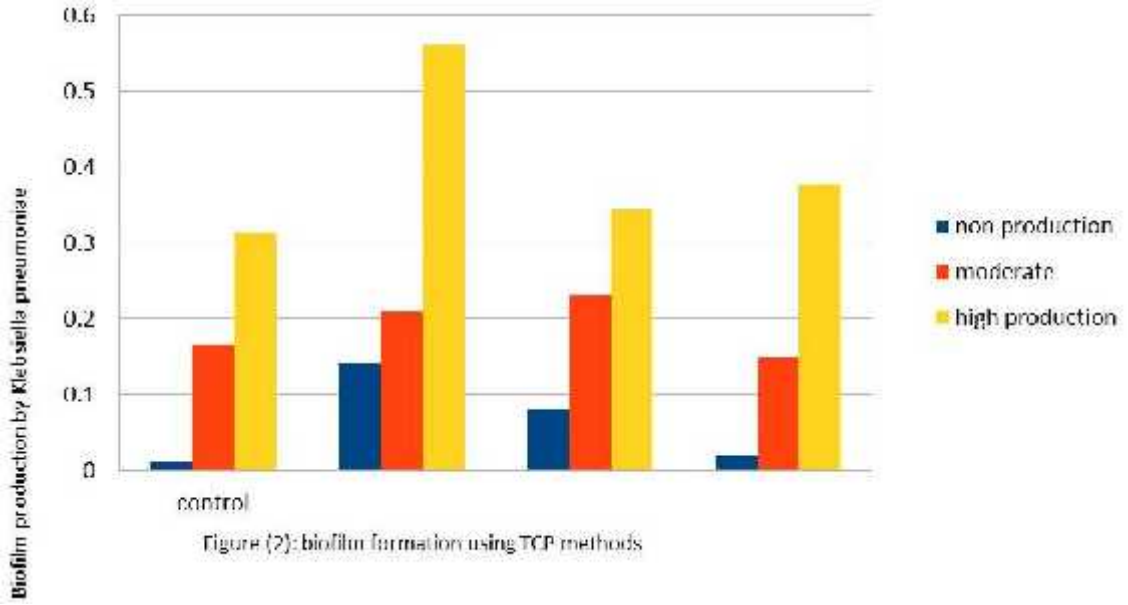

Figure-1: ***Klebsiella pneumoniae*** **in congo red agar (a. non-biofilm production, b.biofilm production, c. non- identification).**

Table-4: Prevalence of biofilm formation in *K.pneumoniae.*

Biofilm formation	**CRA-method**		***TCP-method***	
	NO	**%**	**No**	**%**
High producer	36	72.00%	30	60.00%
Moderate	-	-	10	26.00%
Non-producer	4	20.00%	7	14.00%

Antibiotic Susceptibility Assay:

Bacterial communities (Biofilms) can exhibit tolerance to environmental stress that single cells cannot. Community level resistance adds to the cellular level resistance, thus greatly enhancing the overall antibiotic resistance of the microbial community[23].

To confirm that we proposed this assay for biofilm organized bacteria to compare the antibiotic sensitivity of isolates in both features planktonic and biofilm. Two antibiotics were chosen that are planktonic feature of bacteria was more sensitive (Meropenem and Ciprofloxacin), all isolates became higher resistant in biofilms form than planktonic, about 10 times or more than planktonic, that improve the biofilm role in increasing antibiotic resistance by *K. pneumoniae.*

It is known that antibiotic resistance is usually associated with genetic changes, either to the acquisition of resistance genes, or to mutations in elements relevant for the activity of the antibiotic. However, in some situations, resistance can be achieved without any genetic alteration; this is called phenotypic resistance, non-inherited resistance is associated to specific processes such as growth in biofilms, a stationary growth phase or persistence[24]. The focus of antibiotic discovery has been on discovering compounds that target cellular mechanisms in the planktonic mode of growth, both *in vitro* and *in vivo*, as a result, many antibiotics are less effective against microbes in biofilms[23]. Bacteria organized in biofilm exhibit higher antibiotic tolerance than in planktonic form, as consequence, MIC value for bacterial biofilm can be up to 1000 higher

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than for their relative planktonic bacteria[25]. The main differences between the planktonic and biofilm forms are the structural organization of the bacteria and the presence of extracellular matrix[26]. The strongest isolates were choosen to confirm this study (table-5).

Table-2: Antibiotic susceptibility Assay of *K.pneumoniae*

Strain	Ciprofloxacin (mg/L)		Meropenem (mg/L)	
	MIC	Biofilm susceptibility	MIC	Biofilm susceptibility
Kp 11	0.5	5	2	10
Kp 14	0.25	5	0.25	4
Kp 20	0.5	3	0.25	4
Kp 21	2	12.5	4	10
Kp 31	0.5	3	0.25	4
Kp 32	1	5	0.25	4
Kp 34	2	12.5	0.25	4
Kp 40	0.25	3	0.25	4
Kp 43	0.5	12.5	0.25	4
Kp 45	2	12.5	8	12.5
Kp 47	2	12.5	0.25	4
Kp 48	0.25	3	0.25	4
Kp 50	4	12.5	0.25	4

Kp (*Klebsiella pneumoniae***)**

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