Silver nanoparticle Bacteriostatic activity Assessment against Extended-Spectrum Beta-Lactamase Producing Uropathogenic Klebsiella pneumonia

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Abstract:

The aims: Screen for the Extended-Spectrum Beta-Lactamase producing *Klebsiella pneumonia* from urinary tract infection UTI patients phenotypically and molecularly, also testing different sizes of silver nanoparticles as antibacterial for the resistance isolates.

Methodology: Vitek Extended-Spectrum Beta-Lactamase test , molecular confirmed ESBL-producing isolates were checked by PCR for the presence of *AmpC* and b*la*$_{CTX-M}$ genes. Flat well microtiter plates were used for packing of antibacterial activity of silver nanoparticles . This study included 85 urine samples were collected from patients that admitted to the "Rizgary Hospital" in Erbil province from 15 June to 15 December 2018. Antibiotic sensitivity test of all isolates to (Ampicillin, Ampicillin/sulbactam Cefazolin, Ceftazidime Ceftriaxone, Cefepime, Imipenem, Nafcillin and Nitrofurantoin)antibiotics were tested by the disc-agar method as standardized by the National Committee for Clinical laboratory Standards (2006).

Results: Out of 51 tested isolates of *Klebsiella pneumonia*, 11 (21.6%) were Extended-Spectrum Beta-Lactamase Producers. Their antibiotic resistance profile showed the rate of resistance isolates was (100%) to Ampicillin, Cefazolin, Ceftazidime and Ceftriaxone and (90%) to Cefepime .In addition the susceptibility to Imipenem was (72.7%) of isolates. The bands of the *AmpC* and *bla* $_{CTX-M}$ genes was noted in (42.9%) samples and the remain samples (57.1 %)were negative for both genes. The Minimum Inhibitory Concentration during incubation of Non ESBL producer - ESBL producer isolates in series concentrations of silver nanoparticles size (20 nm) was between (625 -2500) µg/ml and for size (90 nm), it was (1250) µg/ml for non ESBL producer and not affected ESBL producer isolates .The increased percentage rates of β-lactamase producing *Klebsiella* species were seen which was considered as an alarm, due to limitation in treatment options for UTI. It was appeared that Imipenem currently is

DOI:https://doi.org/10.26750/Vol(7).No(2).paper13 http://journal.uor.edu.krd/index.php/JUR Vol.7. No.2, April.2020

the main available antibiotic for UTI treatment as a drug of choice. It was noticed that different sizes of silver nanoparticles showed antibacterial activity for ESBL producing isolates.

Recommendation : Future studies needed on other types and size of nanoparticles on different types of bacterial isolates from different clinical sources which will enable the monitoring of trends overtime.

Keywords: Silver nanoparticle , *Klebsiella pneumonia,* ESBL, UTI.

DOI:https://doi.org/10.26750/Vol(7).No(2).paper13 http://journal.uor.edu.krd/index.php/JUR Vol.7. No.2, April.2020

Introduction :

Extended Spectrum Beta- Lactamase producing organisms causing urinary tract infections (ESBL-UTI) .The spread of ESBL-producing Enterobacteriaceae (ESBL-PE) has dramatically increased worldwide. *E.coli* and *K. pneumonia* are common producers of ESBL, and they generally cause bacteraemia and urinary tract infection .ESBL producing *Klebsiella* species seem to account for most nosocomial outbreaks [1]

The nanoparticles (NPs) are efficiently being used as antibacterial agents. Some studies have been reported which displays the antibacterial nature of NPs against gram negative and gram positive bacteria, mycobacteria and fungi [2]. One of the accepted relationships between NPs and antibacterial activity is NPs can complement and support traditional antibiotics "as a good carrier." [3]. In addition "Nanomaterials as antibacterial complements to antibiotics are highly promising and are gaining large interest as they might fill the gaps where antibiotics frequently fail" [4].

The antibacterial activity of Silver nanoparticles (AgNPs) against gram-negative bacteria, such as *E. coli* was reported [5].Other study was evaluated that AgNPs exhibited high bactericidal activity against ampicillin-resistant *E. coli* O157:H7, multidrug-resistant *Pseudomonas aeruginosa* and erythromycin-resistant *Streptococcus pyogenes* [6]. *Staphylococcus aureus* and *E. coli* also were shown to be substantially inhibited by AgNPs [7].Moreover, the bactericidal activity of AgNPs against the pathogenic, multidrug-resistant (MDR) as well as multidrug-susceptible strains of bacteria was studied by many scientists, and it was proved that the AgNPs are the powerful weapons against the MDR bacteria like *Pseudomonas aeruginosa*, Methicillin-resistant *Staphylococcus aureus* (MRSA), ampicillin-resistant *E. coli*, vancomycin-resistant *Staphylococcus aureus* (VRSA) and erythromycin-resistant *Streptococcus pyogenes* [8]. The antimicrobial activity of AgNPs in strains of *S. aureus* resistant to a large number of antibiotics was tested and underscored [9]. Other study were proved AgNPs as good antimicrobial efficacy againsta broad range of gram-positive and gram-negative bacteria, yeast, molds and viruses [10]. The present study conducted to evaluate the prevalence and antimicrobial resistance pattern of ESBL-producing *K. pneumoniae* among UTI infections patients, determination of the minimum inhibitory concentration (MIC) of different size of silver nanoparticles against these isolates and finally investigation the prevalence of the beta lactamase *AmpC* and $bla_{CTX\text{-}M}$ genes responsible for resistance in isolates under study by PCR .

DOI:https://doi.org/10.26750/Vol(7).No(2).paper13 http://journal.uor.edu.krd/index.php/JUR Vol.7. No.2, April.2020

Materials and Methods

1-Sample collection, Isolation and Identification of K. pneumonia:

A total of 85 midstream urine samples were collected form suspected UTI infected patients (inpatients and out patients) with specialist physicians in Rizgary Hospital in Erbil City during the period between 15 June, to 15 December, 2018. Patients who received antibiotics within the past two weeks were excluded.

A 10 µl (0.01 ml) of mixed urine sample was inoculated on MacConkey agar and incubated at 37°C for 24 hours. Positive culture isolates were preliminarily screened by their colony morphology, Gram-staining techniques Further identifications of isolates were made by conformation of motility ,Catalase ,Oxidase ,Urease ,H2S , Motility ,DNase ,Indole, Citrate, Methyl Red and Voges Proskauer tests.

Subcultures were incubated at 37ºC for 24 hours then single pure colonies were isolated, and transferred to slant nutrient agar tubes for preservation and performing other tests [11].

2- Detection of ESBL producer isolates by VITEK 2 system:

The automated VITEK 2 system was depended for detection of extended-spectrum b-lactamases (ESBLs) producer *Klebsiella pneumoniae* . Moreover, isolate characterization and antimicrobial susceptibility testing were achieved using VITEK 2 system through ID-GNB cards, according to the manufacturer's instructions. The ESBL screening test utilizes the growth response to ceftazidime, cefepime, and cefotaxime in combination with or without clavulanic acid . All results were interpreted by using the Advanced Expert System (software version VT2-R04.03). [12] and [13].

3- Molecular Detection of *AmpC* and *bla*$_{CTX-M}$ genes.

From the sum total of isolates analysed (11 phenotypically confirmed ESBL producers only 6 randomly selected and were subjected to polymerase chain reaction (PCR) analysis. The PCR amplification of bla genes, including *bla*$_{CTX-M}$ bla *AmpC* were carried out with GoTaq® Green Master Mix using primers listed in (Table 1). DNA extraction: A single colony from overnight incubated pure culture was suspended into 1ml of distilled water, centrifuged at 14000xg for 2 min., then the supernatant discarded, after that 120µL of lysostaphin (10 mg/L; Sigma) was added. DNA extracted using mini DNA extraction kit (Promega) according to manufacture instructions. PCR were carried out using thermal cycler (Eppendorf Master cycler Gradient) depending on [14 & 15). The PCR mix was prepared in a volume of 25 µl containing 4 µl DNA template, 12.5 µl Go Taq Green Master (Promega, CA) 2

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ml of 10 X PCR buffer (Sigma, Aldrich), 1.5 mM MgCl2 , 0.6 µl 2 mM dNTPs (ferments), 1µl of (10 Pmol\ µl) of each primer volume made up to 25 µl with distilled water. The cycling conditions used and product size for two different PCR are shown in (Table:2/Fig-1 & 2). The amplified product was subjected to 1.5% agarose gel electrophoresis, and visualized under UV after ethidium bromide staining.

Table 1: Primer used for the amplification of *AmpC* abd bla_{CTX-M} gene.

Primers	Sequence(5'→3')	Length	(bp)
AmpC-F	AATGGGTTTTCTACGGTCTG	20	191
AmpC-R	GGGCAGCAAATGTGGAGCAA	20	
bla_{CTX-M}-F	AATCACTGCGTCAGTTCAC	19	701
bla_{CTX-M}-R	TTTATCCCCCACAACCCAG	19	

Table 2 : PCR Conditions for amplification of *AmpC* and bla_{CTX-M} genes

Gene Detected	Denaturation Time/temp	Annealing Time/temp	Extension Time/temp	No. of cycles	Product	Depending Reference
AmpC	94ºC 1.5 minute	57ºC 30 second	72ºC 10 minute	30	191bp	14
bla_{CTX-M}	94ºC 30 second	50ºC 30 second	72ºC 7 minute	35	701bp	15

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4-Minimum Inhibitory Concentration (MIC) of Nanoparticles against *Klebsiella pneumonia* isolates

Stock solutions of Nanoparticles:

Commercially synthesized AgNPs were purchased from M K Impex Corp., CANADA. The reported ‘‘as manufactured’’ in small glass container labeled with the quantity and size of nanoparticles AgNPs\20, 90 nm. Preparation Stock solution was achieved according to the method mentioned by other investigators. Ten ml of deionizer water was added to100 mg of AgNPs and requested vigorously for 5 minutes to break the bloc of nanoparticles to get a homogeneous solution and then sterilized by autoclaved at 121 C^0 for 15 minutes and cool in temperature room for a final concentration of stocks 10mg / ml[16]. .

Inoculums preparation:

The 18 hours cultures plate from all *Klebsiella pneumonia* isolates were prepared. Single colonies from each isolated plate were transferred to 5 ml sterile suspension media to obtain 106cfu/ml, which were also adjusted with 0.5McFarland tube.

Procedure:

Ninety six flat well microtiter plates were used for packing of bacteriostatic activity of nanoparticles according to the methods described by Amsterdam [17]:-

1- Mueller-Hinton broth 100 µl was putted in each wells of a microtitre plate.

2-One hundred microliters of 5000 µg/ml nanoparticle (size 20 nm) was transferred into the well 1 in row A then mixed with broth by sucking up and down 6-8 times. This makes well 1 a twofold dilution of stock (i.e. 2500µg/ml).

3-From well 1,100 µl was transferred to well 2 in the same row. This makes well 2 a twofold dilution of well 1. This procedure was repeated down to well 10 only and the concentrations (5000, 2500, 1250, 625,312.5,156.25, 78.125, 39.0625, 19.531, 9.531) µg/ml, and100 µl from well 10 was discarded .

4-The same collection of tips was used for the size 90 nm of nanoparticles in different rows with same plate. The plate inoculation was performed by adding 5 µl of bacterial suspension into wells in columns 1 to 10 [well 11 contain broth and bacterial suspension (positive control). well 12 only contain broth without bacterial suspension (negative control). All plates were incubated at 37Cº for 24 hrs. After incubation, the MIC was determined as described previously.

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Results and Discussion

Out of 85 urine sample, 56 (65.9%) showed bacteriuria (dark pink mucoid colonies ,lactose fermentation) and 29 (34.1%) were non-bacteriuria as shown in Table (3).

Table 3: Number and percentage of positive and negative growth cultures in urine samples.

Urine samples Culture	N.	%
Positive	65	65.9
Negative	29	34.1
Total	85	100

phenotypic characteristics of isolates was reported. **VITEK 2 Compact Instrument**, using the GNI (Gram-Negative Identification) card according to the manufacturer instructions was used, and the biochemical characters were summarized in (Table 4).

Table 4:

phenotypic characterizes of *K. pneumonia*

Tests	Results
Gram stain/ Shape	-ve/Rod
Catalase	+ve
Oxidase	-ve
Urease	+ve
H2S	-ve
Capsule	+ve
Motility	-ve
DNase	-ve
Indole	-ve
Citrate	+ve
MR (Methyl Red)	-ve
VP (Voges Proskauer)	+ve
Lactose	+ve

Using the Vitek ESBL test, screening for the resistance profile of ESBL-producing (11 isolates) was obtained and showed in (Table 5). The rate of resistance isolates was (100%) to Ampicillin, Cefazolin, Ceftazidime and Ceftriaxone and (90%) to Cefepime .In addition the susceptibility to Imipenem was (72.7%) of isolates .

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Table 5: MIC and Resistance profile of ESBL- producing *K. pneumonia* isolates by Vitek ESBL test.

Antimicrobial	N(%) of isolates	MIC	Interpretation
ESBL	11/21.6	POS	+
Ampicillin	11(100%)	>= 32	R
Amoxicillin/Clavulanic Acid	3(27.3)	>= 32	R
Ampicillin /Sulbactam	3(27.3)	>= 32	R
Piperacillin/Tazobactam	2(18.2)	32	I
Cefazolin	11(100)	>= 64	R
Ceftazidime	11(100)	16	×R
Ceftriaxone	11(100)	>= 64	R
Cefepime	10(90.9)	2	×R
Ertapenem	5(45.5)	<= 0.5	S
Imipenem	8(72.7)	<= 0.25	S
Gentamicin	5(45.5)	>= 16	R

R: resistant S: sensitive I: intermediate POS:positive

Out of 51 *K. pneumoniae* isolated 16 (31.4%) in males and 35 (68.6%) in females screened for bacteria commonly reported in urinary tract infection most of the isolate were non ESBL producer 40 (78.4%) and ESBL producer were identified at a lower frequency reached 11(21.6%) . From (11) ESBL producers, 7 (63.6%) were isolated from patients older than 50 years and 4 (36.4%) from age groups15- 49 years old as shown in Table (6). Most of ESBL-producing *K. pneumonia* 7(63.6%) were isolated from inpatients who have history of healthcare contact .

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Table 6: Distribution of ESBL-producing and non-ESBL-producing *Klebsiella pneumonia* isolates

Characteristics		Total isolate N (%)	ESBLs-positive (n = 11)	ESBLs-negative (n = 40)
Age groups and sex				
Age	15–49	37 (72.5%)	4 (36.4%)	33 (82.5%)
	≥50	14 (27.5%)	7 (63.6%)	7 (17.5%)
Sex	Female	35 (68.6%)	8 (72.7%)	29 (72.5%)
	Male	16 (31.4%)	3 (27.3%)	11 (27.5%)
Distribution				
Out patients		40 (78.4%)	4 (36.4%)	27 (67.5%)
In patients		11(21.6%)	7 (63.6%)	13 (32.5%)

In the current study, the ESBL-producing phenotype were detected in 21.6% of the obtained isolates, which was higher than results obtained in Taiwan by other investigators [18 & 19] who reported that the ESBL producers from outpatients were 14.3% . In contrast, the percentage of ESBLs observed in our study were lower (23%) than previous observations reported in Southwest Ethiopia [20], also from Saudi Arabia which was (42.38%) [21] moreover, the percentage was higher in Tanzania (45.2%) [22]. The hospital environment has an important role in maintenance of ESBL-producing organism [23]. Moreover, higher rate of ESBLs producers were also observed in inpatients in Saudi Arabia hospital [24] .

polymerase chain reaction assay confirmed that out of the six isolate, 3 (50%) %) harbored *AmpC* and bla_{CTX-M} genes as shown in the Figure (1) and Figure(2). The remainder were negative for *AmpC* and bla_{CTX-M} genes and resistance to many antibiotics this might be due to another gene belonging to the ESBL group but located on plasmids which is not investigated in our study. These finding was in agreement with other resechers who found that the chromosomally encoded AmpC β-lactamases are rare(25). A study on the ability of *Klebsiellae* isolates to *AmpC* production were revealed that 9 (30%) isolates produce AmpC β-lactamase. While confirmed PCR amplification results have shown that only six *Klebsiellae* isolates possess AmpC β- lactamase gene. [26]. PCR remains the gold standard for detection of ESBL genes.

DOI:https://doi.org/10.26750/Vol(7).No(2).paper13 http://journal.uor.edu.krd/index.php/JUR Vol.7. No.2, April.2020

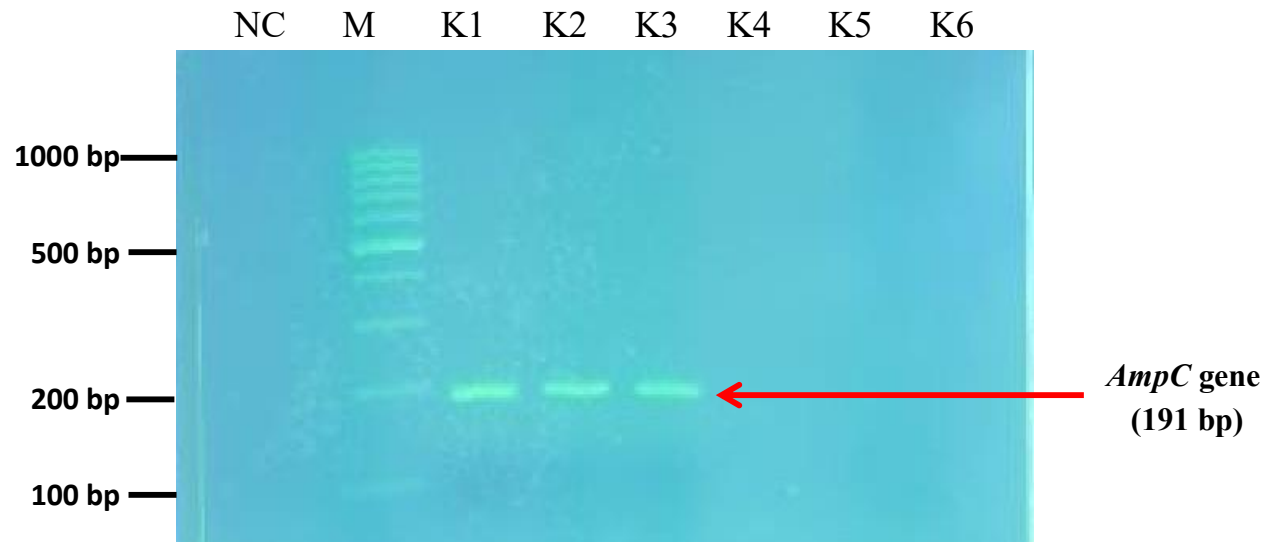

Figure (1) :The band site at the extent of *AmpC* gene in 191bp for *Klebsiella pneumonia* isolates (K1-6). Lane NC: Negative control , Lane M :Ladder (100bp) , Lanes 1,2 and 3 Positive samples , Lanes 4 , 5 and 6 Negative samples

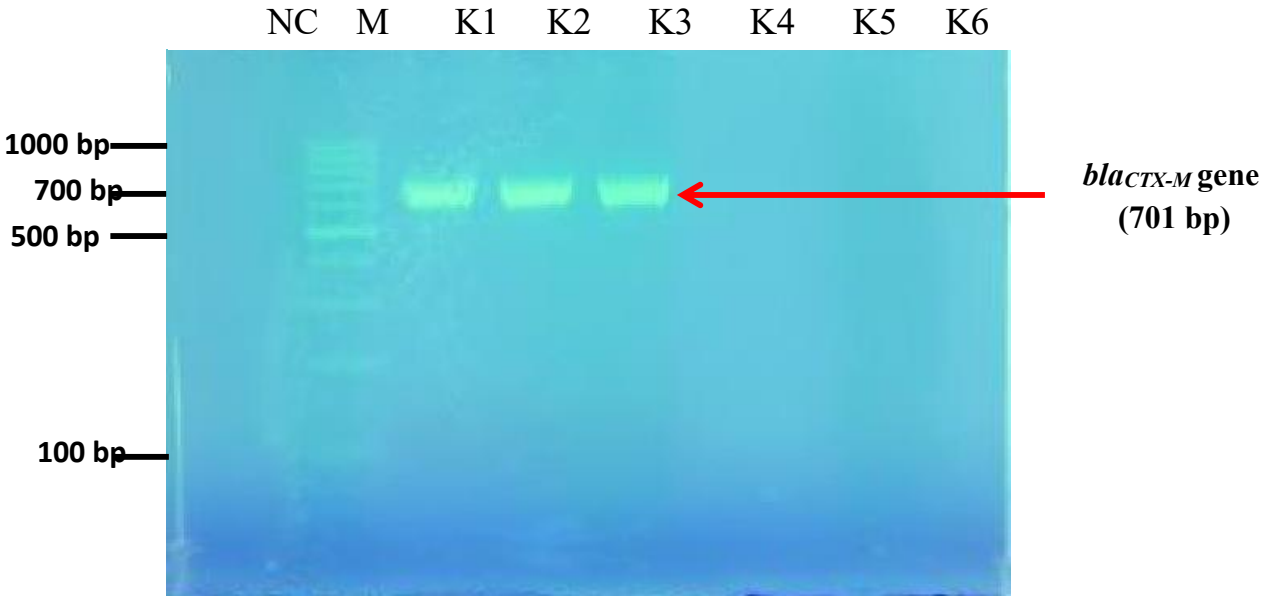

Figure (2) :The band site at the extent of *bla*$_{CTX-M}$ gene in 701bp for *Klebsiella pneumonia* isolates (K1-6). Lane NC: Negative control , Lane M :Ladder (100bp) , Lanes 1,2 and 3 Positive samples , Lanes 4 , 5 and 6 Negative samples

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Incubation of ten isolates of each non ESBL producer and ESBL producer *K. pneumonia* in series concentrations of AgNps size20nm for 48 hours showed that the bacteriostatic effect was between (625 -2500) µg/ml and for AgNP size 90nm was (1250) µg/ml for non ESBL producer while bacterial growth was recorded for ESBL producer isolates as shown in Table (7).

Table (7) The Minimum Inhibitory concentration (MIC) of Silver nanoparticles against *K.pneumonia* .

AgNPs Size	MIC (µg/ml) of isolates
	Non ESBL producer - ESBL producer
AgNPs size 20nm	625 - 2500
AgNPs size 90nm	1250 - +

+ = Bacterial growth

The bactericidal mechanisms of AgNPs are not fully understood [27], although, the antimicrobial activity of AgNps is mainly based on different mechanisms including release of Ag ions which bind to electron donor groups in molecules containing sulphur, oxygen or nitrogen; disruption of DNA replication and oxidative stress through the catalysis of reactive oxygen species (ROS) formation [28& 29] . Silver ions or small AgNPs can easily enter the microbial body causing the damage of its intracellular structures. As a consequence ribosomes may be denatured with inhibition of protein synthesis, as well as translation and transcription can be blocked by the binding with the genetic material of the bacterial cell [30].

DOI:https://doi.org/10.26750/Vol(7).No(2).paper13 http://journal.uor.edu.krd/index.php/JUR Vol.7. No.2, April.2020

هەڵسەنگاندنی چالاکی دژە بەکتریایی تەنۆچکەکانی زیو بە ڕامبەر Extended-Spectrum Beta-Lactamase Producing *Klebsiella pneumonia* جیاکراوە لە میز

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پوختە:

ئامانج لەم توێژینەوەیە، دیارکردنی جیاکراوەکانی Klebsiella pneumonia دەردەری ESBL لەمیزدا ,بەڕێگای ڕووکەش و گەردیلەیی، وە کاریگەری تەنۆچکەکانی زیوی قەبارە جیاواز لە سەر ئەم جیاکراوانە .

ریگاکانی ئیش :تیستی Vitek ESBL, تیستی جیاکراوەکان بە ریگای گەردیلەی بۆ دلنیابوون کە جیاکراوەکان ESBL دەردەدەن لە ڕێگای دیارکردنی هەندێک لە چینەکانی ESBL وەکAMPc و blaCTX بە PCR,ئینجا بەکارهینانی تەنۆچکەکانی زیوی قەبارە جیاواز وەک دژە بەکتریا .

ئەنجامەکان: : لە کۆی51 جیاکراوەی Klebsiella pneumonia 11 (21.6 ٪) جیاکراوە دەردەری ESBL ,بەرگریان هەبوو بە ریژەی (100٪)11 Cefazolin، Ceftazidime، Ceftriaxoneو Ampicillin وە 8 (72.7 ٪) لە جیاکراوەکان هەستیار بوون بۆ Imipene

وە تێبینی باندەکانی چینی AMPc و -blaCTX کرا لە هەندیک لە جیاکراوەکان .

لە کاتی دانانی جیاکراوەکان لە ناو خەستی دوای یەکی تەنۆچکەکەکانی زیوی قەبارە 20 نانومیتر کەمترین خەستی ڕاگری گەشەی بەکتریا MIC لە نیوان (625 -2500) میکروغرام / مل بەلام بۆ قەبارە 90nm لە نیوان (1250 -5000) میکروغرام / مل . زیاد بوون و بلاوبونەوەی Klebsiella - beta-lactamase ئاگادارکردنەوەیەکە جونکە هەلبژاردە جارەسەرەریەکان سنوردارببووە بۆ نەخۆشەکانی هەوکردنی کۆئەندامی میز ،وە دەتوانریت لە ئیستادا دژە تەنی Imipenem وەک جارەسەری سترەکی بەکاربیت بۆ جارەسەری هەوکردنی کۆئەندامی میز. قەبارە جیاوازەکانی تەنۆچکەی زیو ڕاگری گەشەی جیاکراوەکانی دەردەری ESBLبوو .

ڕاسباردەکان : ڕاسباردەی توێژینەوەی داهاتوویی دەکەین لەسەر جۆرەکان و قەبارە جیاوازەکان تەنۆچکەکانی تر بکریت لە سەر جیاکراوەی بەکتریای جیاواز لە سەرچاوەی جیاواز .

کلیلە وشەکان : تەنی نانۆیی زیو , *Klebsiella pneumonia* , ESBL ,هەوکردنی میز .

DOI:https://doi.org/10.26750/Vol(7).No(2).paper13 http://journal.uor.edu.krd/index.php/JUR Vol.7. No.2, April.2020

تقييم النشاط المثبط البكتيري للجسيمات النانوية الفضية تجاه Extended-Spectrum Beta-Lactamase Producing *Klebsiella pneumonia* المعزولة من الإدرار

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الخلاصة :

الهدف من هذه الدراسة :هو الكشف عن عزلات *Klebsiella pneumonia* المنتجة لESBL والمعزولة من مرضى التهاب المسالك البولية، مظهريا و جزيئيا ، وتاثير دقائق الفضة مختلفة الأحجام على هذه العزلات .

طرائق العمل: احتوت فحص Vitek ESBL , **فحص العزلات جزيئيا للتاكد من إنتاجها لـ** ESBL **من خلال التحري عن جين** AMPc **و** blaCTX **بواسطة تفاعل البوليميز المتسلسل. ثم استخدام دقائق الفضة بأحجام مختلفة كمضاد بكتيري بطريقة** microtiter plates.

النتائج : من مجموع51 عزلة *Klebsiella pneumonia* 11 (21.6 ٪) **عزلة كانت منتجة لـ** ESBL**، مقاومة** (100٪)11 **إلى** Cefazolin، Ceftazidime، Ceftriaxone **و** Ampicillin **بينما كانت** 11/8 (72.7 ٪) **من العزلات حساسة لـ** Imipenem **. لوحظت حزم جين** AMPc **و** blaCTX **في بعض العزلات.**

عند حضن العزلات بتراكيز متسلسلة من AgNPs**حجم** 20 **نانوميتر، كان تركيز الأدنى المثبط** MIC **بين** (625 -2500) **ميكروغرام / مل ، بينما بلغ عند حجم** 90nm **بين**

(1250 -5000) **ميكروغرام/مل. إن الانتشار المتزايد لـ** Klebsiella -beta-lactamase **يعد إنذاراً؛ لأن هناك خيارات علاجية محدودة لمرضى المسالك البولية و**

التوصيات: نوصي بدراسات مستقبلية على أنواع وأحجام الجسيمات النانوية الأخرى وتاثيرها على عزلات بكتيرية مختلفة النوع والمصدر .

الكلمات المفتاحية: دقائق الفضة النانوية, *Klebsiella pneumonia*, ESBL, التهاب المجاري البولية.

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