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Detection of Extended Spectrum Beta-Lactamase (ESβL) and Klebocin Production from *Klebsiella pneumoniae* Local Isolates from Urinary Tract Infections

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الخلاصـة:

جمعت (10) عزلات من بكتريا *Klebsiella. pneumoniae* من عينات ادرار من مرضى يعانون من التهابات السبيل البولي من المختبرات التعليمية / مدينة الطب. اعيد تشخيصها اعتمادا على الاختبارات الكيموحيوية و Api 20 E system .

أظهرت نتائج اختبار حساسية العزلات تجاه 23 مضادا حيويا وامتلاك جميع العزلات لنمط المقاومة المتعددة للمضادات الحيوية تراوحت مابين (7−19) مضادا وتضمنت مقاومة 100% لمضادات البنسلين و الامبسلين والاموكسيسلين والتتراسيكلين والسيفالكسين والجنتامايسين والاميكاسين، فضلاً عن امتلاك العزلات مقاومة عالية لمضادات الجيل الثالث من السيفالوسبورينات شملت(60)% مقاومة لمضاد السيفتازديم و (80)% مقاومة لمضادي السيفوتاكسيم و السفتراكسون، في حين اظهرت أغلب العزلات حساسيتها لمضادي Imipenem و Azteronem . وكانت 50% (5عزلات) من هذه العزلات منتجة لانزيمات البيتالاكتاميز بطريقتي اليود القياسية السريعة والانابيب الشعرية. كما اختبرت قابلية العزلات المنتجة لانزيمات البيتالاكتاميز على إنتاج إنزيمات البيتالاكتاميز واسعة الطيف، وقد عد الاختبار التاكيدي من قبل NCCLSمن الطرائق الجيدة المستخدمة في هذه الدراسة للتحري عن إنزيمات البيتالاكتاميزواسعة الطيف. أظهرت نتائج تحديد التركيز المثبط الأدنى لعدد من مضادات البيتالاكتام للعزلات المنتجة لإنزيمات البيتالاكتاميز واسعة الطيف أن جميع العزلات المنتجة كانت ذات مقاومة عالية لمضادي الامبيسيلين والاموكسيسلين, كما إنها كانت مقاومة لمضادات السيفتازديم و السيفوتاكسيم و السفتراكسون اذ تراوح التركيز المثبط الادنى مابين (32−>128) و (32−>256) و (32−>128) مايكروغرام/ مل على التوالي.

أظهرت نتائج الكشف عن قابلية عزلات*Klebsiella* على إنتاج الكليبوسين أن 20 % من العزلات كانت منتجة للكليبوسين تجاه بعض الانواع من بكتريا الاختبار الموجبة والسالبة لصبغة كرام .

تمت دراسة النسق البلازميدي للعزلة المنتخبة 6*K. pneumoniae* ، وأظهرت نتائج الترحيل الكهربائي في هلام الاكاروز امتلاك العزلة حزمتين بلازميدية مختلفة في الموقع والحجم .

اظهرت نتائج تحييد البلازميدات عدم نجاح المحاولات باستخدام العامل الكيمياوي المايتوسين – C للعزلة المنتخبة 6 *K.pneumoniase* المنتجة للكليبوسين وانزيمات البيتالاكتاميز الواسعة الطيف عند استخدام تراكيز تراوحت (10–100)) مايكروغرام/ مل . وقد أشارت نتائج الاقتران إلى أن المورثات المسؤولة عن انتاج انزيمات البيتالاكتاميزو المقاومة لمضادات الامبيسيلين, الاموكسيسلين, السيفتازديم ,السفتراكسون و السيفوتاكسيم هي بلازميدية الموقع.

Abstract:

Ten isolates of *Kebsiella pneumoniae* were isolated from urine samples collected from patient suffering from UTI obtained from Education Labs / medical city, and Re identification according to the biochemical tests and confirmatory by Api 20 E test. Sensitivity of isolates was tested against 23 Antibiotics, results revealed that isolates showed multi resistance to antibiotics ranging between (7-19) antibiotics, and all isolates of *K.pneumoniae* were resistant 100% to Ampicillin, Cephalexin, Amoxicillin, ,Penicillin ,Tetracyclin, Gentamycin and Amikacin, in addition the isolates showed high resistance to third generation of cephalosporines included (60)% resistance to ceftazidime and (80)% resistance to cefotaxime, and ceftriaxone . Imipenem and azteronem was found to be the most effective agents against the isolates.

Detection of Beta–Lactamase by using rapid standard Iodometric assay and capillary tubes method, showed that 50% (5 isolates) of *K.pneumoniae* were B-lactamase producing

All β- lactamase- producing *K.pneumoniae* isolates were also tested for their ability to produce Extended-Spectrum Beta-lactamases (ESBLs), screening and confirmatory tests recommended by NCCLS it considered the most accurate method for detection of ESBL- producing isolates.

Determination the MICs of ESBL-producer isolates against some β-lactam antibiotics showed that five ESBL-producing *K.pneumoniae* isolates was highly resistant for both ampicillin and amoxicillin. They was also resistant to ceftazidime, cefotaxime, and ceftriaxone high MIC results from (32to >128) (32to>256) (32to>128) μg/ml respectively.

Results of detection of klebocin production by *klebsiella* isolates showed that 20 % of them were klebocin-producers against some indicator isolates of G- and G+ bacteria.

Patterns of plasmid was studies of selected isolate *K. pneumoniae* 6, showed its harbor two plasmid bands different in size and position.

Attempts to cure plasmids was unsuccessful from the selected ESBLs and klebsin producer isolate *(K.pneumoniase* 6), in concentration ranged from (10-100) μg/ml when we used mitomycin – C.

Results of conjugation experiment revealed that genes encoding for the production of BLs, and resistance to penicillin, ampicillin, amoxicillin, cefotaxime, ceftraxione and ceftazidime, were located on plasmids.

Introduction:

The Beta-lactam family of antibiotics includes many of the most heavily used antibacterials in clinical medicine. They are important, both historically and currently, because of their effectiveness and generally low toxicity. Resistance to ß-lactam antibiotics in gram-negative bacilli is mainly mediated by the production of ß-lactamases, enzymes which are divided into four major molecular classes A,B,C,and D [1] , that break down the structural β-lactam ring of penicillin -like drugs considered as a predominant mechanism of bacterial resistance to β-lactam antibiotics especially in bacteria (gram negative bacilli) causing clinically significant infections of which there are several classes including plasmid-encoded and chromosomally encoded enzymes [2]. In the current era of increasing use of broad – spectrum antimicrobial agents, the incidence of extended spectrum beta–lactamase (ESBLs)-producing Enterobacteriaceae has increased at an alarming rate [3]. Genes for AmpC (class C) ß-lactamases are generally encoded on the chromosomes in many gram-negative microbes.Chromosomal AmpC enzymes are usually inducible and are often responsible for resistance to cephalosporins as well as to penicillins. Plasmid-mediated class C ß-lactamases have mainly been

described in *Klebsiella spp.*, *Escherichia coli*, and *Salmonella spp.* throughout the world. Plasmid-mediated class C enzymes are currently divided into at least five clusters on the basis of amino acid sequence similarities, together with their putative progenitor chromosomal AmpC enzymes. In Japan, MOX-1 , CMY-8 ,CMY-9 , CMY-2, CFE-1 , and DHA-1 have so far been found as plasmid-mediated AmpC ß-lactamases, mainly in nosocomial isolates of the family Enterobacteriaceae [4], attention has been paid to ESBL production in *Klebsiella* spp. , hence, the present study was carried out to achieve the following aims, the prevalence of β- lactam resistance ,detection of β – Lactamase and ESBL among these 10 clinical local *Klebsiella* isolates were study, as well as their genetic relatedness were elucidated.

Materials and methods:

Bacterial Isolates:

Ten isolates *of Klebsiella pneumonia* were isolated from patient suffered from urinary tract infections, the urine samples were collected in 20.8 from Education Labs / medical city.

Culture Media:

Culture media used in this study included : 1-Brain Heart Infusion agar , 2-Brain Heart Infusion broth , 3-Mueller – Hinton agar, 4- MacConkey agar ,5-Blood Base agar ,6-Mannitol Salt agar, , 7-Nutrient agar, were prepared according to the instructions of the company (Hi-Media-India .XLD) (Oxoid / England), (Difco/USA).

Antibiotic Sensitivity Test:

Twenty-three various available and commonly used antibiotics discs were used in the antibiotic sensitivity test. The susceptibility of ten isolates to different antimicrobials was determined by Kirby-Bauer disk diffusion method on Mueller Hinton media [5]. The sensitivity and resistant were determined by measuring the diameter of inhibition zones around the antibiotic disc. The table below shows the antibiotics disc (BioAnalyse \Turkey) were used in this study.

Antibiotic Disk	Symbol	Disk Content µg / disc	Antibiotic Disk	Symbol	Disk Content µg / disc
Ampicillin	AM	10	Cefepime	FEP	30
Amoxicillin	AX	25	Penicillin	P	10U
Amoxicillin +clavulanic acid	AMC	20/10	Amikacin	AK	30
Piperacillin	PRL	100	Gentamicin	CN	10
Carbenicillin	py	100	Rifampin	RA	5
Cloxacillin	CX	1	Ciprofloxacin	CIP	5
Cephalothin	CL	30	Trimethoprim	TMP	5
Cefotaxime	CTX	30	Chloramphenicol	C	30
Ceftazidime	CAZ	30	Aztreonam	ATM	30
Imipenem	IMP	10	Cefruxime	CFM	30
Ofloxacin	Ofx	30	Tetracycline	TE	30
Tobramycin	TOB	30			

Detection of β- lactamase production

Rapid iodometric method and capillary tubes methods were used for detection of β- lactamase production in β- lactam resistant *Klebsiella pneumoniae* isolates. Both methods depends on detection of penicilloic or cephalosporic acid, resulted from breakdown of amide bond in β-lactam ring for each of penicillins or cephalosporins [7].

A- Rapid iodometric method:

This test was performed for all bacterial isolates that were resistant to β- lactam antibiotics, and it was done according to method described by [8].

B- Capillary tube method:

This test was done to all isolates according to method described by [9].

Detection of Extended Spectrum β- lactamase (ESBL) production:

Three methods were performed for detection of ESBLs in clinical isolates. All bacterial isolates that were positive to β- lactamase production were tested for ESBLs. These tests included:

A- Disk approximation method: This method was carried out as modified by[10].

B- National Committee for Clinical Laboratory Standards (NCCLS)

Screening method : This method described by [11], which included two main procedures**:**

Screening test for ESBLs production:

Antibiotic susceptibility tests were performed using a disk diffusion method on BHIA with antibiotic disks (Ceftazidime, Cefotaxime, and Ceftriaxone). Each bacterial isolate should be considered a potential ESBL- producer, if the test results were as follows: Ceftazidime < 22 mm, Cefotaxime < 27 mm, Ceftriaxone < 25 mm

The phenotypic confirmation of potential ESBL- producing isolates:

This method was performed as follows:

- Using disk diffusion method, each ceftazidime, Ceftriaxone and cefotaxime alone and in combination with clavulanic acid were tested.
- Ceftazidime- clavulanic acid (30 μg/ 10μg), Ceftriaxone - clavulanic acid (30 μg/ 10 μg), and cefotaxime- clavulanic acid (30 μg/ 10 μg) disks prepared. Inhibition zone of ≥ 5 mm increase in diameter for either antibiotic tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL - producing isolate.

Determination of Minimal:

Inhibitory Concentration:

The two- fold agar dilution susceptibility method was used for determination of MICs of a number of β- lactam antibiotics Of ESBL-Producing Isolates according to method described by [12].

Detection of Bacteriocin Production:

Well Diffusion Method was used for detection of bacteriocin (klebocin) production from *K.pneumoniae* isolates against some indicator bacterial isolates, and this method was described by Gupta *et al*., [13].

Total DNA isolation (salting out method):

The method was used in this study described by Pospiech and Neumann [13] to isolate both plasmid and chromosomal DNA.

Agarose Gel Electrophoresis : The method described by [14].

Plasmid Curing Experiment:

In our study we are used Mitomycin C at final concentration as follow: (5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100) µg/ml, and the methods was described by[15].

Bacterial conjugate:

K.pneumoniae 6 isolate which resist to Ceftazidime-and Amikacin were selected (as donor cells) for studying bacterial conjugation with *E. coli* which resist to ciprofloxacin (as recipient cell).

Mating experiments were performed by using filter method as described in[16].

Selectivemedia were prepared for transconjugants selection:

- Brain heart infusion agar supplemented with 30 µg / ml Ceftazidime and Amikacin.
- Brain heart infusion agar supplemented with 5 µg / ml ciprofloxacin

Brain heart infusion agar supplemented with ml Ceftazidime, Amikacin .and ciprofloxacin (Oxoid / England), (Difco/USA).

Results and Discussion:

Bacterial Isolates:

Ten *K. pneumonia* isolates were identified according to morphology, characteristics with some biochemical tests (Table-1). Phenotypic identification of each isolates was performed by using a commercial identification system (Api 20 system) according to the instructions of the manufactures.

Test	*K. pneumoniae*	Test	*K. pneumoniae*
Growth on MacConkey agar	Growth (pink mucoid)	Acid from : Glucose	+
Growth on Blood agar	Growth creamy	Lactose	+
Hemolysis	Non hemolysis	*Sucrose*	+
Catalase	_	Salicin	+
Oxidase	+	D- Sorbitol	+
H2S production	+	D- xylose	+
IMViC tests	- -++	Mannitol	+

Table -1: Morphological and biochemical tests of *Klebsiella pneumoniae*

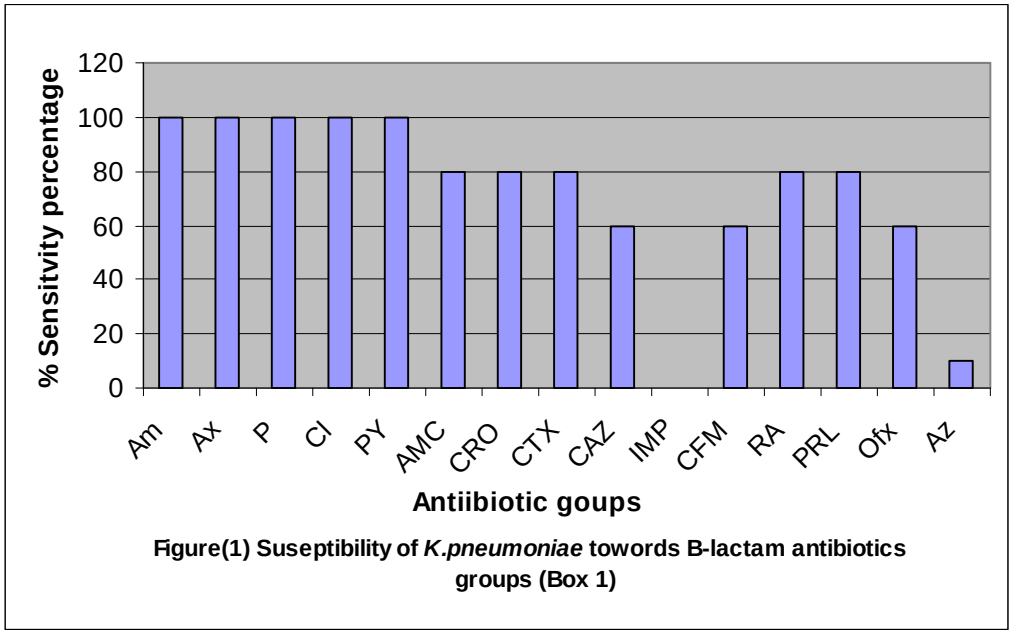
Detection of Antibiotic Resistance:

All *Klebsiella* isolates were tested for their sensitivity toward twenty three antibiotic discs using disk diffusion method. All isolates were found to be resistant to at least 7 antibiotics tested. Hence all the isolates were considered to be multidrug resistant.

These isolates were 100 % resistant to some antibiotics related to Beta-lactam group (box 1) such as penicillin , ampicillin, cephalexin, carbenicillin and amoxicillin .It was found that major mechanism of resistance in gram- negative bacteria causing clinically significant infection is the expression of β-lactamases, of which there are several classes including plasmid-encoded and chromosomally encoded enzymes [17].

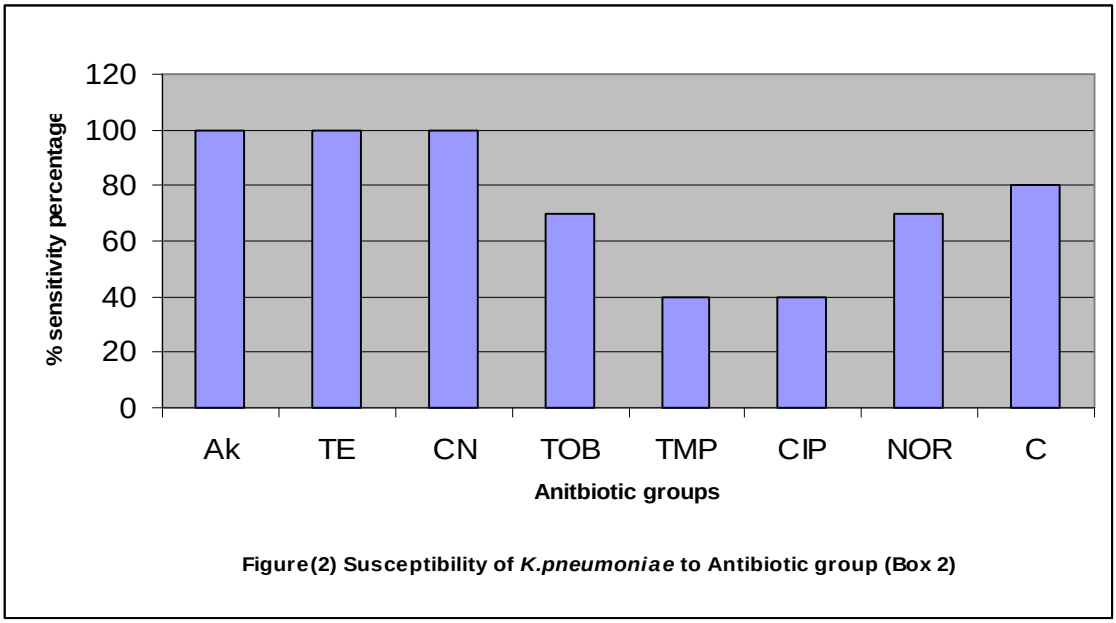
It was also clear from figure-1 that 80 % of *K.pneumoniae* isolates were resistant to piperacillin.

In the present study For first generation cephalosporins, although *K.pneumoniae* isolates were highly resistant to cephalexin (100 %), one of the most striking findingswas that the high level resistance to third generation cephalosporins (3GC) among *K.pneumoniae* isolates which were resistant to cefotaxime and ceftriaxone in a percentage of (80)% but showed low level of resistance to ceftazidime (60 %).Ceftazidime resistance markers for the presence of extended spectrum β-lactamases (ESBLs). Since all the isolates showed multidrug resistance, the therapeutic strategies to control infections due to *K. pneumoniae* isolates has to be carefully formulated, and since most of the isolates were sensitive to imipenem and azteronam, they might serve as the drug of choice for the treatment of infections caused by *Klebsiella*.



Figure(1) Suseptibility of *K.pneumoniae* towords B-lactam antibiotics groups (Box 1)

Results of figure (2) showed that *K. pneumoniae* isolates were highly resistant to antibiotic groups (box 2) such as gentamycin, tetracyclin and amikacin (100%) with moderately resistance (40 %) to Trimethoprim and ciprofloxacin to highly resistance to chloramphenicol, tobramycin and norfloxacin (80, 70,70) % *respectively* .



Figure(2) Susceptibility of *K.pneumoniae* to Antibiotic group (Box 2)

Conjugal transfer of genes and genetic elements between different strains of *Klebsiella* and between environmental and clinical strains in both directions under natural conditions is a probable issue**,** [18]. ESBLs are usually plasmid mediated, since these plasmids are easily transmitted among different members of the Enterobacteriaceae, accumulation of resistance genes results in strains that contain multiresistant plasmids [19]. Prevalence of ESBL enzymes has been increased in many parts of the world. Infections caused by ESBL producing isolates are difficult to treat, because they confer resistance to all currently available β-lactam agents, except imipenem, and in some cases piperacillin–tazobactam [20]. In addition, ESBL production is usually associated with resistance to other classes of antimicrobial agent, such as aminoglycosides and fluoroquinolones [21].

Detection of β- Lactamase Production:

Results of (Table-2) shows that 5 isolates of *K.pneumoniae* (50 %) gave positive results with rapid iodometric method anD 4 isolates (40 %) showed positive reaction with acidometric method.

According to high ratio of resistance (100 %) to more than antibiotics that related to beta – lactam group this indicated that these β-lactamase-producing isolates have an enzymatic mechanism of resistance represented by the productionof β-lactamases.

Method assay	No. of β-lactamase Producers (%)	No. of non-producers %
iodometric assay	5(50)	5 (50)
capillary tubes method	4(40)	6(60)

Table-2: β-lactamase producing and non-producing *K.pneumoniae* isolates

Many studies reported that carrier rates of gram-negative bacteria increases during staying in hospital, and of persons outside hospitals especially in patients receiving antibiotic treatment [22].The transfer of bacteria from the hospital environment would appear to be the most likely source involved [23].The interpretation of these results depends on the concentration of β-lactamase enzyme in the periplasmic space and based on concentration of the released penicillinase or cephalosporinase enzyme. Factors such as temperature and pH also play an important role in enhancement or reduction of enzyme activity

Detection of Extended-Spectrum β- Lactamases :

Disk Approximation Method:

In this test, results were determined depending on enhancement of the inhibition zone between a beta- lactam disks (CTX, CAZ, and CRO) and augmentin disk (amoxicillin-clavulanate – 20/10 µg/m), as indication for the presence of an ESBL. On the other hand another side of this test was determined depending on enhancement of the inhibition zone between a beta- lactam disks (CTX, CAZ, COR, AZT and CL) and augmentin disk (amoxicillin-clavulanate – 20/10 µg/m), From five β- lactamase producing isolates, only 4 (40%) isolates of K.. *pneumoniae* were ESBL–producers. These were; *K. pneumoniae* 1*;K.. pneumoniae* 3*; K.. pneumoniae 5;* and *K.. pneumoniae* 6.

Results of the present study showed that the prevalence rate of ESBL-mediated resistance to third-generation cephalosporins (3GC) was 80 % to *K.pneumoniae*. Livermore and Yuan [24] mentioned that ESBLs occured in about 20 to 25 % of *klebsiella* isolated from patients in the intensive care units in Europe, although they found that up to 30 to 40 % of those were from France alone. The

low prevalence rate, when they compared with other studies, can also be attributed to the fact that presence of ESBLs in a bacterial cell does not always produce a resistance phenotype when using the disk diffusion interpretive criteria published by the NCCLS (11)

Screening and confirmatory tests recommended by NCCLS :

Results in (Table-3) show that 4 isolates (80%) of *K.pneumoniae* were ESBL - producing isolates which were detected by this method. These isolates were found to produce ESBLs, and they are considered as non- susceptible to all penicillins, cephalosporins (including expanded-spectrum cephalosporins), regardless of the susceptibility test (11). Although National Committee for Clinical Laboratory Standards recommendations exist for detecting ESBL-producing isolates of *Escherichia coli* and *Klebsiella* spp., no recommendations existed for detecting ESBLs in other organisms or for detecting plasmid-mediated AmpC beta-lactamases in any organisms .

No. of β-lactamase Producers isolates	Inhibition zone (mm)					
	Cefotaxime (≤ 27)	Cefotaxime + clavulanic acid	Ceftazidime (≤22)	Ceftazidime + clavulanic acid	Ceftriaxone (≤ 25)	Ceftriaxone + clavulanic acid
K.pneumoniae 1	10	20	R	30	10	20
K.pneumoniae 2	16	22	13	30	R	20
K.pneumoniae 4	15	25	R	25	12	17
K.pneumoniae 5	21	22	18	20	25	25
K.pneumoniae 6	R	20	R	25	R	20

Table-3: Initial screening test for ESBL–producing *Klebsiella* Isolates.

MICs Determination of ESBL –producing Isolates:

Two-fold agar dilution susceptibility method was used to determine the MICs of ESBL-producer isolates according to results in fig (1) against five β-

lactam antibiotics :Ampicillin, Amoxicillin, Cefotaxime, Ceftazidime, and Ceftriaxone.

The MIC values were based on break point recommended by NCCLS (11), for estimation of the response. Results in (Table -4) show that all the four ESBL-producing *Klebsiella* isolates were highly resistant for both ampicillin and amoxicillin with concentrations reached >2048 , and the lowest MIC for the isolates was for Cefotaxime and Ceftriaxone as it ranges (32 - 128) μg/ml **.**

On the other hand the MIC values of cefotaxime and Ceftriaxone for 4 isolates of *K. pneumoniae* was 32 μg/ml, which was less than the break point value (≥ 64 μg/ml).Only two isolate of *K. pneumoniae* were able to grow in a concentration equal to break point to the Ceftazidime.

Results from a local study showed that gram-negative enteric rods were 100 % resistant to ampicillin and amoxicillin [25]. Tullus *et al*., [26] showed that there was an increasing incidence of ampicillin- resistant strains of *E. coli* and *Klebsiella* isolated from stool samples of neonates, they also found that the MIC value for *Klebsiella* reached >1024 μg/ml, and that was attributed to the frequently and commonly use of this antibiotic. It was also shown by other investigators that TEM or SHV enzymes of gram-negative bacteria confers resistance to ampicillin, amoxicillin, ticarcillin, and carbenicillin, with MICs exceeding 256 μg/ml compared to (1 – 4) μg/ml for *E. coli* non-producer of enzyme isolates(7). Resistance of gram-negative enteric rods to first generation cephalosporins, was due to the plasmid-mediated β-lactamases TEM-1, TEM-2, and SHV-1. [27]. High MIC values of *Klebsiella* isolates in the present study suggests that ESBL enzymes is endemic in study area.

Isolate	MIC (µg/ml) of :				
	AMP (≥32µg/ml)	Amx (≥32µg/ml)	CTX (≥64µg/ml)	CAZ (≥32µg/ml)	CRO (≥64µg/ml)
K.pneumoniae **1**	128	128	> 128	> 256	> 128
K. pneumoniae **2**	128	128	32	32	32
K. pneumoniae **4**	64	64	32	32	32
K. pneumoniae **6**	> 2048	>2048	>128	64	>128

Table-4: **MICs of number of β- lactam antibiotics for ESBL-producing *Klebsiella isolates***

***Numbers between backets refer to break points recommended by NCCLS (2003b).**

AMP, Ampicillin; Amx, Amoxicillin; CTX, Cefotaxime; CAZ, Ceftazidime; CR0, Ceftriaxone

Klebocin- producing isolates:

Results of (**Table- 5**) show that 2 isolates (20 %) from 10 *Klebsiella* isolates, were klebocin- producers. Some Gram-negative rods were affected by klebocins of *Klebsiella* isolates**.** *Pseudomonas aeruginosa* isolate was very sensitive to klebocin of *K pneumoniae* 6 which gives an inhibition zone of (18 mm). Two Klebocin-producing isolates **(Table 4)** had bacteriocinic effects on *E.coli ATCC but do not effectE.coli type.* While *Acinetobater baumonnii* was very sensitive to klebocin of *K. pneumoniae* 6 giving a large inhibition zone (22 mm). Results of (**Table -5**) also showed that all Gram- positive bacteria were resistant to the tested klebocins,

Test organism	1	2	3	4	5	6	7	8	9	10
E. coli type 1	–	–	–	–	–	–	–	–	–	–
E. coli ATCC 25923	–	–	–	+	–	+	–	–	–	–
Pseudomonas aeruginosa	–	–	–	–	–	++	–	–	–	–
Acinetobacter baumannii	–	–	–	–	–	+++	–	–	–	–
Staphylococcus aureus	–	–	–	–	–	–	–	–	–	–
Enterococcus faecalis S10	–	–	–	–	–	–	–	–	–	–
Proteus mirabilis	–	–	–	–	–	–	–	–	–	–
Streptococcus pneumonia	–	–	–	–	–	–	–	–	–	–

Table-5: Activity of klebocins of *Klebsiella pneumoniae* isolates on some pathogenic bacterial species.

[b] +, sensitive to klebosin(+(10-15)mm, ++(16-20)mm, and +++(21-25)mm , resistant to klebosin.

In the study reported by Opal *et al.*,[28] obtained the bacteriocin activity of *E. coli* strains, isolated from urine of patients suffering of UTI and from stool of healthy individuals ,found that no significant difference in ability of these strains to produce bacteriocin, between those isolated from urine or stool samples, which indicates that the bacteriocin is not a virulence factor but it aids them in their competition.

Isolation of Plasmid DNAL:

The results of Plasmid profile of ESBL-producing isolate as in figure (3) (lane 1 and 2) showed that the selected isolate of *K. pneumoniae6* harbors two plasmid bands, This isolate was also β-lactamase, kleboocin producers and resistant to most antibiotics that were used in this study especially the β-lactam antibiotics related to 3GC, such as ceftazidime, ceftraxone and cefotaxime which are markers for the presence of extended-spectrum β-lactamases. They were also rapid β-lactamase producers within few seconds. The production of β-lactamase as well as resistance to β-lactam antibiotics may indicate that these properties may be carried on plasmids that were detected in this isolate. Studies worldwide showed that *K. pneumoniae* isolates harbor more than one plasmid encoded resistance to large

number of antibiotics and most of these markers were associated with conjugative plasmid s which encode SHV- or TEM- type β-lactamases [29].

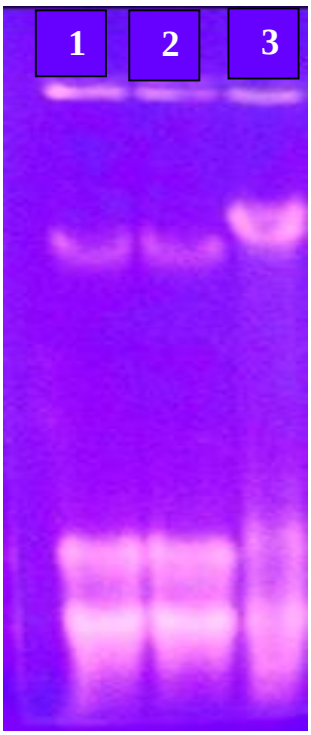


Figure-3: Agarose gel electrophoresis of plasmid DNA extracted from *K. pneumoniae* with agarose concentration (0.7%),voltage 5volt/cm, during 2hr
Lane 1 and 2: plasmid profile of isolate *K. pneumoniae* 6
Lane 3: plasmid profile of isolate *E.coli*

Plasmid curing:

Attempts were made to lose the plasmids from the selected B-Lactamase producer isolate of *K. pneumoniae6* by using mitomycin – c to detect the role of plasmids in production of ESB-lactamase , and resistance to most antibiotics that are used in this study . Attempts to cure plasmids were unsuccessful when Several concentrations of mitomycin – c were used ranged from (10 - 100) µg/ml , it was observed that the MIC for mitomycin –c was 90 µg/ml, however ; it was unable to obtain any cured single colony by using antibiotic selective media with defined concentrations and the antibiotics used were :ampicillin , amoxicillin , cefotaxime, ceftriaxone and ceftazidime . After curing the isolate the resistance remands to

these antibiotics. Mitomycin –c composes of hydroquinone compound that undergoes changes to produce an intermediate compound that interfere purine bases which leads to cross linkage between the double – stranded DNA and finally stops its transcription by RNA polymerase[15].

Bacterial Conjugatio

The bacterial conjugation was carried out in order to detect the role of plasmids in transferring of drug resistance as well as ESBL and klebocin producing isolates (*K.pneumoniae 6*) used which harbor more than one plasmid band, this isolate was considered as donor cells (resistant to Ceftazidime and Amikacin) and the isolate *E. coli*

Type1not producer of beta lactamase enzyme and susceptible to AMK, CAZ, CTX, AMX and CL, (but resistant to ciprofloxacin) as a recipient. Results of (Table-6) reveal that the conjugation between the *E. coli* and *K.pneumoniae* 6 isolate was successful. The conjugation frequency for this transconjugants was relatively high and ranged from 1.9 x $^{10-3}$ in isolate *E. coli*. The transconjugants expressed their antibiotic resistance when they were able to grow in selective medium containing Ceftazidime and Amikacin (at final concentration of 30µg/ml), ciprofloxacin (at final concentration of 5µg/ml). The acquisition of Ceftazidime and Amikacin resistance in recipient cell indicated that this property was plasmid-mediated.

Isolate Donor cells	**No. of** Recipient cells *(E. coli)*	**No. of** Transconjugants	**Conjugation frequency**
k.pneumoniae 6	**57×10^{-4}**	**300×10^{-6}**	**1.9×10^{-3}**

Table-6: Results of conjugation between ESBl- producing *k.pneumoniae 6* isolate with the *E. coli* isolate.

Figure (4) shows results of bacterial conjugation of isolate *K.pneumoniae 6* and *E coli* which revealed the plasmid bands were transferred from donor to recipient cells. Lane A represents the standard strain *E. coli,*. Lane B represents plasmid profile of ESBL-producing isolate *K.pneumoniae 6* which possesses two small plasmids. Plasmids were transferred to the recipient cell (lane) during

conjugation. The transconjugants(Lane C) in the present study, were able to produce ESβ-lacatamase enzyme when detected for their ability to produce it, and this prove the property is a plasmid-mediated. Transconjugants were also tested using antibiotic susceptibility test .Results showed that compared with the original donor cells. The resistance to these antibiotics (CTX, CAZ, ,AMX and AM), appeared in the transconjugants which refers to the presence of plasmid- encoded ESBLs in these isolates. The transconjugants resulted from conjugation between klebocin-producing isolates and the standard *E. coli* type 1, were detected and results showed that the transconjugants were not able to produce klebocin , and this may be due to the characteristics of klebocin production harbor on mega plasmid and in our study we couldn't extract mega plasmid, or may be harbored on a chromosome.

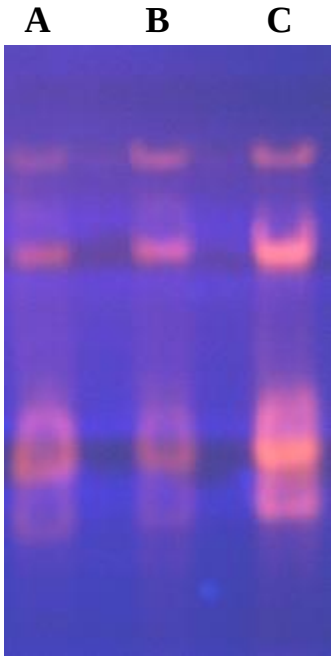


Figure (4) : Agarose gel electrophoresis of plasmid DNA from isolates of *K. pneumoniae6* and theirtransconjugants (in *E. coli type 1*) *pneumoniae* with agarose concentration (0.7%),voltage 5volt/cm,during 2hr

Lane A: DNA content of *K. pneumoniae6* isolate
Lane B : plasmid profile of *E. coli* isolate.
Lane C : plasmid profile of transconjugant resulting from conjugation between *K.pneumoniae 6* with *E. coli* isolate .

It can be concluded from the results above, that the expression of ESBL, production, were plasmid-encoded, and transferred to the recipient isolate, which received these properties, were carried on a non-conjugative plasmid that was transferred by mobilization, by the aid of first plasmid, to the recipient cell. It is also important to know, that the particular plasmid carrying particular property, can be detected by performing transformation process. Because of their transfer among bacterial genera as well as their facilitating transfer of non-conjugative plasmids, these plasmids are considered very dangerous, being able to confer resistance to β-lactam and very large numbers of other antibiotics. Gene transfer may occur across a very broad host range, such as between Gram-negative and Gram-positive bacteria [30,31].

References:

1- Bush, K., G. A. Jacoby,and A.A.Medeiros. (1995). A functional classification scheme for ß-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. 39:1211-1233.

2- Wachino , J.; Kurokawa,H.; Suzuki , S.; Yamana,K.;Shibata,N.; Kimura,K.; Ike,K. and Arakawa,Y. (2006). Horizental transfer of bla CMY- bearing plasmid among clinical E.coli and K.pneumoniae isolates and emergence of cefepime – hydrolyzing CMY-19.Antimicrobial agent and chemotherapy .50(2): 543-541.

3- Ku,Y.H.; Chuang,Y.C. and Yu,W.L.(2008). In vitro activity of tigecycline against clinical isolates of extended – spectrum beta – lactamase – producing Klebsiella pneumoniae ,Serratia marcescens and Enterobacter cloacae . J.Microbiol Immunol Infect.41: 332-336.

4- Jun,I.W.; Hiroshi, K.; Satowa ,S.; Kunikazu ,Y.; Naohiro ,S.; Kouji ,K.; Yasuyoshi ,I. and Yoshichika, A. (2005) . Horizontal Transfer of blaCMY-Bearing Plasmids among Clinical Escherichia coli and Klebsiella pneumoniae Isolates and Emergence of Cefepime-Hydrolyzing CMY-19 . Antimicrobial agent and chemotherapy .50(2).534-541.

5- Bauer,A.W.; Kirby,W.M.M.; Sherries,J.C. and Turk,M. (1966). Antibiotic Susceptibility Testing by a Standardized Single Disc Method. Am.J. Clin. Pathol, 45: 493 – 496..

6- National Committee for Clinical Laboratory Standards(NCCLS). (2003a). Performance standards for disk susceptibility tests, 8th ed. Approved standard M2-A8. National Committee for Clinical Laboratory Standards, Wayne, Pa.

7- Livermore, D. M. 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev., 8: 557–584.

8- WHO.(1978). Techniques for the detection of β- lactamase producing strains of Neisseria gonorrhoeae. 616 : 137- 143.

9- Sykes, R. B., and M. Matthew. (1976). The β-lactamases of Gram-negative bacteri and their role in resistance to β-lactam antibiotics. J. Antimicrob. Chemother. 2:115–157.

10- Coudron, P. E., E. S. Moland, and C. C. Sanders. (1997). Occurrence and detection of extended-spectrum β-lactamases in members of the family Enterobacteriaceae at a Veterans Medical Center: seek and you may find. J. Clin. Microbiol., 35: 2593–2597.

11- National Committee for Clinical Laboratory Standards(NCCLS). (2003b.) Performance standards for antimicrobial susceptibility testing. approved standard M 100-S13. National Committee for Clinical Laboratory Standards, Wayne, Pa.

12- National Committee for Clinical Laboratory Standards(NCCLS). (2003c). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, 6th ed. M7-A6.

13- Gupta, U. ; Radramma, A. ; Rati, E.R. and Joseph, R. (1998). Nutritional quality of lactic acid fermentel bitter international . J. Food. Sci. and Nutrition. 49(2) : 101–108.

14- Sambrook J, and Rusell DW. 2001. Molecular cloning. A laboratory manual. Third ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press, N.Y.

15- Trevorse, J.T. (1986) . Plasmid curing in bacteria . FEMS. Microbiol . Rev . 32 : 149– 157.

16- Shiono, A. and Ike,Y. (1999). Isolation of Enterococcus faecalis clinical isolates that efficiently adhere to human carcinoma T24 cells and inhibition of adhesion by fibrenoctin and types in treatment. Infect. Immun. 67(4): 1585–1592.

17- Livermore, D.M. 1998. β- Lactamase-mediated resistance and opportunities for its control. J. Antimicrob. Chemother., 41, Suppl. D, 25–41.
18- Al-Yasseen, A.K.N. 2004. Bacteriological and genetical study to evaluate the relationship between hospital effluent and distribution of multiple antibiotic resistant bacteria. Ph.D. thesis. College of Science. Babylon University
19- Bradford, P. A. 2001. Extended -spectrum β-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev., 14: 933-951.
20- Johnson, D. M., Bieddenbach, D. J. & Jones, R. N. 2002. Potency and antimicrobial spectrum update for piperacillin/tazobactam (2000): emphasis on its activity against resistant organism populations and generally untested species causing community acquired respiratory tract infections. Diagnostic Microbiol. Infect. Dis., 43, 49–60.
21- Gales, A. C., Bolmstrom, A., Sampaio, J. and Sader, H. S. (1997). Antimicrobial susceptibility of Klebsiella pneumoniae producing extended spectrum β-lactamase (ESBL) isolated in hospitals in Brazil. Brazilian Journal of Infectious Diseases, 1, 196–203.
22- Haverkorn, M.J. and Michel M.F. (1979). Nosocomial klebsiellas: Colonization of hospitalized patients. J. Hyg., Camb, 82: 177-193.
23- Haverkorn, M.J. and Michel M.F. (1979). Nosocomial klebsiellas: Transfer in a hospital ward. J. Hyg., Camb, 82: 195-205.
24- Livermore, D. M. and M. Yuan. (1996). Antibiotic resistance and production of extended-spectrum β-lactamases amongst Klebsiella spp.from intensive care units in Europe. J.Antimicrob. Chemother., 38:409–424.
25- Al-Jubouri, S.S. (1997). Genetical and molecular study of β-lactamase produced by local isolates of gram-negative bacteria. Ph.D.thesis. College of Science. Al-Mustansiryia University.
26- Tullus, K.; Berglund, B. and Burman L. (1990). Emergence of cross-resistance to β-lactam antibiotics in fecal E. coli and Klebsiella strains from neonates treated with ampicillin or cefuroxime. Antimicrob. Agents Chemother., 34 (2): 361-362.
27- Chang, F. Y., L. K. Siu, C. P. Fung, M. H. Huang, and M. Ho. (2001). Diversity of SHV and TEM beta-lactamases in Klebsiella neumoniae: gene

evolution in northern Taiwan and two novel beta-lactamases, SHV-25 and SHV-26. Antimicrob. Agents Chemother. 45:2407–2413.

28- Opal, S.M., Cross A.S., Gemski P., and Lyhte L.W. (1988). Survey of purported virulence factors of Escherichia coli isolated from blood, urine and stool. Eur. J. Clin. Microbiol. Infect. Dis., 7: 425- 427.

29- Alvarez, M., J. H. Tran, N. Chow, and G. A. Jacoby.(2004). Epidemiology of conjugative plasmid-mediated AmpC β-lactamases in the United States. Antimicrob. Agents Chemother., 48:533–537.

30- Salyers, A.A. and Amabile-Cuevas, C.F. (1997). Why are antibiotic resistance genes so resistant to elimination ? Antimicrob. Agents Chemother., 41: 2321–2325.

31 - Kaftandzhieva, A.; Kotevska, V.; Jankoska, G.; Kjurcik-Trajkovska, B.; Cekovska, Z. and Petrovska, M. (2009). Extended-Spectrum Beta-Lactamase Producing *E. Coli* and *Klebsiella Pneumoniae* in Children at University Pediatric Clinic in Skopje. Macedonian Journal of Medical Sciences. 15; 2(1):36-41.