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RESEARCH ARTICLE

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DETECTION OF EXTENDED SPECTRUM AND AMBLER CLASS C BETA LACTAMASES AMONG BETA-LACTAM RESISTANT KLEBSIELLA SPECIES: GENETIC ASPECTS

ABSTRACT:
Background and objective: It is well recognized that life infections due to variety of β-lactamases producing isolates of *Klebsiella* spp. are the major problematic in the treatment of these types of infections due to their expression high level of resistance to the most effective antimicrobial agents. This study has been undertaken for detecting the ability of *Klebsiella* species to produce extended-spectrum β-lactamases (ESBLs) and Ambler class C (AmpC). Further, for determining the genetic elements encoding for such enzymes whether chromosomally or conjugated plasmid mediated. The study was performed on 110 wound patients admitted to Department of Surgery, Ramadi Teaching Hospital.Rapid iodometric β-lactamase production test, double disk synergy test, and phenotypic and confirmatory tests for production of extended spectrum and AmpCβ-lactamases were performed. Plasmid DNA extraction, conjugation and curing experiments were performed. Fifty isolates were bacteriologically identified as *Klebsiellae* spp. including 36 (72%) *Klebsiellae pneumoniae* and 14 (28%) *Klebsiellae oxytoca.* In double disk synergy test, out of the 25 β-lactamase producer isolates, 12 (48%) and 7 (70%) isolates of *K. pneumonia* and *K. oxytoca* respectively demonstrated a clavulanic acid effect with cefotaxime or ceftazidime.In the plasmid profile analysis, the results revealed that 7 (87.5%) of Eight ESBLproducer isolates of *Klebsiella* spp. respectively were conjugative plasmid mediated. The result of curing experiments showed successful curing for all the study isolates by both methods. The study suggested that the production of β-lactamases of type ESBLs and Amp C, appeared to be the major mechanisms ofresistance of *Klebsiellae* spp. to β-lactams. In addition, conjugative plasmids were found to be the main genetic elements encoding for ESBLs produced by *Klebsiellae* spp. Finally, the study suggested that sodium dodecyl sulphate was of higher potency than elevated growth temperature in the curing of plasmid DNA.

KEY WORDS:
β-lactamases, *Klebsiella*, Plasmids, Conjugation, Curing

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INTRODUCTION:

Extended-spectrum beta (β)-lactamases (ESBLs) are enzymes conferring broad resistance to penicillins, cephalosporin and monobactams, but not carbapenems. ESBLs are often plasmid-mediated and most are members of the TEM and SHV families of enzymes. Plasmids containing genes encoding for ESBLs often contain resistance determinants for other classes of antimicrobial agents and are readily transmissible from strain to strain and between different species of enteric Gram-negative bacilli (Paterson, 2006; Mehrgan *et al.*, 2010). It is well recognized that life infections due to variety of β-lactamases producing isolates of *Klebsiella* spp. are the major problematic in the treatment of these types of infections due to their expression high level of resistance to the most effective antimicrobial agents (Babini and Livermore, 2000).

With the widespread use of extended-spectrum cephalosporins throughout the world, strains that produce ESBLs have been detected on every inhabited continent. These enzymes are most commonly found in K. pneumoniae, and other gram-negative bacilli. The emergence and spread of ESBL-producing strains have led to questions regarding the optimal therapy for infections caused by ESBL-producing strains (Kim *et al.*, 2002). The correlation between in vitro susceptibility of ESBL-producing bacteria to noncarbapenem antimicrobials and clinical efficacy has been questioned by two recent observational studies which documented an advantage of imipenem treatment over either ciprofloxacin (CIP) or any other noncarbapenem treatment of bacteremia caused by ESBL-producing Klebsiella pneumoniae (Zimhony *et al.*, 2006). This advantage was unrelated to an apparent susceptibility of the respective isolates to noncarbapenem agents (Endimiani *et al.*, 2004; Paterson *et al.*, 2004).

On the other hand, AmpC β-lactamases are also mediate resistance to extended-spectrum cephalosporins and aztreonam in addition to cephamycins, such as cefoxitin. Unlike ESBLs, however, AmpC-β-lactamases are not inhibited by clavulanic acid or other similar compounds (Tenover *et al.*, 1999). Detecting and reporting isolates producing plasmid mediated AmpC beta-lactamases are more difficult issues than those associated with ESBLs. Detection is technically difficult in organisms that also produce a chromosomal AmpC, since proving that an AmpC is plasmid mediated, andnot the usual chromosomal enzyme, is necessary. This determination is beyond the capabilities of most clinical laboratories. However, *Klebsiella* spp. does not possess a chromosomal AmpC. This makes them convenient indicator organisms to screen when attempting to detect plasmid mediated AmpCs (Thomson, 2001).

The concern for the detection and occurrence of ESBLs, and AmpC β-lactamases is due to main reasons. Firstly, the ubiquitous prevalence in nosocomial infections intensive care unit (ICU) and its association with therapeutic failure especially in the life-threatening infections. Secondarily, many strains producing these enzymes demonstrate an inoculum effect in that the MICs of antimicrobials rise as the inoculum increases. Therefore, this study has been under taken for screening and phenotypic confirmatory tests for extended-spectrum β-lactamases (ESBLs) and Ambler class C (AmpC) isolates of *Klebsiellae* spp. Further, in the genetic part, for determination the genetic elements coding for these enzymes whether determining chromosomally or plasmid mediated. Furthermore, for determining whether the plasmid mediated resistance is curried by conjugative plasmid or not and to confirm the plasmid profile phenomenon by curing experiments.

PATIENTS AND METHODS:

Swabs or aspirates were taken from different anatomical sites of 110 wound patients like Bone, Joints, and Connective tissues. The swabs were taken during the period from January to September, 2009 from patients admitted to Ramadi General Hospital. Out of 110 isolates, 50 (45.5%) isolates were bacteriologically identified as *Klebsiellae* spp. The clinical data regarding the distribution of *Klebsiellae* isolates, type of specimens and type of infection are presented in table 1.

Table 1. The distribution of clinical isolates of *Klebsiellae pneumoniae, Klebsiellae oxytoca* according to the type of specimens and type of infection

Study isolates no (%)	Type of specimen	Type of infection
	Klebsiellae pneumoniae 36 (72%)	
16(44.4)	Wound swab	Osteomyelitis
2(5.6)	Wound swab	Diabetic foot infection
9(25)	Wound swab	Burn
4(11.1)	Knee aspirate	Septic arthritis
2(5.56)	Hip aspirate	Septic arthritis
2(5.56)	Mid-stream urine	Urinary tract infection
1(2.8)	Blood culture	Bacteraemia
	Klebsiellae oxytoca 14 (28%)	
3(21.4%)	Wound swab	Osteomyelitis
4(28.6%)	Wound swab	Diabetic foot infection
2(14.3%)	Wound swab	Burn
2(14.3%)	Knee aspirate	Septic arthritis
2(14.3%)	Hip aspirate	Septic arthritis
1(7.1%)	Blood culture	Bacteraemia

The swabs and aspirate were inoculated immediately on routinely used culture media nutrient agar, blood agar, MacConkey agar and chocolate agar plates and incubated overnight at 37°C. The suspected Klebsiellae colonies were identified bacteriologically according to the following confirmatory methods mentioned by Baron *et al.* (1994) which include staining with Gram stain, biochemical tests like IMViC, Urease test, Motility test and Ornithine decarboxylation .

Bacterial Standardization:

The inoculum was adjusted by using McFarland 0.5 turbidity standard 'barium sulfate' (NCCLS, 2004). It was prepared by adding 0.5 ml of 0.048 M Bacl2 (1.175 % w/v $Bacl_2$. H_2O) to 99.5 ml of 0.36N H2SO4 1% v/v) in Bush and Lamb glass tubes. This

standard was agitated on a vortex mixer prior to use.

The tube which contained bacterial suspension was compared with the turbidity standard and the density of the test suspension was adjusted visually to that of the standard by adding more bacteria or sterile saline (Vandepitte *et al.*, 1996). Moreover, for each test suspension, fine adjustment of turbidity was performed until the O.D. value for the test suspension corresponded to O.D value for 0.5 McFarland standard solution which was read previously.

Beta-Lactamase production test:

To determine the production of β-lactamase enzymes by the local clinical isolates of *Klebsiellae* species, the cell suspension rapid iodometric method described by Miles and Amyes (1996) was used as follows:

From an overnight culture of the test organism, a heavy suspension was made (containing 10^9 colony-forming units/ml) in 100mM sodium phosphate buffer at pH 7.3 containing penicillin at 6g/liter. A negative control test had been done without the organisms. An organism known to produce beta-lactamase was often tested in parallel as a positive control.The test and controls in parallel were setting up by placing 0.1 ml aliquots into the wells of a microtiter plat. After incubation for 1 hour at 37°C, two drops of the freshly prepared 1% soluble starch solution were added to each well. A drop of the iodine reagent was added. If the blue colour is lost within 10 minutes, the presence of the beta-lactamase is inferred. If the blue colour persists, the culture is considered to be beta-lactamase negative.Negative control strain (*Escherichia coli* ATCC 35218) was used in this experiment.

Determination of minimal inhibitory concentration (MIC):

The double fold dilutions of antimicrobial in five ml volumes of broth were prepared. A starting range of about eight fold higher than the normal MIC for the species has been tested and extended to at least one dilution below that of the control organism. A drug free control tube was included, thereafter one set of tubes was inoculated with a drop of well grown broth culture of the test organism diluted one in hundred (about 10^5organism) and the other with the control organism similarly diluted and then incubated overnight. After incubation, the last tube which shows no growth will represent the minimal inhibitory concentration "MIC" (Al-Ouqaili, 1999).

Screening for AmpC cephalosporinase and extended spectrum β-lactamase:

A. Standard disk diffusion technique: The β-lactamase producer isolates were screened for their susceptibility to (ceftazidime, ceftriaxone, cefoxitin) and carbapenems represented by imipenem (NCCLS, 2004; Mehrgan *et al.*, 2010). 5ml of Brian heart infusion broth medium was inoculated with the bacterial isolates, incubated at 37ºC for 3-4 hr. (mid log phase). Ten µl of broth suspension to 10 ml normal saline, 0.1 ml of the suspension was transferred to Muller-Hinton agar plates and spreaded with sterile swab on the agar surface in three different planes by rotating the plate approximately 60º each time to obtain an even distribution of the inoculum.

The inoculated plats were placed in room temperature for 30 min to allow absorption of excess moisture. With sterile forceps the selected antibiotic disks (CAZ, CTX, FOX, IMP) were placed on the inoculated plates and incubated at 37 Cº for 18 hours in inverted position. After incubation, the diameter of inhibition zones was noted and measured by a ruler and scored as sensitive, intermediate susceptible and resistant according to NCCLS (2004). *Escherichia coli* 25922 and *Escherichia coli* MM294 were used as the reference strains.

Phenotypic Confirmatory Testing Part I:

Extended Spectrum Cephalosporinase Production:

Double disk synergy test:

All isolates that showed negative result for AmpC production test were submitted to this test. Double disk synergy test was used to demonstrate the production of extended spectrum cephalosporinase: In this test, 5ml of sterile nutrient broth was inoculated with the bacterial isolates, incubated at 37ºC for 4 hours. 10µL of suspension broth to 10 ml normal saline, 0.1 ml of this dilution was spread by sterile spreader on the entire surface of Muller-Hinton agar in three different planes by rotating the plate approximately 60º each time to obtain an even distribution of the inoculum (Al-Ouqaili, 2002). The inoculated plates were left at room temperature for 30 min. to allow absorption of excess moisture. Using sterile forceps, the selected antibiotic disks containing (30 µg of ceftazidime and 30 µg of cefotaxime with and without 10 µg of clavulanic acid) were used for testing. Disks containing clavulanic acid were prepared by applying 10 µl of a 1000 µg /ml clavulanic acid stock solution to each disk 122. The prepared disks were allowed to dry for 30 min.

The disks are arranged so that the distance between them is approximately twice the radius of the inhibition zone produced by the later generation cephalosporins test on its own. After overnight incubation, the inhibition zones were measured and synergism occurred in some isolates between CAZ and CA and CTX. The results were interpreted according to the recommendations of the CLSI (NCCLS, 2004; Mehrgan *et al.*, 2010). *E. coli*

ATCC 25922 was used as negative controls for ESBL production.

Phenotypic Confirmatory Testing Part II For ESBLs and AmpC Production:

In this test, MIC tubes containing ceftazidime or cefotaxime with and without clavulanic acid were prepared in nutrient broth. 5 ml of sterile nutrient broth medium was inoculated with the bacterial isolates, incubated at 37ºC for 4 hours. 10 µl from suspension broth was placed in 10 ml normal saline, 10 µl from the suspension broth distribution to the tubes containing the ceftazidime, cefotaxime (in concentration of 0.5 to 256 µg/ml) were tested combination with 4 µg of CA per ml. After incubation for overnight at 37ºC, the lowest concentration was measured visually and compared with positive control (bacteria in broth), negative control (broth only).The results were interpreted according to the criteria established by NCCLS (2004).

Plasmid Profile:

DNA isolation was performed using alkaline-lysis technique of Birnboim and Doley (1979). This technique was mentioned with some modifications by Sambrook *et al.* (1989) as follow: Bacterial isolate was grown in blood agar plate for 18 hr. at 37ºC.The growth was collected with sterile swab, then, inoculated in 50 ml brain heart infusion broth supplemented with 100 µg/ml ampicillin. After that, incubated over night at 37ºC.The growth was distributed in tubes (10 ml), and then centrifuged 10000 rounds per minute (r.p.m) for 15 minutes at room temperature. Then the pellet was resuspended in 1.5 ml of TEG-solution, mixed by gentle inversion and stood on crushed ice for 30 min. After that, 2.5 ml of freshly prepared 0.2N NaOH containing 1% SDS was added and then mixed by gentle inversion and stood on crushed ice for 5 min. 1.5 ml of chilled 5M potassium acetate was added, mixed inversion, stood on crushed ice for 1 hr. Then, the supernatants were centrifuged at 14000 r.p.m for 15 min at room temperature and transferred to new micro-centrifuge tubes. Phenol-chloroform solution was added to DNA extract, centrifuged by micro-centrifuge at 10 min. The water layer was drawn and transferred to new micro-centrifuge tube, and then precipitated with ice-cold absolute ethanol and incubated over night at 37ºC. The solution was centrifuged for 10 min. by micro-centrifuge, then supernatants were discarded, and the extracted DNA was dried and dissolved in 10 µl TE buffer. The resulting extracts were electrophoresed in 1% agarose gels at 50 volt for 1.5 hr. Afterthat, The samples were stained with ethidium bromide and they were visualized with UV illumination.

Conjugation experiment:

Mating experiments were performed by using conjugation in broth medium method described by O'Connell (1984). Escherichia coli MM294 was used as the recipient strain and donors strain was separately inoculated into 5ml brain heart infusion broth, then incubated at 37ºC for overnight. The mixture was centrifuged at 10000 r.p.m for 10 min, washed for one time by brain heart infusion broth removing the supernatant (O.D 0.5 at 540 nm). One ml from donor strain and 1ml from recipient strain were placed in sterile tube, incubated 1-2 hr. at 37ºC. Then the mixture was mixed on the vortex shaker for 1 min only.0.1 ml of 10-1 to 10-6 dilutions were plated by spreading on appropriate selective media (containing rifampicin and ampicillin 100 µg/ml) and incubated for 24 hr. at 37ºC. Afterthat, 0.1 of 107, 108 dilutions were plated by spreading on appropriate selective media (containing rifampicin only) and incubated for 24 hr. at 37ºC.Conjugation Frequency was determined as follows:

Conjugation frequency = No. of transconjugation cell /No. of recipient cell.

Finally, β-lactamase test was used to confirm the transferring of plasmid from donor to recipient cell.

Plasmid Curing:

Curing experiments were performed by using two types of curing agents sodium dodecyl sulphate according to Salzano *et al.* (1992) and for elevated growth temperature according to AL-Saeed (1997).

1-Sodium Dodecyl Sulphate:

Cells were grown in 5 ml of brain heart infusion broth to mid log phase (3.5 hrs). 0.1 ml of young culture of *Klebsiella* spp. were inoculated in a series of 5 ml fresh brain heart infusion broth universals containing various concentration of SDS (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2400, 2800, and 3000) µg/ml. Then, the growth density of different universals was measured visually and compared with the control to determine the effect of SDS on bacterial growth.The lowest concentration of SDS that inhibited bacterial growth was considered as the minimal inhibitory concentration (MIC). Samples were taken from universals containing the highest concentration of each curing agent that still allows bacterial growth which is known as (subminimal inhibitory concentration) and serially diluted.0.1 ml of 10^4, 10^5, 10^6, 10^7, and 10^8 dilution was spreaded on nutrient agar plates and incubated over night at 37ºC to score the survived colonies.

2- Elevated growth temperature:

Cells were grown in 5 ml of Brain heart infusion broth for 24 hr. at 37º for 3 hr. at 44°C. This step was repeated for 5 to 7 times. Then, 0.1 ml of 10^4-10^8 dilution was spreaded

on nutrient agar plates and incubated overnight at 37°C.

Selection of cured cells:

Sterile toothpicks picked the obtained isolated single colonies by procedure (Picking and patching) on plates of nutrient agar, incubated overnight at 37°C. These were regarded as master plates and from nutrient agar plates containing 100µg/ml ceftazidime and incubated overnight at 37°C and the number of colonies that lost resistance phenomenon to this antibiotic was determined. Finally, Curing frequency was determined from the following equation:

Curing frequency = Induced – spontaneous / total count.

RESULTS:

Out of 110 isolates, 50 (45.5%) isolates were *Klebsiellae* spp., 21 (19.1%) *Staphylococcus aureus*, 11 (10.0%) *Pseudomonas aeruginosa*, 7 (6.4%) *Escherichia coli*, 5 (4.6%) Enterobacter cloacae while the others 16 (14.6%) were sterile specimens. Out of 50 isolates of *Klebsiellae* spp. isolated from wound infections, 36 (72%) were *Klebsiellae pneumoniae* while the others 14 (28%) were *Klebsiellae oxytoca*. In the routinely β-lactamase production test, (rapid iodometric method), out of 50 isolates of *Klebsiellae* spp. 35 (70%) were β-lactamase producer isolates, 10 (28%) of them were *Klebsiellae oxytoca* and 25 (71.4%) were *Klebsiellae pneumoniae*.

In the screening tests for extended spectrum β-lactamase production test (ESBL), (double disk synergy test), out of 25 isolates of *Klebsiellae pneumoniae* 12 (48%) demonstrated a clavulanic acid (CA) effect with cefotaxime (CTX) or ceftazidime (CAZ) (an increase in cefotaxime or ceftazidime zone diameters of ≥5 mm in the presence of clavulanic acid. Of these, 8 (66%) isolates of *Klebsiellae pneumoniae*, showed this effect with ceftazidime alone and 4 (33.4%) with cefotaxime alone. Further, out of 10 isolates of *K. oxytoca* 7 (70%) demonstrated a clavulanic acid effect when it is combined with CAZ or CTX. Of these 6 (85.7%) showed this effect with CAZ alone and 5 (71.4%) showed this effect with CTX alone (Tables 2&3).

Table 2. Screening and phenotypic confirmatory tests for extended spectrum beta-lactamase production against beta-lactamase producer isolates of *Klebsiellae* spp.

No. of isolates	Clavulanic acid + CAZ or CTX		Clavulanic acid + CAZ		Clavulanic acid + CTX	
	DDS	MIC	DDS	MIC	DDS	MIC
K. pneumoniae 25(71.4%)	12 (48%)	16 (64%)	8 (66.6%)	16 (100%)	4 (33.4%)	13 (81.2%)
K. oxytoca 10(28.0%)	7 (70%)	9 (90%)	6 (85.7%)	9 (100%)	5 (71.4%)	8 (88.8%)

DDS: Double disk synergy test

MIC: Minimal inhibitory concentration, CAZ: ceftazidime, CTX: cefotaxime

Table 3. Screening test for potential ESBLs producing isolates of *Klebsiellae* spp .

Study isolates no.	MIC screening test (µg /ml)			Disk diffusion screening test (mm)			
	CAZ	CTX	CRO	CAZ	CTX	IMP	FOX
SKP1	128	16	32	6	10	12	9
SKP2	32	256	256	6	6	12	13
SKP3	64	8	32	6	8	8	18
SKO4	8	128	64	6	12	15	14
SKP5	64	128	256	6	12	12	10
SKO6	16	128	32	7	6	8	6
SKO7	32	128	128	6	6	12	7
SKP8	16	256	16	6	6	8	16
SKP9	32	16	256	6	6	10	6
SKP10	16	16	32	6	6	7	18
SKP11	64	32	32	6	6	6	20
SKP12	64	128	32	6	6	16	6
SKP13	64	16	4	6	6	14	6
SKP14	64	64	128	6	12	16	15
SKP15	256	256	32	6	7	6	19
SKO16	32	128	32	6	12	14	14
SKO17	64	128	64	9	14	16	15
SKP18	32	32	64	9	13	14	14
SKP19	8	4	32	9	12	15	16
SKP20	32	32	128	7	6	20	8
SKP21	16	64	32	6	6	20	7
SKP22	16	128	32	6	6	6	8
SKP23	8	32	32	6	6	18	10
SKO24	4	64	32	10	12	19	9
SKP25	16	32	64	6	8	19	8
SKO26	8	32	4	12	11	19	16
SKP27	16	64	32	6	6	17	8
SKP28	64	64	128	6	6	7	6
SKO29	32	16	32	6	6	9	11
SKP30	4	128	32	8	7	19	11
SKO31	4	128	128	8	9	19	6
SKO32	4	32	16	10	10	20	15
SKP33	8	16	32	6	6	6	16
SKP34	8	128	128	7	6	20	12
SKP35	32	32	64	6	6	19	12

CAZ: Ceftazidime, CTX: Cefotaxime, CRO: Ceftriaxone, FOX: Cefoxitin, IMP: Imipenem

In the confirmatory test for (ESBL), (broth microdilution technique), 16 (64%) isolates of *K. pneumoniae* demonstrated a clavulanic acid effect (reduction of CAZ or CTX MICs by ≥ 3 dilutions). Of these 16 (100%) showed this effect when a clavulanic acid combined with ceftazidime alone and 13 (81.2%) demonstrated the effect with cefotaxime alone. Further, out of 10 (28.0%), of *Klebsiellae oxytoca*, 9 (90%) demonstrated the effect with CAZ or CTX. Of these 9 (100%) with CAZ alone and 8(88.8%) with CTX alone.

The cefoxitin (FOX) zone diameter for 2 (8%) *K. pneumoniae* that failed to show CA effect by broth microdilution was ≤14 mm (Table 4).

Table 4. Screening tests (Broth microdilution and disk diffusion techniques) for potential AmpC producer isolates of *Klebsiellae* spp.

Study isolates no.	MIC screening test (µg/ml)			Disk diffusion test (mm)				Effect of CA			
	CAZ	CTX	CRO	CAZ	CTX	FOX	IMP	CAZ	CTX	CAZ	CTX
SKP12	64	128	32	6	6	6	16	64	128	-ve	-ve
SKP13	64	16	4	6	6	6	14	32	8	-ve	-ve

CAZ: ceftazidime, CTX: cefotaxime, CRO: ceftriaxone, FOX: cefoxitin, IMP: imipenem,

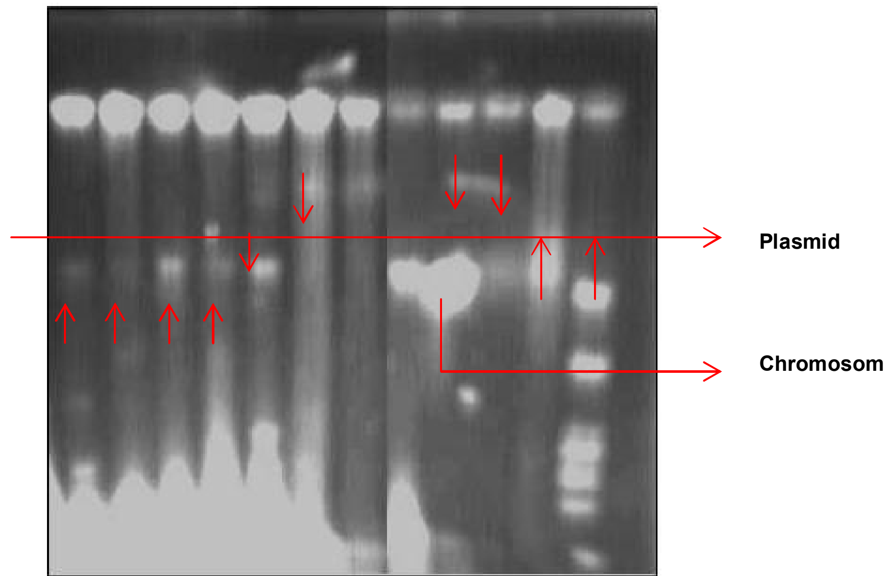

Fig. 1. The left five bands in addition to 9th and 10th represent plasmid; encoding for ESBLs production of *Klebsiellae*. Each of the 1st, 2nd and 5th bands harbored two plasmids one large in molecular weight and the other was small in molecular weight while the others harbored single plasmid. The genetic elements coding for 8th and 11th bands were chromosomes. The first right band represents DNA marker (λ DNA phage). Agarose concentration is 1% (W/V), voltage 50 and time is 1.5 hr.

Conjugation experiments were performed by using broth mixture technique for 5 (83.3%) of ESBL enzymes, respectively (Table 5).

Table 5. Conjugation between multi-resistant bacterial donor strains and the recipient *Escherichia* coli MM 294.

Donor strains	Mechanism of resistance	Recipient and its resistance pattern	No. of transconjugants Cells/ml	No. of recipient cell	Conjugation frequency
SKP1	ESBL production	*Escherichia coli* MM 294 RF^r	30 X 10^{-5}	3 X 10^{-9}	10 X 10^{-4}
SKO17	ESBL production	*Escherichia coli* MM 294 RF^r	16 X 10^{-7}	5 X 10^{-9}	3.2 X 10^{-2}
SKP8	IRT production	*Escherichia coli* MM 294 RF^r	9 X 10^{3}	3 X 10^{8}	3 X 10^{-5}
SKP9	Metallo production	*Escherichia coli* MM 294 RF^r	5 X 10^{4}	7 X 10^{8}	7.1 X 10^{-5}
SKP2	ESBL production	*Escherichia coli* MM 294 RF^r	3 X 10^{3}	9 X 10^{7}	1.4 X 10^{-4}
SKO24	ESBL production	*Escherichia coli* MM 294 RF^r	4 X 10^{4}	7 X 10^{8}	5.7 X 10^{-5}
SKO31	ESBL production	*Escherichia coli* MM 294 RF^r	16 X 10^{3}	2 X 10^{8}	8 X 10^{-5}

Curing experiment showed successful curing for all the study isolates by using SDS and elevated growth temperature as curing methods (Tables 6, 7, 8, &9).

Table 6. Effect of Sodium Dodecyl Sulphate as curing agent

Bacterial strains	Sub MIC	Dilution (no. of colony)				
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
SKP1	3000µg/ml	95	40	20	2	8
SKO17	2800µg/ml	160	60	25	20	15
SKP8	2800µg/ml	120	110	15	8	2
SKP9	2800µg/ml	110	40	9	4	2
SKO24	3000µg/ml	90	43	15	1	1
SKP2	3000µg/ml	200	120	50	10	1

SKO31	3000µg/ml	200	60	15	6	9

Table 7. Effect of elevated growth temperature as curing agent

Bacterial strains	Temperature	Dilutions (no. of colony)				
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
SKP1	44ºC for 6 times	250	100	25	4	1
SKO17	44ºC for 6 times	43	30	25	8	4
SKP8	44ºC for 6 times	150	40	10	Zero	Zero
SKP9	44ºC for 6 times	70	25	12	10	5
SKO24	44ºC for 6 times	170	50	5	7	3
SKP2	44ºC for 6 times	8	4	2	1	Zero
SKO31	44ºC for 6 times	150	20	10	1	Zero

Table 8. Number of bacterial cell that lost resistance to antibiotic (ceftazidime) after treatment with sodium dodecyl sulphate

Bacterial strains	Total number of colonies	Number of bacterial cells that lost resistance to CAZ (%)
SKP1	15	3(20%)
SKO17	15	9(60%)
SKP8	15	4(26.6%)
SKP9	15	9(60%)
SKO24	15	13(86.6%)
SKP2	15	10(66.6%)
SKO31	15	5(33.3%)

Table 9. Number of bacterial cell that lost resistance to antibiotic (ceftazidime) after treatment with elevated growth temperature (44°C) for 6 times

Bacterial strains	Total number of colonies	Number of bacterial cells that lost resistance to CAZ (%)
SKP1	15	2 (13.3%)
SKO17	15	4 (26.6%)
SKP8	15	2 (13.3%)
SKP9	15	2 (13.3%)
SKO24	15	12 (80%)
SKP2	15	Zero

DISCUSSION:

It is well known that life threatening infections due to extended spectrum β-lactamases (ESBLs) and Ambler class C (AmpC) producing isolates of Klebsiellae spp. are continuing to be one of the major leading causes of morbidity and mortality in the community and hospital setting. On the other hand, the accurate detection nevertheless is important because clinical failures arise even when the MICs of cephalosporins for ESBL producers are only 1µg/ml. Two detection strategies are in common use: I. using ceftazidime or cefpodoxime as an indicator drug, and considering Klebsiellae with reduced susceptibility to these drugs to be resistant to all oxyimino-aminothiazolyl cephalosporins or II. Screening for synergy between extended-spectrum cephalosporins and clavulanic acid. Synergy can be detected by double-disk synergy tests (Carter *et al.*, 2000). Thus, this study has been undertaken to detect accurately the occurrence of these enzymes in vitro. It must be stressed that precise placement of the disks, correct storage of the clavulanate-containing disks, and performance of appropriate control tests are critical to the sensitivity of the disk approximation method. Any enhancement of the zone of inhibition between a beta-lactam disk and that containing the beta-lactamase inhibitor is indicative of the presence of ESBLs. A > = 5 mm increase in a zone diameter for an antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone indicates probable ESBL production (NCCLS, 2004).

In broth microdilution technique, the phenotypic confirmatory test for ESBLs, 16 (64%) isolates of *K. pneumoniae* demonstrated a clavulanic acid effect (reduction of CAZ or CTX MICs by ≥ 3 dilutions). Of these 16 (100%) showed this effect when a clavulanic acid was combined with ceftazidime alone and 13 (81.2%) demonstrated the effect with cefotaxime alone. Further, 9 (90%) of *Klebsiella oxytoca* demonstrated the effect with CAZ or CTX. Of these 9 (100%) with CAZ alone and 8 (88.8%) with CTX alone. It must be stressed that the range of CAZ concentration in this study was extended beyond 128 µg/ml to improve performance of the broth microdilution test among isolates with higher MICs (the antimicrobial result to be on-scale). In another study, when break point of CAZ concentration is 128 µg/ml, testing cefepime (fourth generation cephalosporins) in conjugation with CA can serve as a secondary indicator of ESBL production, particularly in organisms containing AmpC-type β-lactamases like *Klebsiella* and *Enterobacter* spp. (Steward *et al.*, 2001).

Many investigators prefer imipenem and meropenem as the drugs of choice for life-threatening infections due to ESBL-producing Enterobacteriaceae or in an outbreak setting (Giamarellou, 2005). It is difficult to detect ESBLs because they have different levels of activity against various cephalosporins. Thus, the choice of which antimicrobial agents to test is critical. For example, one enzyme may actively hydrolyze ceftazidime, resulting in ceftazidime minimum inhibitory concentrations (MICs) of 256 µg/ml but have poor activity on cefotaxime producing MICs of only 4 µg/ml. If an ESBL is detected, all penicillins, cephalosporins, and aztreonam should be reported as resistant even if *in vitro* test results indicate susceptibility (Kim *et al.*,

2002; NCCLS, 2004) documented that the high ESBL frequency may have been caused by the excessive use of broad-spectrum antibiotics in Tehran hospital and to a higher level in the community setting in this city, together with a lack of attention to laboratory screening of ESBL production by clinical isolates (Mehrgan *et al.*, 2010).

The current National Committee for Clinical Laboratory Standard (NCCLs) documents do not indicate the screening and confirmatory tests that should be used for the detection of AmpC beta-lactamase in *Klebsiellae pneumoniae* and *Escherichia coli*. Our study used the standardized disk diffusion break point for cefoxitin (zone diameter of 14 mm as the criterion for the screening of isolates). Cefoxitin resistance in non-AmpC producers may be due to a lack of permeation of porin135. Although screening methods which use cefoxitin in standardization method to detect AmpC-harboring isolates are useful, they are not perfect (Courdon *et al.*, 2000). Our result showed that the cefoxitin zone diameters for 2(8.0%) K. pneumoniae that failed to show CA effect by broth microdilution were <14mm suggesting either the presence of AmpC type β-lactamases or porin changes that could mask clavulanic effect (Steward *et al.*, 2001).

Recently, clinical isolates producing class A extended-spectrum-β-lactamases (ESBLs) that differ by a few point mutations. The level of resistance is determined by the efficiency of the enzyme for hydrolyzing the drug and by the number of resistance mechanisms present in the organism. Organisms can produce more than one hydrolyzing enzyme and may show modifications in more than one porin, producing high-level resistance to the carbapenems (MIC >16 g/ml). Organisms with decreased susceptibility produced by porin changes alone often have lower MICs (2-8g/ml). Most other species of Enterobacteriaceae are very susceptible "0.5g/ml" (NCCLS, 2004).

In the genetic part of this study, the result of plasmid profile analysis revealed that out of 8 ESBL producer isolates of *Klebsiellae* spp. 7 (87.5%) were plasmid mediated (the genes coding for these enzymes were located on the plasmid). However, 1 (12.5%) and 2 (100%), of ESBL and AmpCenzymes respectively failed to show plasmid bands in agarose gel electrophoresis.It is well recognized that conjugation is considered a major pathway for horizontal gene transfer among bacteria. Conjugation requires cell-to-cell contact and operates by DNA replication resulting in unidirectional transfer of genetic material from a donor to a recipient cell. It is mediated mainly by conjugative plasmids, although conjugative transposons are also capable of triggering the process of conjugation[25]. ESBLs are often plasmid-mediated and most are members of the TEM and SHV families of enzymes. Plasmids containing genes encoding for ESBLs often contain resistance determinants for other classes of antimicrobial agents and are readily transmissible from strain to strain and between different species of enteric Gram-negative bacilli (Paterson, 2006; Mehrgan *et al.*, 2010).

Detecting and reporting isolates producing plasmid mediated AmpC beta-lactamases are more difficult issues than those associated with ESBLs. Detection is technically difficult in organisms that also produce a chromosomal AmpC, since proving that an AmpC is plasmid mediated, and not the usual chromosomal enzyme, is necessary. This determination is beyond the capabilities of most clinical laboratories. However, *Klebsiella* spp. do not possess a chromosomal AmpC. This makes them convenient indicator organisms to screen when attempting to detect plasmid mediated AmpC (Thomson, 2001).

Transfer of antibiotic resistance markers from one bacterial strain into another by conjugation is another method to determine whether the antibiotic resistance genes are located on plasmid or not. For this reason, conjugation experiments were performed between multi-resistant bacterial strains and *E. coli* MM 294 containing the plasmid RFr as recipient. The failure to detect transconjugants can be either due to the fact that the β-lactam antibiotic resistance markers of *Klebsiella* spp. are located on non-conjugative plasmid or because conjugation between different strain of different species is not always possible[26].Successful conjugation between isolates of *K. pneumoniae* and *Klebsiella oxytoca* with *E. coli* MM 294 was observed in this study, clearly indicated that conjugation is one of the important factors in spreading plasmids coding for antibiotic resistance among bacteria and that such transfer can occur not only by the strains of the same species but also between strains of closely related species especially those belong to the family of enterobacteriaceae. Their ability to pass between different bacterial strains or species has been considered to be of lower efficiency than their ability to pass between similar bacteria, due to diverse barriers such as restriction systems (Sanders, 1996).

Our results on curing experiments revealed that *Klebsiellae* spp. SKP1, SKP8, SKP9, SKP2, SKO17, SKO24, and SKO31 were very resistant and the MIC was higher than the highest concentration used in this study (3000 µg/ml). Samples from sub-minimal inhibitory concentrations sub MIC (the highest concentration allows bacterial growth) were taken to select cured cells. It is

well recognized that Sodium dodecyl sulphate (SDS) is capable of curing certain plasmids. Some plasmid containing cells are presumed to be more sensitive to SDS because of plasmid-specific pili on their cell surface.

The concentrations of SDS used in this study were (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2400, 2800, and 3000 µg/ml). However, Trevors (1998) reported that the most effective concentration of a particular curing agent could vary considerably in the range of 100-to 1000-fold depending on the species being treated, curing agent efficiency and the mode of action of curing agent. After treatment of bacterial strains with curing agents, survivors were analyzed for the loss of resistance to antibiotics by plating them on agar media containing the proper antibiotic. Then the curing percentage and efficiency of each agent were analyzed.

Elevated incubated temperature (5-7°C) above the normal or optimal growth temperature was also used in this study as a curing method. Results of this method revealed that Amp C producing isolates of *Klebsiellae* spp. were cured at higher ratio than ESBLs producing isolates. However, Trevors (1998) observed that these cells did not appear until after several cell generations at the elevated temperature. Further, Trevors141 showed that *Escherichia coli* K12 has also been cured of F plasmids by elevated growth at 42-44°C. This temperature also prevented the re-establishment of plasmids in cured cells if the culture was permitted to reach cell numbers >10^5 ml.

The study concluded that the production of β-lactamases of the types extended spectrum and Ambler class Cappear to be the major mechanisms of resistance of *Klebsiellae* spp. to β-lactams. Further, ceftazidime was proved to have adequate sensitivity to detect the extended spectrum β-lactamases and Ambler class C cephalosporinase producing isolates. Furthermore, isolation of plasmid DNA revealed that most of the isolates examined harbored more than one plasmid. Furthermore, conjugative plasmids were found to be the main genetic elements encoding for extended spectrum β-lactamases produced by *Klebsiellae* spp. while conjugative plasmids were not found in Ambler class C cephalosporinasesuggesting that the genes encoding for resistance may be located on the chromosome. The study suggested that sodium deodecyl sulphate was of higher potency than elevated growth temperature in the curing of plasmid DNA of the study isolates.

Finally, the molecular studies including Polymerase chain reaction technique for detection and amplification of the genes responsible for mutants transformation leading to arising new types of enzymes is recommended to be achieved.

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الكشف عن البيتا لاكتاميز واسع المفعول الممتد فئة ج بين أنواع الكليبسيلا المقاومة للبيتا لاكتام: الجوانب الوراثية

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أن هدف الدراسة هو تحديد قدرة بكتريا الكلبسيلا لإنتاج إنزيمات البيتالاكتاميز واسعة النطاق ونوع أمبلر صنف C، بالاضافه إلى تحديد العناصر الوراثية المشفرة لإنتاج الإنزيمات. تم إجراء الدراسة ل110 مريض من مرضى الجروح الراقدين في قسم ألجراحه في مستشفى الرمادي التعليمي،تم إجراء اختبار إنتاج إنزيم البيتالاكتاميز بطريقه اليود السريعة ،أجراء المسح الأولي بطريقه تآزر الأقراص الثنائية وأجراء الفحص التأكيدي لإنتاج إنزيمات البيتالاكتاميز واسعة النطاق ونوع أمبلر صنف C. كذلك تم تحليل المحتوى الوراثي بطريقه عزل الDNA وإجراء تجارب الاقتران البكتيري وتجارب التحييد. أظهرت النتائج عزل 50 عزله من الكلبسيلا *Klebsiellae* spp تتضمن 36 (72%) *Klebsiellae pneumoniae* و *Klebsiellae* (28%) 14 *oxytoca* . كانت نتائج تآزر الأقراص الثنائية من بين 35(70%)عزله منتجه لإنزيم البيتالاكتاميز توزعت كالتالي *K. pneumonia* (71.4%) 25 , *K. oxytoca* (28%) 10 كما أظهرت نتائج المسح الأولية بطريقه التآزر الأقراص الثنائية تأثير حامض الكلافيونيك على 8(66%) , 12 (48%) of *K. pneumonia* and *K. oxytoca* على التوالي أظهرت نتائج تحليل البلازميدات بأن 7(87.5%) من 8 عزلات من بكتريا الكلبسيلا المنتجة لإنزيمات البيتالاكتاميز كانت مشفرة بواسطة بلازميدات .وأظهرت نتائج نجاح تجارب الاقتران الوراثي و تجارب التحييد لجميع عزلات الدراسة. تستنتج الدراسة أن إنتاج إنزيمات البيتالاكتاميز واسعة النطاق ونوع أمبلر صنف C من قبل بكتريا الكلبسيلا كانت مقاومه مضادات البيتالاكتام بالاضافه إلى أن جينات المقاومة لمضادات البيتالاكتام لعزلات الدراسة كانت موجودة على بلازميد واحد أو أكثر ذاتي الانتقال أو قابل للتحريك قابله للتحييد بسلفات دودسيل الصوديوم وبنسب اقل في استعمال درجه الحرارة ألمرتفعه كعامل محييد.

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