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Full Length Research Paper

Occurrence and detection of extended-spectrum β-lactamases in *Klebsiella* isolates in Hilla, Iraq

Alaa H. Al-Charrakh[1]*, Samira Y. Yousif[2] and Hussein S. Al-Janabi[3]

[1]Department of Microbiology, College of Medicine, Babylon University, Babylon Province, Iraq.
[2]Department of Biotechnology, College of Science, Baghdad University, Baghdad, Iraq.
[3]Department of Internal medicine, College of Medicine, Kufa University, Al-Najaf Province, Iraq.

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A total of 88 *K. pneumoniae* strains were isolated from different environmental and clinical samples in Hilla/Iraq during the period from January to July 2003. Primary screening of β-Lactam resistant isolates showed that 73.8% (65 strains) were resistant to β-Lactam antibiotics. 58.4% of these strains were β-lactamase-producers. All the β- lactamase-producing *Klebsiella* strains showed multiple-drug resistance to least 8 antibiotics. *Klebsiella* strains were also tested for their ability to produce extended-spectrum beta-lactamases (ESBLs) using three methods. Determination of minimum inhibitory concentration (MIC) with and without clavulanate was the most accurate method for detection of ESBL-producing isolates, by which 8 (21%) ESBL-producing isolates were detected. Plasmid profile of *Klebsiella* strains (including ESBL-producers) was detected. The genes encoding for the production of ESBLs and resistance to penicillin, ampicillin, amoxicillin, tetracycline, refampin, and erythromycin were located on conjugative plasmids whereas genes encoding for resistance to cephalothin, cefazolin, cephalexin and gentamycin were located on the chromosome.

Key words: Antimicrobial resistance, β-lactamases, ESBLs, plasmid profile, *Klebsiella pneumoniae*.

INTRODUCTION

Extended-spectrum beta-lactamases (ESBLs) have been found in many pathogenic gram-negative bacteria, but they are most common in nosocomial isolates of *Klebsiella pneumoniae* (Philippon et al., 1989; Medeiros, 1993), giving a proportion of 75% of ESBL-producing strains (Sirot, 1995). ESBLs were first recognized in a single strain of *K. pneumoniae* isolated in Germany (Knothe et al., 1983). Since then several types of ESBLs have been described (Jacoby and Medeiros, 1991). Hospital outbreaks of ESBL-producing *K. pneumoniae* have been reported all-around the world and recently ESBL-producing *K. pneumoniae* isolates were reported from different countries in the world (Pagani et al., 1994; Galas et al., 1999; Kawakami et al., 2000; Kariuki et al., 2001; Pai et al., 2001; Bedenic et al., 2001b; Subha and Ananthan, 2002; Mendelson et al., 2005).

Although *K. pneumoniae* is one of the well known bacterial species in which the ESBLs have been most commonly reported around the world (Sirot, 1995), little or no studies are available in Iraq concerning ESBL production in *Klebsiella* spp. To our knowledge, the present study is the first to investigate the occurrence of ESBLs and detection of genetic factors controlling ESBL production in *Klebsiella* isolates in the area of the study.

MATERIALS AND METHODS

Bacterial isolates

Eighty eight strains of *Klebsiella* spp. were isolated from different environmental and clinical samples in Hilla/Iraq during the period from January to July 2003. The environmental *Klebsiella* isolates (89 isolates) were as follows: sewage (48), still water (10), fountain water (6), tap water (8), toilette seat (9), and skin ointment (8). The clinical *Klebsiella* isolates (89 isolates) were as follows: urine (108), stool (50), blood (6), in addition to swabs from ear (27), wound (3), burn (2), skin (4), vagina (5), and throat (4). Clinical samples were collected from the main three hospitals in Hilla (Teaching hospital, Margan hospital, Maternity and pediatric hospital), in addition to some private laboratories. Bacteria were cultured on blood and

*Corresponding author. E-mail: aalcharrakh@yahoo.com. Tel.: 00964030262194.

MacConkey agar in aerobic condition at 37°C for 24 h. Then identified to the level of subspecies with conventional biochemical tests (Finegold and Baron, 1998; MacFaddin, 2000).

Standard bacterial strains

The following standard strains are gift from Dr. George Jacoby, Massachusetts, USA.
Escherichia coli J53, *E. coli* pMG223, and *E. coli* ATTC 25922.

Antimicrobial resistance testing

Preliminary screening of *klebsiella* strains resistance to β-lactam antibiotics was carried out using pick and patch method on Muller-Hinton agar plates supplemented with Ampicillin and Amoxicillin (each alone) at final concentrations of 100 and 50 µg/ml, respectively (NCCLS, 2003a). *Klebsiella* strains that were resistant to β-lactam antibiotics were tested for their ability to produce β-lactamase enzyme using rapid iodometric method (WHO, 1978).

The antimicrobial resistance patterns of strains to antimicrobial agents was determined using disk diffusion method and interpreted according to national committee of clinical laboratory standards documents (NCCLS, 2003a). The following antimicrobial agents were obtained (from Oxoid, U.K) as standard reference disks as known potency for laboratory use: penicillin (P, 10 IU), ampicillin (AMP, l0 µg), amoxicillin (Amx, 25 µg), piperacillin (PIP, 100 µg), carbencillin (CB, 100 µg), cloxacillin (CX, 1 µg), amoxicillin-clavulanate (20/10 µg), cephalothin (CF, 30 µg), cefazolin (CZ, 30 µg), cefixime (CFM, 5 µg), cefaclor (CEC, 30 µg), cephalexin (CN, 30 µg), cefuroxime (FUR, 30 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), ceftizoxime (COX, 30 µg), ceftriaxone (CTR, 30 µg), gentamycin (GN, l0 µg), rifampin (RD, 30 µg), erythromycin (E, 15 µg), and tetracycline (TE, 30 µg).

β-lactamase-producing strains were also tested for their ability to produce ESBLs. Three methods were used for this purpose using third generation cephalosporins (3GC) as representatives of β-lactam antibiotics and clavulanic acid as β-lactamase inhibitor: Disk approximation method (Jarlier et al., 1988), NCCLS screening method (NCCLS, 2003a), and determination of MIC with and without clavulanate (Bedenic et al., 2001a).

Disk approximation method was performed on Muller- Hinton agar plate inoculated with the test bacterial strain, by placing disks containing 30 µg ceftazidime, cefotaxime, and ceftriaxone 15 mm (edge to edge) from a disk of augmentin (20 µg amoxicillin plus 10 µg of clavulanic acid). Following incubation for 16 - 20 h at 35ºC, any enhancement of the zone of inhibition between a β-lactam disk and augmentin disk, was indicative of the presence of an ESBL.

NCCLS screening method included two main procedures: (i) Screening test for ESBLs production. This test was performed using a disk diffusion method on Muller-Hinton agar with antibiotic disks (CTX, CAZ, and CTR). Each bacterial strain should be considered a potential ESBL-producer if the inhibition zones (mm) of the antibiotics were: Ceftazidime < 22 mm, Cefotaxime < 27 mm, Ceftriaxone < 25 mm. (ii) Phenotypic confirmatory test. Using disk diffusion method, each ceftazidime and cefotaxime alone and in combination with clavulanic acid were tested. 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 MIC with and without clavulanate: Minimum inhibitory concentrations of cefotaxime, ceftazidime, and ceftriaxone with and without clavulanic acid (4 µg/ml) were determined, using agar dilution method. The test was considered positive (ESBL-producing isolate), if the MIC against the tested β-lactam antibiotic was reduced ≥ 8- fold by clavulanate.

Determination of MICs of ESBL-producing strains

All ESBL-producing strains were also tested to determine the minimal inhibitory concentrations (MICs) of a number of β-lactam antibiotics (NCCLS, 2003b) using two- fold agar dilution susceptibility method. Appropriate dilutions of β-lactam antibiotic solutions were prepared according to the report of international collaborative study by (Ericsson and Sherris, 1971), in which one part of the antimicrobial solution was added to nine parts of liquid Muller-Hinton agar. The MIC was recorded as the lowest concentration of the antimicrobial agent (in µg/ml) at which no more than two colonies was detected. The MIC values were compared with the break points recommended by NCCLS (2003b).

Results of all of the experiments above were compared with both, the negative control (*E. coli* ATTC 25922) and the positive control (*E. coli* pMG223).

Bacterial conjugation

Klebsiella strains showing resistance to ≥ 1 antibiotic were selected (as donor cells) and examined for their ability to transfer the resistance. Conjugation was performed using the standard strain *E. coli* J53 (as recipient cell) (O'Connell, 1984). Aliquots of overnight cultures of donor and recipient organisms were mixed in a final volume of 4 ml Luria-Bertoni medium. The mixture was incubated at 37°C for 2 h. Ten-fold serial dilutions of conjugation mixture were made, and 0.1 ml of each dilution was spreaded on the agar surface of Muller-Hinton agar supplemented with ampicillin (100 µg/ml) and sodium azide (200 µg/ml), for counting the total number of transconjugants. The plasmid DNA of transconjugant was analyzed on agarose gel in order to see whether the presence of the requisite plasmids correlated with the phenotype. The transconjugants were tested for their ability to produce β- lactamase enzyme, ESBL (using determination of MIC with and without clavulanic acid), and also tested for their antibiotic resistance and MICs to β-lactam antibiotics. The results were compared with those obtained from original isolates.

Plasmid profiles

Plasmid DNA was extracted from cultured cell using alkaline lysis method (Pospiech and Neumann, 1995) and the plasmid DNA of the strains was analyzed on agarose gel. Because of unavailability of standard plasmids of known molecular weight (size markers), the molecular weights of plasmids isolated in the present study were not estimated.

RESULTS

Eighty eight strains of *Klebsiella* spp. were isolated from different environmental and clinical samples. Out of the 59 isolates from environmental samples, 51 isolates (86.4%) were isolated from sewage samples. Other environmental samples revealed low ratios of recovered *Klebsiella* isolates. No *Klebsiella* isolates were recovered from samples of tap water and skin ointment. Out of the 209 clinical samples collected during this study, 29 *Klebsiella* isolates were recovered, 14 isolates (48.3%) were isolated from stool samples, 11 isolates (37.9%) from urine, 4 isolates (13.8%) from ear swabs, but none was isolated from the other clinical samples.

Out of the 88 isolates, 84 isolates (95.5 %) belonged to

Table 1. Antimicrobial resistance patterns of *Klebsiella* isolates from clinical sources.

Isolate designation*	Antimicrobial resistance pattern	
	Original	**Transferred**
1 C	P, AMP, Amx, CB, CX, FUR, ZOX, CFM, CN, GN, TE	P, AMP, Amx, CB, CX, ZOX, GN
2 C	P, AMP, Amx, PIP, CX, CZ, FUR, CTX, CTR, CEC, CN, GN, RD,E,	-
4 C	P, AMP, Amx, CB, CF, CZ, ZOX, CFM, CN, TE	-
6 C	P, AMP, Amx, PIP, CF, CZ, FUR, CTX, CAZ, ZOX, CTR, CFM, CN, GN, RD,E, TE	P, AMP, Amx, PIP, FUR, CTX,CAZ, ZOX, CTR, RD E, TE
7 C	P, AMP, Amx, CX, FUR, ZOX, CFM, CN,	-
9 C	P, AMP, Amx, PIP, CB, CF, CZ, CTX, CTR, CEC, CN, GN, E, TE	P, AMP, Amx, PIP, CB, CTX, CTR, CEC, GN, E, TE
10 C	P, AMP, Amx, CB, CX, FUR, ZOX, CN, GN, E	P, AMP, Amx, CX, FUR, ZOX, CN, E
11 C	P, AMP, Amx, PIP, CZ, FUR, CTX, ZOX, CTR, CFM, CEC, GN, RD,E, TE	P, AMP, Amx, PIP, CTX, ZOX, CTR, CFM, GN, E, TE
20 C	P, AMP, Amx, CX, FUR, CFM, CEC, CN, E, TE	P, AMP, Amx, CX, CEC, CN, E, TE
21 C	P, AMP, Amx, CX, CF, CZ, CTX, CAZ, ZOX, CTR, CFM, CEC, CN, GN, RD,E	P, AMP, Amx, CX, CF, CZ, CTX, CAZ, ZOX, CTR, CFM, CEC, CN, GN, RD,E
22 C	P, AMP, Amx, PIP, CB, CF, CZ, FUR, CTX, CAZ, ZOX, CTR, CFM, CEC, CN, GN, RD, TE	P, AMP, Amx, PIP, CB, FUR, CTX, CAZ, ZOX, CTR, CFM, CEC, RD,TE
25 C	P, AMP, Amx, CX, FUR, CFM, CEC, CN, GN, E, TE	P, AMP, Amx, CX, FUR, CFM, CEC, CN, GN, E, TE
27 C	P, AMP, Amx, PIP, CB, CX, CF, CZ, FUR, CTX, ZOX, CTR, CFM, CEC, CN, GN, RD,E, TE	P, AMP, Amx, PIP, CB, FUR,CTX, ZOX, CTR, CEC, GN, E, TE
28 C	P, AMP, PIP, CB, CF, CZ, FUR, ZOX, CFM, CEC, CN, GN, TE	-

*Isolates in bold are ESBL-producers.

K. pneumoniae and 4 isolates (4.5 %) were *Klebsiella oxytoca*. All known subspecies of *K. pneumoniae* were isolated in the present study. *K. pneumoniae* subsp. *pneumoniae* was predominant (87%) among the subspecies of *K. pneumoniae* followed by *K. pneumoniae* subsp. *ozaenae* (9.5%) and then *K. pneumoniae* subsp. *rhinoscleromatis* (3.5%).

Antimicrobial drug resistance

Primary screening of β-Lactam resistant isolates showed that 65 strains (73.8%) were resistant to β-Lactam antibiotics. 58.4% of these β-Lactam resistant strains were β-lactamase-producers.

All 38 β-lactamase-producing *Klebsiella* isolates were tested for their antibiotic resistance against 21 antibiotics. 100% of these strains were found to be multi-drug resistant to least 8 antibiotics. Multiple drug resistance was also common (90%) in *K. pneumoniae* strains isolated from nosocomial infections. Tables 1 and 2 summarize the antimicrobial susceptibility patterns of clinical and environmental *Klebsiella* isolates. Antimicrobial resistance was not distributed uniformly. Twenty six (68.4%) of 38 resistant strains of *Klebsiella* transferred their drug resistance to the recipient cells. 100% of *Klebsiella* isolates were resistant to penicillin, ampicillin, and amoxicillin and 73.6% of them were resistant to ceftizoxime and 50% to cefotaxime. Very low level of resistance was found (18.4%) in strains to ceftazidime and (34.2%) of them to both of ceftriaxone, and piperacillin. Results also showed that *Klebsiella* isolates were highly resistant to gentamycin (81.5%), tetracycline (63.1%), and erythromycin (52.6%) but they were highly sensitive to rifampin.

Three methods were used for detection of ESBLs production. Out of the 38 β- lactamase producing isolates, only 4 (10.5%) ESBL–producers were detected by disk approximation method, 6 (15.7%) isolates were detected by screening tests of NCCLS, and 8 (21%) ESBL–producers were detected by determination of MIC with and without clavulanate. Figure 1 illustrates the production of ESBL in clinical isolate *K. pneumoniae* 6C, by enhancement of the zone of inhibition between central disk of augmentin and the disks containing cefotaxime, ceftazidime, and ceftriaxone. Using determination of MIC with and without clavulanate (Table 3), number of fold decrease of β-lactam MICs (especially ceftazidime) in the presence of clavulanate, ranged from 12 to 16- fold, indicating that these isolates were ESBL-producers.

Table 2. Antimicrobial resistance patterns of *K. pneumoniae* isolates from environmental sources.

Isolate designation*	Antimicrobial resistance pattern	
	Original	Transferred
E 4	P, AMP, Amx, CB, FUR, ZOX, CFM, CN, GN, TE	-
E 5	P, AMP, Amx, CB, CX, CZ, CTX, ZOX, CEC, GN, E	-
E 6	P, AMP, Amx, CX, CZ, FUR, CTX, ZOX, CFM, CN,GN	P, AMP, Amx, CX, CTX, ZOX, CFM, GN
E 12	P, AMP, Amx, CX, FUR, ZOX, CFM, CEC, CN, GN, E, TE	-
E 16	P, AMP, Amx, PIP, CB, CX, CZ, CFM, CN, GN	-
E 22	P, AMP, Amx, CX, CZ, FUR, CTX, ZOX, CN, GN	P, AMP, Amx, CZ, FUR, CTX, CN, GN
E 32	P, AMP, Amx, CB, CZ, FUR, ZOX, CEC, CN, TE	P, AMP, Amx, CB, FUR, ZOX, CN, TE
E 33	P, AMP, Amx, CX, ZOX, CFM, CN, GN, E	P, AMP, Amx, CX, ZOX, CFM, GN, E
E 34	P, AMP, Amx, CX, CZ, FUR, CTX, ZOX, CFM, GN, E, TE	P, AMP, Amx, CX, FUR, CTX, ZOX, GN
E 35	P, AMP, Amx, CB, CX, FUR, ZOX, CFM, CEC, CN, GN, TE	-
E 36	P, AMP, Amx, CB, CX, FUR, ZOX, CFM, CEC, CN,GN, RD, E, TE	-
E 37	P, AMP, Amx, CX, CZ, FUR, CTX, CFM, CN, GN, TE	-
E 38	P, AMP, Amx, PIP, CB, FUR, ZOX, CTR, CFM, CN, GN, E	P, AMP, Amx, PIP, CB, CFM, CN, GN, E
E 39	P, AMP, Amx, CX, CZ, FUR, CTX, ZOX, CEC, CN, GN, RD, E, TE	-
E 40	P, AMP, Amx, CB, FUR, CFM, CEC, GN	P, AMP, Amx, CB, CEC, GN
E 46	P, AMP, Amx, CX, CF, CZ, FUR, CTX, CAZ, ZOX, CTR, CEC, CN, GN, RD, E, TE	P, AMP, Amx, CTX, CAZ, ZOX, CTR,, CEC, RD, RD,TE
E 49	P, AMP, Amx, CB, CX, CZ, CTX, ZOX, CTR, CFM, CN, TE	P, AMP, Amx, CX, CTX, ZOX, CTR, TE
E 51	P, AMP, Amx, PIP, CF, CZ, ZOX, CN, GN, RD, E, TE	P, AMP, Amx, ZOX, CN, RD, E, TE
E 52	P, AMP, Amx, PIP, CX, CF, CZ, FUR, CTX, CAZ, CTR, CFM, CN, GN, RD, TE	P, AMP, Amx, CX, CZ, FUR, CTX, CAZ, CTR, CN, RD, TE
E 54	P, AMP, Amx, PIP,CB, CF, CZ, FUR, CTX, CAZ, ZOX, CFM, CEC, CN, GN, RD, E	P, AMP, Amx, PIP, CB, FUR, CTX, CAZ, ZOX, CFM, RD, E
E 55	P, AMP, Amx, CX, FUR, ZOX, CN, GN	P, AMP, Amx, CX, ZOX, CN, GN
E 56	P, AMP, Amx, CB, CF, CZ, FUR, CTR, CFM, CEC, GN, E, TE	P, AMP, Amx, CB, FUR, CTR, CEC, GN, E
E 58	P, AMP, Amx, CX, CZ, CTX, ZOX, CFM, CN, TE	P, AMP, Amx, CTX, ZOX, CFM, CN
E 59	P, AMP, Amx, PIP, CX, CF, FUR, CTX, CAZ, ZOX, CTR, CFM, CN, GN, RD, E	P, AMP, Amx, PIP, FUR,CTX,CAZ, ZOX, CTR,CFM, CN, RD,E

* Isolates in bold are ESBL-producers.

However, all the producing isolates belonged to *K. pneumoniae*. Among the three ESBL detection methods mentioned previously, determination of MIC with and without clavulanate, was the most accurate method in detection of ESBL-producing isolates where 8 isolates were obtained (Tables 1, 2).

Results of determination of MICs of ESBL-producer isolates against eight β-lactam antibiotics showed that all 8 ESBL-producing *Klebsiella* isolates were highly resistant for both ampicillin and amoxicillin (Table 4). They

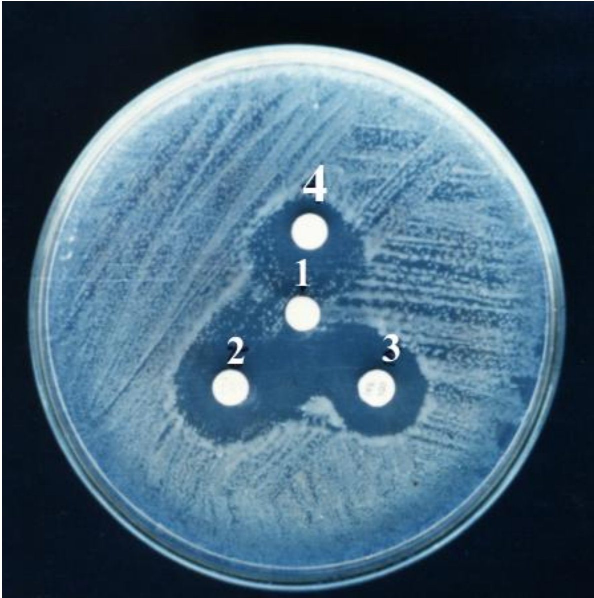

Figure 1. Detection of ESBL production in *Klebsiella pneumoniae* 6 C by disk approximation method: 1. Amoxicillin-clavulanate disk (20/10 µg); 2. Cefotaxime disk (30 µg); 3. Ceftazidime disk (30 µg); and 4. Ceftriaxone disk (30 µg).

were also resistant to ceftazidime, ceftizoxime, and ceftriaxone but not for cefotaxime.

Plasmid DNA studies and bacterial conjugation

In most of *Klebsiella* strains isolated from different environmental and clinical samples, plasmids of different molecular size were found (Figure 2). In several cases different isolates showed very similar plasmid profiles. Among 38 isolates contained plasmids, the presence of transferable R plasmid was detected in 26 (68.4%). Fifteen of these strains harboured a conjugative mega plasmid. The mega plasmid conferring resistance to extended-spectrum cephalosporins was repeatedly found in transconjugants. There was no consistent relationship between plasmid profile and antimicrobial resistance pattern.

Figure 3 shows results of bacterial conjugation of isolate *K. pneumoniae* 6C and E 38 which revealed that all the plasmid bands were transferred from donor to recipient cells. Lane B represents plasmid profile of ESBL-producing strain *K. pneumoniae* 6C, which possesses large and small plasmids. Some plasmids were transferred to the recipient cell (lane C) during conjugation which indicated that the plasmids were conjugative, conferring β-lactamase and ESBL production trait to the recipient cell. The same result was obtained with isolate E 38 which possesses one large plasmid (Lane D) that transferred to the recipient cell (lane E) during conjugation. This result indicates that this large plasmid was a conjugative plasmid conferring resistance to P, AMP, Amx, PIP, CB, CFM, CN, GN, E to the recipient cell (Table 2). It can be concluded from the results above, that the expression of β-lactamase and ESBL production, were plasmid-encoded transferred to the standard strain during conjugation.

The transconjugants were also detected for their ability to produce ESBL by using determination of MIC with and without clavulanic acid. Transferring this plasmid to recipient cells reduced the MIC values for CTX, CAZ, and CTR > 12 fold indicating that these transconjugants were ESBL-producers. The transconjugants expressed their antibiotic resistance when they were able to grow in selective medium containing ampicillin (at final concentration of 100 µg/ml) and sodium azide (at final concentration of 200 µg/ml). The acquisition of ampicillin resistance in recipient cell, in the present study, indicated that this property was plasmid-mediated. These transconjugants were also able to give positive result in β-lactamase production test.

DISCUSSION

The high detection rate of environmental *Klebsiella* spp. strains in sewage samples was expected because these samples provide an excellent growth conditions for these bacteria (Rose and Schreier, 1968; Podschun and Ullmann, 1998). The absence or the low ratios of recovered *Klebsiella* strains in other environmental samples could be due to the low number of samples taken in this study. The absence recovered *Klebsiella* strains in clinical samples of blood, and that of wound, burn, skin, vagina, and throat swaps could be also due to the low number of samples taken in this study.

The relatively high ratio of resistance to β-Lactam antibiotics in primary screening was not attributed only to production of β-lactamase enzyme, but it could be also due to the decreased affinity of the target PBPs (penicillin binding proteins) or decreased permeability of the drug into the cell (Piddock and Wise, 1985; Sanders and Sanders, 1992; Jacoby and Munoz-Price, 2005).

The production of β-lactamase by 58.4% of β-Lactam resistant strains indicates that the enzymatic resistance was prevalent among more than half of β-lactam resistant *Klebsiella* isolates. 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 (Piddock and Wise, 1985; Sanders and Sanders, 1992; Livermore, 1998). Results showed that all 38 β-lactamase-producing *Klebsiella* isolates were found to be multi-drug resistant to least, 8 antibiotics. Antibiotic resistance among isolates of *Klebsiella* in the present study was comparable to reports from other parts of the world, which also revealed multiple drug resistance among gram-negative rods (Oplustil et al., 2001; Winokur et al., 2001).

Table 3. MICs of β-lactam antibiotics with and without clavulanic acid for ESBL-producing *Klebsiella* isolates.

Isolate designation	MICs (mg/L) with and without clavulanic acid								
	Cefotaxime			Ceftazidime			Ceftriaxone		
	Without	With	No. of fold decrease	Without	With	No. of fold decrease	Without	With	No. of fold decrease
K. pneumoniae E 46	16	0.008	12	32	0.004	14	32	0.008	13
K. pneumoniae E 51	32	0.016	12	32	0.004	14	32	0.016	12
K. pneumoniae E 52	32	0.008	13	32	0.008	13	64	0.004	15
K. pneumoniae E 54	16	0.008	12	64	0.002	16	64	0.004	15
K. pneumoniae E 59	16	0.008	12	32	0.008	13	128	0.008	15
K. pneumoniae 6C	64	0.008	14	32	0.002	15	64	0.008	14
K. pneumoniae 21C	16	0.008	12	32	0.002	15	64	0.008	14
K. pneumoniae 22C	32	0.016	12	32	0.002	15	›128	0.008	15

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

Isolate designation	MIC (µg/ml) of:							
	AMP (≥32µg/ml)	Amx (≥32µg/ml)	PIP (≥128µg/ml)	CF (≥32µg/ml)	CTX (≥64µg/ml)	CAZ (≥32µg/ml)	ZOX (≥64µg/ml)	CTR (≥64µg/ml)
K.pneumoniae E 46	> 128	> 128	32	64	16	32	64	32
K. pneumoniae E 51	> 128	> 128	16	32	32	32	32	32
K. pneumoniae E 52	64	64	64	32	32	32	64	64
K. pneumoniae E 54	> 128	64	> 128	32	16	64	> 128	64
K. pneumoniae E 59	> 128	32	64	64	16	32	64	> 128
K. pneumoniae 6 C	64	> 128	> 128	> 128	64	32	> 128	64
K. pneumoniae 21 C	> 128	> 128	32	64	16	32	32	64
K. pneumoniae 22 C	> 128	64	> 128	32	32	32	64	> 128

*Numbers between brackets refer to break points recommended by NCCLS (2003b).
AMP, Ampicillin; Amx, Amoxicillin; PIP, Piperacillin; CF, Cephalothin; CTX, Cefotaxime; CAZ, Ceftazidime; ZOX, Ceftizoxime; CTR, Ceftriaxone.

Only 34.2% of isolates were resistant to piperacillin. Although this antibiotic was first introduced in clinical therapy in 1978 (Fu and Neu, 1978), the low level of resistance to this drug may be attributed to the fact that this antibiotic was recently introduced in Iraq, so the emergence of resistance has not been established yet. One of the most striking findings in present study was the high level of resistance to third generation cephalosporins (3GC) among *Klebsiella* isolates. Half of the isolates were resistant to cefotaxime, and more than 73% of them were resistant to ceftizoxime, but they showed low level of resistance to ceftazidime (18.4%) and ceftriaxone (34.2%). Ceftazidime and cefotaxime resistance are markers for the presence of extended-spectrum β-lactamases (ESBLs).

The prevalence rate of ESBL-mediated resistance to third-generation cephalosporins (3GC) using disk approximation method was 10.5% and this result is higher than those results reported by (Subha and Ananthan, 2002), who found that 6.6% of *Klebsiella* isolates resistant to 3GC antibiotics, were ESBL-producers, but it is much lower than that reported by other studies (Abigail et al., 1995; Desimoni et al., 2004). 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 (2003a). Several studies have also shown that the disk approximation test failed to detect some ESBL-producing strains (Thomson and Sanders, 1992; Coudron et al., 1997).

Results of this study were in agreement with those reported by Bedenic et al. (2001a) who compared five different methods for detection of different types of SHV ESBLs, and they found that the MIC determination of β-lactam with and without clavulanate was the most sensitive method for detection of ESBL-producing strains, regardless to the type of β-lactamase. Although MIC determination of β-lactam with and without clavulanate provides an accurate detection of ESBLs, it is unfortunately time consuming and for that reason it is very rarely used in the clinical laboratories. In addition to that, the disadvantage of all methods relies on enzyme inhibition by clavulanic acid is that the inhibitor resistant

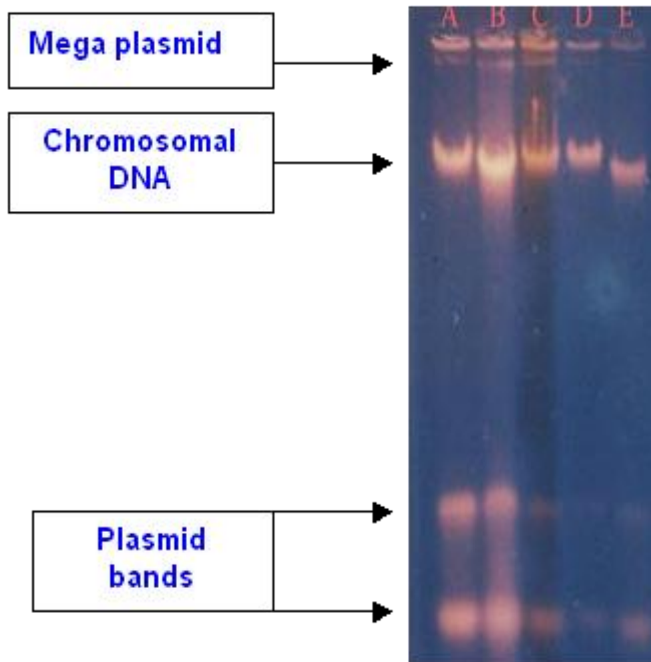

Figure 2. Agarose gel electrophoresis of plasmid profiles of *K. pneumoniae* strains isolated from clinical and environmental samples: Lanes: A, 6 C; B, E 54; C, 22 C; D, E 38; and E, E 40.

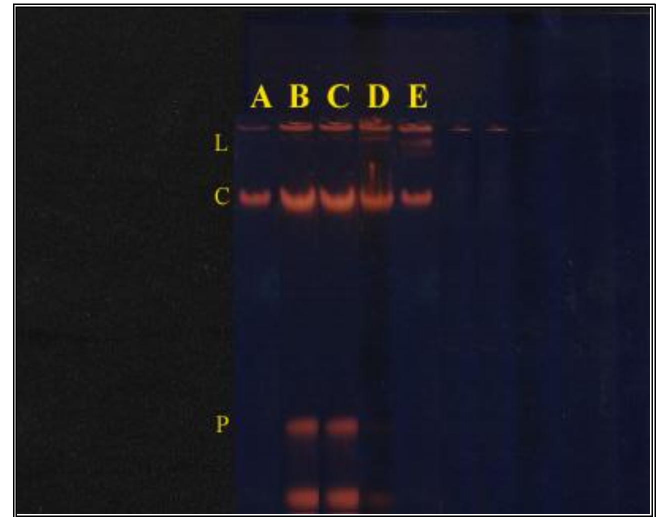

Figure 3. Agarose gel electrophoresis of plasmid DNA from wild type isolates of *K. pneumoniae* and their transconjugants (in *E. coli* J53). Lanes: A, standard strain J53; B, 6 C; C, transconjugant resulting from conjugation between *K. pneumoniae* 6 C with standard strain *E. coli* J53; D, E 38 isolate; E, transconjugant resulting from conjugation between *K. pneumoniae* E 38 with standard strain J53.

groups of ESBLs cannot be detected.

The high MIC values of ESBL-producing *Klebsiella* isolates in the present study suggests that ESBL enzymes are endemic in the study area. Higher MICs of ceftazidime and ceftriaxone compared to those of cefotaxime suggested the ceftazidimase activities of the ESBLs in *Klebsiella* clinical isolates in this study.

Difference in plasmid profiles and in size of plasmids of *K. pneumoniae* isolates in the present study was in agreement with that reported by (Podschun et al., 1986) who showed that plasmids of *K. pneumoniae* isolated from human patients were distributed widely and showed great diversity. The occurrence of conjugative large plasmids in *K. pneumoniae* was reported by several authors who isolated *Klebsiella* strains harboring large plasmids encoding β-lactamase enzyme. In most cases, these plasmids were conjugative type that encoded resistance to multiple antibiotics and metal ions (Yuan et al., 2000; Horii et al., 1993; Kikuchi et al., 1995).

In spite of the wide range of plasmids present in the bacterial isolates from environmental and clinical samples, there was no consistent correlation between plasmid profiles and antibiotic resistance pattern. This is not unexpected since the same antimicrobial resistance pattern can be encoded by unrelated plasmids, transposons, phages and chromosomal genes (Karbasizaed et al., 2003).

The transfer of resistance to the transconjugants is a serious and dangerous indication of emergence of enzymatic resistance among *K. pneumoniae* isolates in the area of study. Results also showed that non-β-lactam antibiotics were also transferred from donor to recipient cells (Tables 1 and 2). Resistance to tetracycline and refampin appeared in all ESBL-producing transconjugants, which indicated that the resistance to these antibiotics was carried on a plasmid that transferred during conjugation. The resistance to tetracycline, refampin, and erythromycin was co-transferred with the gene encoding ESBL resistance, and the genes encoding resistance to these non-β-lactam antibiotics were transferable (mediated by a plasmid) to *E. coli*. Resistance to gentamycin was not appeared in the ESBL-producing transconjugants and was not co-transferred with the gene encoding ESBL resistance which may indicate that this resistance was chromosomal-mediated (Tables 1 and 2).

Several studies reported that the β-lactamase gene, in ESBL-producing *K. pneumoniae* strains, was co-transferred with the non-β-lactam antibiotics (Sirot et al., 1988; Petit et al., 1988; Livermore, 1995). ESBL production is encoded by genes that are prevalently located on large conjugative plasmids and since these plasmids are easily transmitted among different members of the Enterobacteriaceae, accumulation of resistance genes results in strains that contain multi-resistant plasmids. For this reason, ESBL-producing isolates are resistant to a variety of classes of antibiotics (Sirot, 1995; Bradford, 2001).

In conclusion, our data indicate that multi-drug resistance patterns of clinical *Klebsiella* isolates particularly for expanded-spectrum beta-lactams is becoming an impor-

tant problem, especially taking into account the limited choice of antimicrobial agents for treatment and the possibility of transfer of resistance to other enteric organisms.

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