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A Study of Antibiotic Resistant *Klebsiella Pneumoniae* Isolates Producing B-Lactamases in Some Baghdad Hospitals and the Transfer of Antibiotic Resistance by Conjugation

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□ ABSTRACT □

Fifty klebsiella *pneumoniae* isolates from different infections were collected from three hospitals in Baghdad. Sensitivity of those isolates was tested against Ceftazidime. Results revealed that 24 isolate(48%) were resistant to Ceftazidime. Ceftazidime resistant isolates were tested against ten Antibiotics. Results showed that all local isolates were resistant to Cefuroxime and Amoxycillin. 15 isolates (62.5 %) had the ability to produce B-lactamase enzymes and 8 isolates (33.3%) were able to produce Extended-Spectrum B-lactamases (ESBLs). 87.5 % of isolates contained plasmid. Transconjugants were able to resist Cefotaxime, Ceftazidime and Amoxicillin. One transconjugant isolate was able to transfer the production of ESBLs.

Key Words: *Klebsiella pneumoniae*, Ceftazidime resistant, Extended-Spectrum B-lactamases.

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دراسة حول مقاومة بعض عزلات جراثيم *Klebsiella pneumoniae* المنتجة لأنزيمات البيتالاكتاميز لبعض أنواع المضادات الحيوية في عدد من مستشفيات مدينة بغداد وأنتقال صفة المقاومة بالأقتران البكتيري

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□ الملخّص □

جمعت 50عزلة تعود لبكتريا ***Klebsiella pneumoniae*** من إصابات مختلفة من ثلاثة مستشفيات في بغداد. أختبرت حساسية هذه العزلات تجاه مضاد السفتازديم، وأظهرت النتائج وجود (24) عزلة (% 48)مقاومة لهذا المضاد. اختبرت بعدها حساسية العزلات المقاومة للسفتازديم تجاه عشرة مضادات حيوية وقد أظهرت النتائج أن جميع هذه العزلات مقاومة لمضادي السيفيوركسيم والاموكزسلين، وأن أفضل هذه المضادات تأثيرا هي الأمبنيم والسفبيم .وكانت 15عزلة (% 62.5)من العزلات المقاومة للسفتازديم منتجة لأنزيمات البيتالاكتاميز، وأظهرت ثمانية عزلات منها قابليتها على إنتاج أنزيمات البيتالاكتاميز واسعة الطيف (% 33.3)، احتوت معظم العزلات (% 87.5)حزم بلازميدية مختلفة الأحجام.وعند إجراء تجارب الاقتران البكتيري، أظهرت النتائج انتقال صفة المقاومة المتعددة للمضادات الحيوية إذ لوحظت صفة مقاومة السيفوتاكسيم والسفتازديم والأموكزسسلين وصفة إنتاج أنزيمات البتالاكتاميز واسعة الطيف في إحدى العزلات الاقترانية.

الكلمات المفتاحية : كلابسلا نيمونيا، مضاد السفتازديم، البيتالاكتاميز واسعة الطيف.

*مدرس في قسم علوم الحياة □ كلية العلوم □ الجامعه المستنصريه □ بغداد □العراق.

Introduction:

Klebsiella pneumoniae is a common cause of serious nosocomial gram-negative infections, including ventilator-associated pneumonia, urinary tract infection, and bloodstream infection (BSI) [1]. Infections due to *K. pneumoniae* occur in both outbreak settings and settings of endemicity [2].

K. pneumoniae is considered an extracellular pathogen whose virulence is linked with the production of a polysaccharide capsule and type 1 fimbriae [3]. *K. pneumoniae* are becoming increasingly resistant to antibiotics. Infections due to multidrug-resistant *K. pneumoniae* are associated with increased morbidity and mortality compared to susceptible strains [4,5].

Since 1982, strains that produce Extended-Spectrum ß-lactamases (ESBLs), which render them resistant to extended-spectrum cephalosporins, have evolved [6,7]. The hallmark of these strains, resistance to ceftazidime, is observed in both *K. pneumoniae* and *K. oxytoca* isolates [8]. In U.S. intensive care units (ICUs); 20.6% of *K. pneumoniae* infections were resistant to expanded-spectrum cephalosporins in 2003. Compared to the mean rate of resistance for the preceding 5 years, resistance to expanded-spectrum cephalosporins among tested *K. pneumoniae* isolates increased 47% in 2003 [2].

Over the last 20 years, there has been an increasing resistance to ß-lactams because of the secretion of extended-spectrum ß-lactamases (ESBLs) mediated by plasmids [9,10]. This type of resistance is now observed in all species of *Enterobacteriaceae* and is currently disseminated throughout the world [11].

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. For this reason, ESBL-producing isolates are resistant to a variety of classes of antibiotics. Moreover, the emergence of these multiply resistant *Klebsiella* strains is unfortunately accompanied by a relatively high stability of the plasmids encoding ESBLs [6,12].

The aims of this study:

The aims were to determine the distribution of Ceftazidime -resistant **K. pneumoniae** isolates producing ESBLs in Baghdad hospitals, and studying the transfer of genetic determinantes for Ceftazidime and ESBLs production and multiple antibiotic resistance during the conjugation process.

Materials and Methods:

1) **Clinical isolates:** Fifty Clinical *K. pneumoniae* isolates were collected from different infections sources from three hospitals in Baghdad (Central Child hospital, Central Medicine City hospital and Al-Noman hospital). Isolates were identified according to [13] by classical microbiological methods and API 20-E system.

2) **Antimicrobial susceptibility:** Sensitivity of *K. pneumoniae* isolates was tested against Ceftazidime by disk diffusion testing on Muellar-Hinton agar. All Ceftazidime -resistant *Klebsiella pneumoniae* isolates were tested for susceptibility by disk diffusion testing on Muellar-Hinton agar by use of the antibiotics: Amoxicillin(AML),Cefotaxime(CTX),Imipenem(IPM),Cefipeme(FEP), Ciprofloxacine(CIP), Aztreoname(ATM), Cefuroxime(CXM), Ceftriaxone(CRO),

Pipracillin(PRL), and Gentamicin(G).

3) **Detection of beta-lactamases:** The ability of Ceftazidime - resistant *K. pneumoniae* Isolates for beta-lactamases production were tested. Iodometric method described by [14] was used as follows: Test *K. pneumoniae* were removed with a loop from an overnight culture on solid medium and suspended with Penicillin solution, at 1 h. two drops of starch indicator were added to the suspension, followed by one drop of iodine reagent and were mixed thoroughly. A blue colour developed immediately, persistence of the blue colour for longer than 10 min. constitutes a negative result.

4) **Detection of Extended-Spectrum beta-lactamases (ESBLs):** This test was done according to [15] by using a clavulanate double –disk diffusion method: synergy between cefotaxime and Clavulanate was detected by placing a disk of amoxicillin / clavulanate (20 µg/10 µg. respectively) and a disk of cefotaxime(30 µg), 30 mm a part (center to center) on an inoculate agar plate. A clear extension of the edge of the cefotaxime inhibition zone toward the disk containing Clavulanate was interpreted as synergy, indicating the presence of an ESBLs.

5) **Hemolysin production:** The ability of Ceftazidime - resistant *K. pneumoniae* Isolates for hemolysin production were tested according to [16]. The washed human RBCs(5%) was used to prepare blood agar medium in order to observe the capability of isolates to lyse the blood. All the isolates were cultured on previously the prepared blood agar medium and incubated at 37 C for 24 h.

6) **Adherance factors:** Ceftazidime – resistant *K. pneumoniae* Isolates were investigated for their ability to adhere with the epithelial cells according to [17] by using human uro-epithelial cells.

7) **Haemoagglutination test:** This test was done according to [18] by slide agglutination test. 0.05 ml of test *K. pneumoniae* suspension were mixed with 0.05 ml of human RBCs (3 %), haemoagglutination was observed under light microscope.

8**) Plasmid extraction**: Plasmid DNA was extraction according to [19] by Alkaline method. Electrophoresis was conducted at 5 V/cm in TBE buffer. Plasmid DNA bands were observed under U.V. light (Transilluminator) with wave length of 340 n.m.

9) **Bacterial conjugation:** The Bacterial conjugation tests were done between Ceftazidime - resistant *K. pneumoniae* isolates as a donor cells and *E. coli* MM 294 as a recipients cells according to [20]. Recipients cells, un like donor cells, grew onto culture media containing only 100 µg /ml Rifampicin, while donor cells grew onto 100 µg /ml of another antibiotic that they resist, but recipients cells was unable to grow onto this media. The frequency of conjugation was calculated as follows:

Conjugation frequency = transconjugants cells / recipients cells

Results and Discussion:

Infections caused by Extended-Spectrum-ß-lactamases (ESBLs)-producing *Klebsiella pneumoniae* isolates are a major concern for clinicians, since they markedly increase the rates of treatment failure and death [6]. Sensitivity of 50 *Klebsiella pneumoniae* isolates was tested against Ceftazidime, results revealed that 24 isolate (48 %) were resistant to Ceftazidime

A total of 24 Ceftazidime - resistant *Klebsiella pneumoniae* isolates were tested against (10) antibiotics. The percentages which were resistance were as follows: for amoxicillin and Cefuroxime, 100%; Ceftriaxone, 95.8%; Pipracillin,62.5%; Gentamicin,58.3%;Cefotaxime, 50%,Ciprofloxacine,41.6%; Aztreoname,25%; Cefipeme, 16.6 % and no Imipeneme resistance found in this survey' s isolates (figure 1).

Imipenem and Cefepime were found to be the most effective two agents against the isolates,and all the isolates have showen multiple resistance for antibiotics.

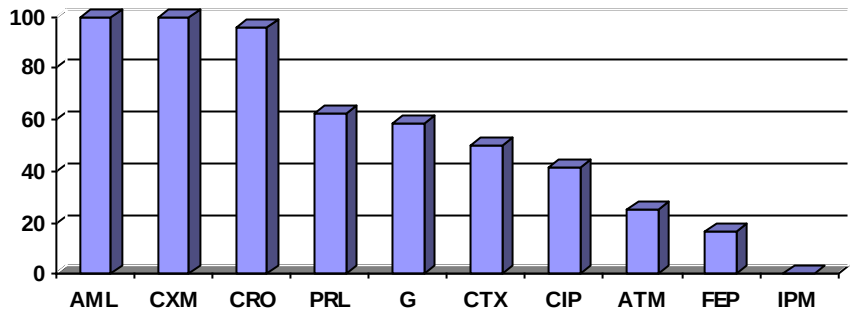
Ceftazidime- resistant *K. pneumoniae* is increasing in prevalence, and infections caused by this organism present a therapeutic challenge. While expanded-spectrum cephalosporins remain an important choice for the treatment of many gram-negative infections, these agents are not appropriate choices for the treatment of Ceftazidime-resistant *K. pneumoniae*. Currently, many consider carbapenems to be the treatment of choice for infections due to ESBL-producing Ceftazidime- resistant *K. pneumoniae*. Increasing drug resistance trend has been reported earlier by (4, 5). Our results, however, were similar to those of [21].

The results showed also that 15 Ceftazidime- resistant *K. pneumoniae* isolates (62.5 %) had the ability to produce B- lactamase enzyme, 8 isolates (33.3 %) were able to produce ESBLs.

Since ESBL production frequently is accompanied by multi-resistance to antibiotics, therapeutic options become limited. In 1988, isolates of *Klebsiella pneumoniae* from China which contained SHV-2 were reported, further reports of other SHV-2-producing organism in China occurred in 1994. In a major teaching hospital in Beijing, 27% of *Escherichia coli* and *Klebsiella pneumoniae* blood culture isolates collected from 1997 through 1999 were ESBL producers [15].

By the early 1990s, 25 to 35% of nosocomially acquired *Klebsiella pneumoniae* isolates in France were ESBL producing. However, it has been reported that 36.1% of *Klebsiella pneumoniae* isolates collected in a single South African hospital in 1998 and 1999 were ESBL producers. ESBLs have also been documented in Saudi Arabia, and a variety of North African countries [15]. Rates of ESBL production by *Klebsiella pneumoniae* clearly differ from hospital to hospital.

Resistance%



-Antibiotics-

Figure (1): Antibiotic resistance of Ceftazidime- resistance *K. pneumonia* isolates.

On the other hand, results showed that all Ceftazidime- resistance *K. pneumonia* isolates have ability for haemoagglutination and able to adhere the epithelial cells. None of these isolates were able to produce hemolysis on blood agar containing human RBCs.

In our study, DNA analysis showed that 87.5 % of the isolates contained plasmid of different molecular weights (figure 2).

However, isolates no. 1,12,19,13,23,24,9 harboured a single plasmid bands, while Isolates no. 5,11,16 don't harboured any plasmid bands, another isolates harboured more than one plasmid bands (Table 1). Moreover, again it seems interesting that Ceftazidime-resistance *K. pneumonia* isolates showed previously multiple antibiotic resistance in addition to production of B-lactamase, ESBLs enzyme and some virulence factors (hemolysin, adherence factors). So, we can conclude that in our local isolates, the genetic determinants may be carried on the same plasmid.

Regarding plasmids existence, our finding may be of epidemiological significance since the high possibility of dissemination of these genetic elements among other microbial populations.

Plasmid conjugation is an important mechanism of disseminating drug resistance among bacterial populations; conjugations are a convenient method of transferring drug resistant genetic determinants among intra and inter generic bacterial populations. During the present study the transconjugants formed during the conjugation process had shown the stable transfer of the resistance markers of Ceftazidime, Cefotaxime and Amoxicillin, One isolate was able to transfer the production of ESBLs. All these resistance characters were transferable to *Escherichia coli* by conjugation, and the frequencies of conjugation ranged from 10^{-5} to 10^{-4} (Table 2). Similar results were showed by (12) which indicated that the multiple resistances were mediated by a 95kb plasmid-mediated ß-lactamase with a pI of 6.3 that was much more active against third-generation cephalosporins.

Table (1) : Plasmid content for Ceftazidime- resistance *K. pneumonia* isolates.

Plasmid content	***K. pneumonia* isolate**	**No. (%)**
Without plasmid band	K5,K 11, K16	3 (12.5%)
One Plasmid band	K1, K12, K19,K13,K23,K24,K9	7 (29.17%)
More than one plasmid	K6, K20, K3,K8, K2,K15,K21,K29,K31,K33,K18,K30, K28,K29	14 (58.33%)

K= *Klebsiella pneumoniae* isolate

The ESBLs are frequently plasmid encoded [22]. Plasmids responsible for ESBLs production frequently carry genes encoding resistance to other drug classes (for example, aminoglycosides) [15]. A recent report from Taiwan has described a *Klebsiella pneumoniae* isolate with a novel IMP-type carbapenemase (IMP-7), which was encoded on a plasmid also harboring genes encoding TEM-1 and the ESBLs SHV-12. Transfer of genotypically related ESBLs from hospital to hospital within a single city from city to city and from country to country, has been documented. A notable clone has been an SHV-4-producing, serotype K-25 isolate of *Klebsiella pneumoniae* which has spread to multiple hospitals in France and Belgium [15].

Table (2) : Frequency of conjugation of selected isolates.

Isolate No.	**Frequency of conjugation**
K1	4×10^{-5}
K12	3×10^{-5}
K 20	2×10^{-5}
K 6	1.2×10^{-5}
K 13	1×10^{-5}
K 19	3×10^{-4}
K 8	2.2×10^{-4}
K 3	1×10^{-4}

K = *Klebsiella pneumoniae* isolate

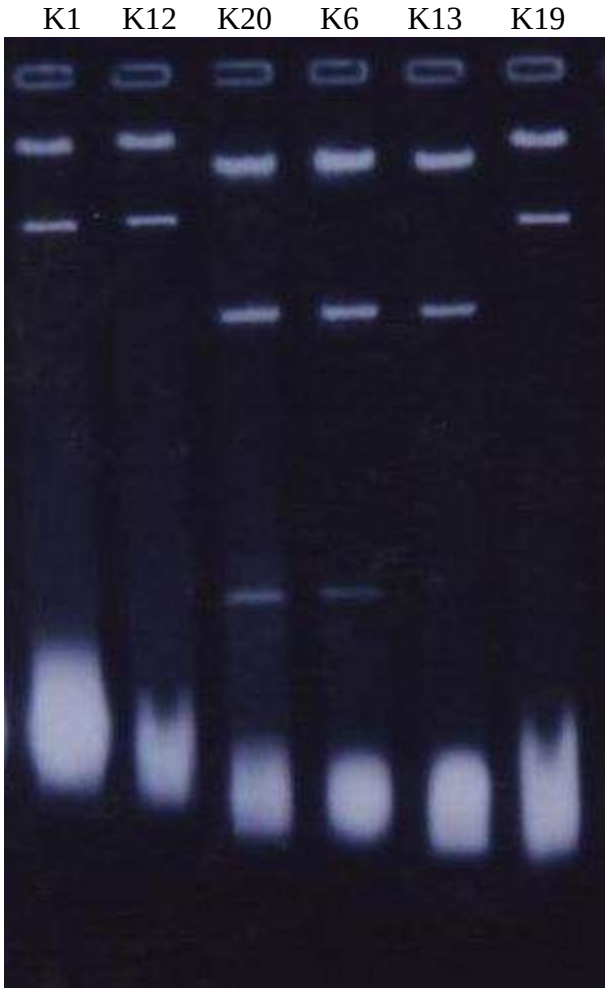


Figure (2) : Plasmid profile for Ceftazidime- resistance *K. pneumonia* isolates.

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