Mechanism of ESBL Production of *Klebsiella pneumoniae* Isolated from Egypt

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1. Abstract: Extended spectrum Beta-lactamases (ESBL) of *Klebsiella pneumoniae* was studied by investigation the plasmid mediated AmpC, and genes of SHV and TEM enzymes as well as INT and Delta T-cells virulence genes. Out of 92 *Klebsiella pneumoniae* strains 26 (23.2%) were plasmid mediated AmpC and 10 (10.9%) strains were ESBLs producer. Four clusters of either two or three strains demonstrated more than 50% similarity in their PFGE patterns. The strains with similar PFGE patterns did not have any relationship with each other in time and place of occurrence.

[Hesham M. Mahdy, Abdel-Moniem M. Sharaf, Mahmoud M. Al-Aaser and Hamdy M. El-Sayed. **Mechanism of ESBL Production of *Klebsiella pneumoniae* Isolated from Egypt.** *N Y Sci J* 2013;6(1):1-8]. (ISSN: 1554-0200). http://www.sciencepub.net/newyork. 1

Keywords: ESBL, *Klebsiella pneumoniae*, plasmid mediated AmpC, PFGE

2. Introduction

Beta-lactamases represent the most common mechanism of β-lactam resistance. Extended-spectrum β-lactamases (ESBLs) represent a major group of β-lactamases currently being identified worldwide in large numbers along with inducible AmpC β-lactamases and derepressed mutants. β-lactamases continue to be the leading cause of resistance to β-lactam antibiotics in Gram negative bacteria (Bradford, 2001).

Acquired resistance to β-lactams is mainly mediated by ESBLs that confer bacterial resistance to all β -lactams except carbapenems and cephamycins, which are inhibited by other β -lactamase inhibitors such as clavulanic acid. The most well-known of the "newer" β-lactamases was first described in 1983 and have been named the ESBLs. These enzymes have the ability to hydrolyze the penicillins, cephalosporins, and monobactams, but not the cephamycins and carbapenems, and are inhibited by "classical" β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Lascols *et al*., 2012).

Plasmid is extrachromosomal small, ring-shaped molecule of DNA present in almost all bacteria. Although most of them are covalently closed circular double strand DNA molecules, now linear plasmids have been isolated from different bacteria (Letunic, 2007). Resistance plasmids enable bacteria to degrade or inactivate antibiotics used to halt bacterial growth. Resistance plasmids are currently a topic of intense research because of the growing problem with disease-causing bacteria that are resistant to penicillin and other commonly used antibiotics. This process may be repeated several times, giving rise to a plasmid that will make bacteria resistant to a number of different antibiotics. Infections caused by such bacteria are extremely difficult to treat.

ESBL enzymes are plasmid mediated, TEM and SHV derived enzymes, resulted of mutations of TEM-1, TEM-2 and SHV-1 enzymes which confer high level resistance to early penicillins and low-level resistance to first generation of cephalosporins. Widespread use of third generation cephalosporins is believed to be the major cause of the mutations in these enzymes that has led to the emergence of the ESBLs (Nathisuwan *et al*., 2001). However, ESBL enzymes of *Klebsiella pneumoniae* are plasmid mediated or genes of SHV and TEM enzymes, and INT and Delta T-cells virulence genes or both of them could be ascertain in this paper.

2. Materials and Methods

2.1. Sources of organisms: A total of 112 *Klebsiella pneumoniae* strains were obtained from different clinical specimens as described before in Mahdy *et al.* (2012).

2.2. Screening of ESBLs: Screening the presence of Extended Spectrum β-Lactamases (ESBLs) was done by double disc synergy test and Vitek machine according to CLSI guidelines, 2008. Sixteen antibiotics were tested: amikacin (AK), ampicilin (AMP), ampicillin/sulbactam (SAM), aztreonem (ATM), cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone (CRO), cephalothin (CF), chloramphinicol (C), ciprofloxacin (CIP), gentamycin (CN), imipenem (IPM), nalidixic acid (NA),

tetracycline (TE), ticracillin/clavulanic acid (TIM) and trimethoprim/ sulphamethoxazole (SXT).

2.3. Mechanism of ESBL production: It may be necessary to screen the strains for the presence of plasmid mediated AmpC from ESBLs to avoid therapeutic failures and to implement appropriate infection control measures. A simple application of phenotypic method was used for detection of the mechanism of ESBL production. We have used some criteria for deciding an organism to be either ESBL producer; Plasmid mediated AmpC β-Lactamase or a derepressed mutant. *Klebsiella pneumoniae* ATCC 700603 was used as quality control for ESBL test as described by National Committee for Clinical Laboratory Standards (NCCL, 2001).

Procedure: This test was applied for strains which were positive for ESBLs production by Vitek ESBL test. Using the standard disc diffusion method to apply the discs of cefoxitin 30µg (FOX) and cefipime 30µg (FEP). The data recorded for antimicrobial discs testing and also data for Vitek ESBL test were collected. Resistance to cefotaxime would suggest being either a derepressed AmpC or an ESBL producer. Combination with clavulanic acid bringing it back to completely susceptible level would indicate an ESBL alone. If there is an improvement with clavulanic acid, but not to the completely susceptible range, it would suggest either a derepressed AmpC + ESBL or could also suggest the presence of an ESBL with several other (non-AmpC) enzymes. If a strain is susceptible to cefepime and resistant to cefotaxime and cefoxitin then AmpC is a likely player. If it is cefotaxime resistant, cefoxitin sensitive and cefipime resistant or sensitive then probably it is an ESBL producer.

Interpret the results: Strains with zone diameters <18mm of both 30 µg cefoxitin disc and 30µg cefipime were considered resistant.

a. **ESBL producer:** Zone diameters for various 3rd generation cephalosporins, susceptible to cefoxitin and increasing in zone size with addition of an inhibitor by ≥ 5 mm.
b. **Plasmid mediated AmpC β-Lactamase:** Resistant to cefoxitin, blunting of zone towards inducer, no increase in zone size with addition of an inhibitor, and susceptible to cefipime.
c. **Derepressed mutants:** Resistant to cefoxitin and cefotaxime and no increase in zone size with addition of an inhibitor.
d. **Multiple mechanisms:** Resistant to cefoxitin, blunting of zone towards inducer and increase in zone size with addition of an inhibitor by ≥ 5 mm.

2.4. Plasmid patterns and analysis of some multi-resistant bacterial isolates: This test was carried out for the plasmid mediated AmpC β-Lactamase (Resistant to cefoxitin, blunting of zone towards inducer, no increase in zone size with addition of an inhibitor and susceptible to cefipime).

Procedure: The isolation of plasmid was carried out using high pure plasmid isolation kit (Qiagen, British) which includes the following components: suspension buffer, RNase A (dry powder), lysis buffer, binding buffer, wash buffer I, wash buffer II, elution buffer, high pure filter tubes and collection tubes. Electrophoresis of plasmid DNA was done on a horizontal gel apparatus (Sambrook *et al*., 1989).

Agarose gel electrophoresis for isolation of plasmid DNA:

Ultra-pure agarose; ethiduim bromide; ethylene diamine tetra acetic acid (EDTA); Tris-base; boric acid (Amresco, USA); DNA Step Ladder 50bp (15 fragments with molecular weight ranged from (250–12000) bp precisely 250–1000 bp by 250, to 12000 by 1000) was purchased from Sigma; Tris– borate buffer (10X) and Tris Boric EDTA (TBE) buffer (Tris-base 59 g, Boric acid 27.5 g and EDTA 20ml of 0.5 M (pH: 8.3).

Agarose (1%) in 1X TBE buffer was prepared. Ten microliters of plasmid DNA and 2µl of loading buffer dye were mixed well and loaded into the gel containing 10µl ethiduim bromide (1µg/ml in water). The electrophoresis was conducted for 90–120 min. at constant voltage 75V in trisborate buffer according to the method of Meyers *et al*. (1976). The gel was examined on UV transilluminator (Cole-Parmer, USA) at wavelength 312nm. Photography was carried out by a Polaroid Camera (DS–34 Polariod, USA) with Digital 0.01 g balance model SBA 51 (Scaltec, Germany). The data obtained from the scanning process of each gel were analyzed using (Gel documentation system (Alpha-chem Imager, USA) determine the degree of similarity and dissimilarity between the plasmid profile of the different tested isolates.

2.5. Genotypic analysis for TEM, SHV, INT and Delta T-cells genes by Polymerase Chain Reaction (PCR): PCR were performed to determine the presence of plasmid mutation of SHV and TEM enzymes and presence of INT and Delta T-cells virulence genes of the tested strains. DNA extraction was performed by using the QIAamp DNA Blood Mini Kit (QIAGEN, USA).

Gel electrophoresis: Gel electrophoresis was performed in power pac 300 (BIORAD, Hercules, CA, USA) at 100 volt for 40 min. In the presence of 100 pb DNA ladder as a marker, the PCR product was electrophoresed in 1.5% low malt agarose gel using Tris/Borate/EDTA (TBE) buffer and stained with ethidium bromide. The product was visualized under ultraviolet light and photographed by a digital

camera (Kodak DC 290) as described by Sambrook *et al* (1989). DNA molecular weight marker 100 pb Ladder was obtained from BIORAD laboratories, Hercules, CA, USA.

2.6. Genotypic analysis relationships of ESBL-producing strains by Pulsed-Field Gel Electrophoresis (PFGE): Pulsed-field gel electrophoresis (PFGE) was performed with a CHEF Mapper XA system (Bio-Rad Laboratories, Inc.), as described by Gautom (1997).

3. Results

3.1. The mechanism of ESBL production: Out of 92 *Klebsiella pneumoniae* strains which were positive screening of ESBL production, 48(52.2%) were derpressed mutants while 10(10.9%) were plain ESBL producers and 26(28.3%) were plain for the presence of Plasmid mediated AmpC β-Lactamase (Table 1).

Table 1. Screening for the mechanism of ESBL production.

Percentage of different mechanisms of β-lactam resistance				
No. of tested strains	Plasmid mediated AmpC β-Lactamase	ESBL producers	ESBL producers + Derepressed mutant	Derepressed mutant
92	26(23.2%)	10(10.9%)	8(8.7%)	48(52.2%)

3.2. Plasmid profiles analysis of the isolated bacteria:

According to the results of screening for the mechanism of ESBL production that showed 26(23.2%) were plain for the presence of Plasmid mediated AmpC β-Lactamase (Table 1) and also, the results of antibiograms of the majority of the tested strains (Mahdy *et al.* 2012), their plasmid's profiles were studied. The plasmid of antibiotics resistant 26 bacterial strains were extracted as and then analyzed in agarose gel electrophoresis for detection the differences among the plasmid profiles among the isolated bacteria (Figures 1 and 2). The obtained gel electrophoretic photograph was subjected to the scanning program to determine the degree of similarity between the plasmids profile of the different tested bacterial isolates.

Manual scoring of the isolated plasmid DNA indicated that only one DNA band are observed for each twenty six bacterial strains, out of ninety two bacteria have been isolated during the present study. The results obtained in Figure (1) and Table (2) showed that differences on the plasmid profiles of these strains according to the scoring process based on the scanning program. Four plasmids particles are observed in the first set of bacterial isolates with different molecular weights as follows: 13662.67 (K-1, H-11, D-47, H-19, K-8, H-2, H-25 & H-5), 13961.37 (K-36, H-4, K-44, D-39, D-6, H-43, D-41 & D-3), 14266.60 (K-19 & D-25) & 13370.36 (D-21).

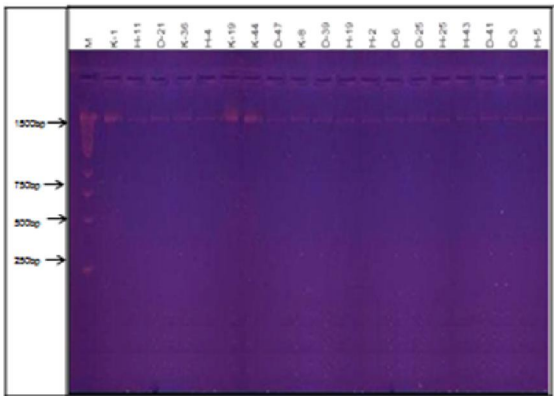

Figure 1. Electrophoretic micrograph of the extracted plasmids DNA from the bacterial isolates (first group).

Table 2. Properties of the bacterial isolates according to their plasmid profile analysis and antibiotics resistance (first group).

Lane	Bacterial strain	R.F	M.W	Antibiotic resistance
K-1	*Klebsiella pneumoniae*	0.0898	13662.67	Ampicillin
H-11	*Klebsiella pneumoniae*	0.0898	13662.67	Ampicillin
D-21	*Klebsiella pneumoniae*	0.0939	13370.36	Ampicillin
K-36	*Klebsiella pneumoniae*	0.0857	13961.37	Ampicillin
H-4	*Klebsiella pneumoniae*	0.0857	13961.37	Ampicillin
K-19	*Klebsiella pneumoniae*	0.0816	14266.60	Ampicillin
K-44	*Klebsiella pneumoniae*	0.0857	13961.37	Ampicillin
D-47	*Klebsiella pneumoniae*	0.0898	13662.67	Ampicillin
K-8	*Klebsiella pneumoniae*	0.0857	13961.37	Ampicillin
D-39	*Klebsiella pneumoniae*	0.0898	13662.67	Ampicillin
H-19	*Klebsiella pneumoniae*	0.0898	13662.67	Ampicillin
H-2	*Klebsiella pneumoniae*	0.0898	13662.67	Ampicillin
D-6	*Klebsiella pneumoniae*	0.0857	13961.37	Ampicillin
D-25	*Klebsiella pneumoniae*	0.0816	14266.60	Ampicillin
H-25	*Klebsiella pneumoniae*	0.0898	13662.67	Ampicillin
H-43	*Klebsiella pneumoniae*	0.0857	13961.37	Ampicillin
D-41	*Klebsiella pneumoniae*	0.0857	13961.37	Ampicillin
D-3	*Klebsiella pneumoniae*	0.0857	13961.37	Ampicillin
H-5	*Klebsiella pneumoniae*	0.0898	13662.67	Ampicillin

Based on the data shown in Figures (2) and Table (3), four different plasmids of different molecular weights as follows: 13247.67 (H-46, H-12 & D-12), 13735.03 (D-43), 13038.24 (H-28 & H-50) and 13052.52 (H-37).

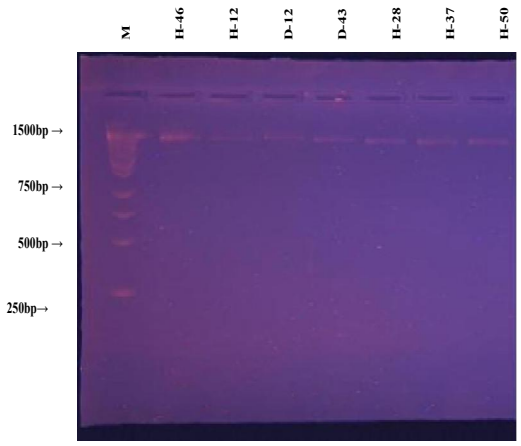

Figure 2. Electrophoretic micrograph of the extracted plasmids DNA from the bacterial isolates (second group).

Table 3. Properties of the bacterial isolates according to their plasmid profile analysis and antibiotics resistance (second group).

Lane	Bacterial strain	R.F	M.W	Antibiotic resistance
H-46	*Klebsiella pneumoniae*	0.0743	13247.67	Ampicillin
H-12	*Klebsiella pneumoniae*	0.0743	13047.67	Ampicillin
D-12	*Klebsiella pneumoniae*	0.0743	13147.67	Ampicillin
D-43	*Klebsiella pneumoniae*	0.079	13735.03	Ampicillin
H-28	*Klebsiella pneumoniae*	0.0941	13038.24	Ampicillin
H-37	*Klebsiella pneumoniae*	0.099	13052.52	Ampicillin
H-50	*Klebsiella pneumoniae*	0.0941	13038.24	Ampicillin

3.3. Genotypic analysis for SHV and TEM enzymes; and INT and Delta T-cells virulence genes by using Polymerase Chain Reaction (PCR):

PCR were performed to determine the presence of plasmid mutation of SHV and TEM enzymes and presence of INT and Delta T-cells virulence genes of the *K. pneumoniae* strains. From previous results (Mahdy *et al*., 2012), it was found that 76 (67.86%) strains were positive for ESBLs; 60 (53.57%) strains were resistant for equal or more than eight antibiotics of different groups including cefotaxime, ampicillin, cephalothin, ceftriaxone, tetracycline. Only ten strains (8.93%) are ESBLs producers and have all these virulence factors, which selected for genotypic analysis (Table 4).

PCR demonstrate different banding patterns of *K. pneumoniae* strains for genes of SHV and TEM enzymes, and INT and Delta T-cells virulence genes (Figures 3, 5, 7, and 9), and show dissimilarities in dendograms in figures (4, 6, 8, and 10) respectively. Lane 1, 100 pb DNA ladder; lane 2, blood culture isolate (D-2) showing pattern II; lanes 3 and 4, urine culture isolates (K-29, K-43) showing pattern III and IV; lane 5, blood culture isolate (H-14) showing pattern V; lane 6, wound swab isolate (K-6) showing pattern VI; lane 7, sputum culture isolate (H-30) showing pattern VII; lane 8, urine culture isolate (D-45) showing pattern VIII; lane 9, pus culture isolate (D-38) showing pattern IV; lanes 10 and 11, blood culture isolates (D-27, K-13) showing pattern XIII and XI.

Table 4. Collects the data of strains that selected for genotypic analysis.

Antibiotic	Strain codes									
	D-2	K-29	K-43	H-14	K-6	H-30	D-45	D-38	D-27	K-13
AK*	I	S	R	S	S	S	R	S	S	S
SAM	R	R	R	R	R	R	R	R	R	S
AMP	R	R	R	R	R	R	R	R	R	R
ATM	R	R	R	R	R	R	I	R	I	R
CTX	R	R	R	R	R	R	R	R	R	R
CAZ	I	R	R	R	R	R	S	I	S	R
CRO	R	R	R	R	R	R	R	R	R	R
CF	R	R	R	R	R	R	R	R	R	R
C	S	S	R	R	S	S	R	R	S	R
CIP	R	S	R	R	S	R	S	R	R	S
CN	R	R	R	R	R	S	S	R	S	S
IPM	S	S	S	S	S	S	S	S	S	S
NA	R	S	R	R	S	R	S	R	R	I
TE	R	R	R	R	R	R	R	R	R	R
TIM	R	R	R	R	R	R	I	R	R	I
SXT	S	S	R	R	S	R	I	R	R	S
SBLs producer	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve

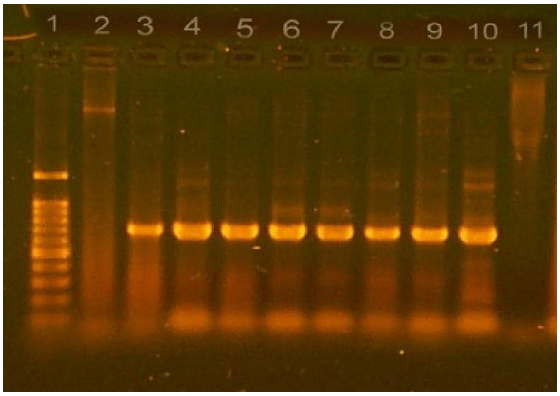

Figure 3. PCR patterns for SHV gene of *K. pneumoniae* strains.

A phenetic dendrogram in figure (4) represent a correlation similarity between bands of SHV gene in 10 tested *K. pneumoniae*

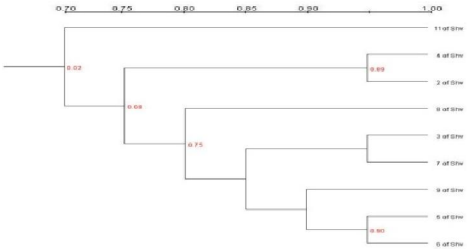

Figure 4. Dendrogram of PCR results for SHV gene from 10 isolates of multi-antibiotic resistant *K. pneumoniae*. Strains were clustered by Dice Coefficient. The scales indicate the percentage of genetic similarity.

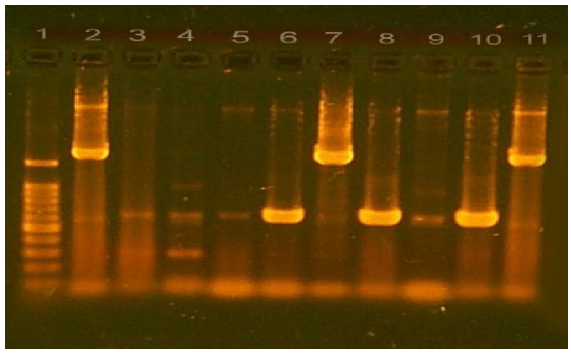

Figure 5. PCR patterns for TEM gene of *K. pneumoniae* strains.

A phenetic dendrogram in figure (6) represent a correlation similarity between bands of TEM gene in 10 tested *K. pneumoniae*

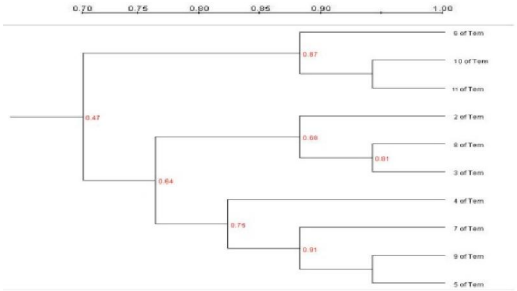

Figure 6. Dendrogram of PCR results for TEM gene from 10 isolates of multi-antibiotic resistant *K. pneumoniae*. Strains were clustered by Dice Coefficient. The scales indicate the percentage of genetic similar.

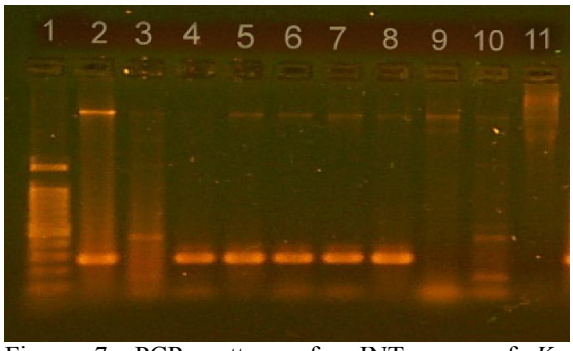

Figure 7. PCR patterns for INT gene of *K. pneumoniae* strains.

A phenetic dendrogram in figure (8) represent a correlation similarity between bands of of INT gene in 10 tested *K. pneumoniae*

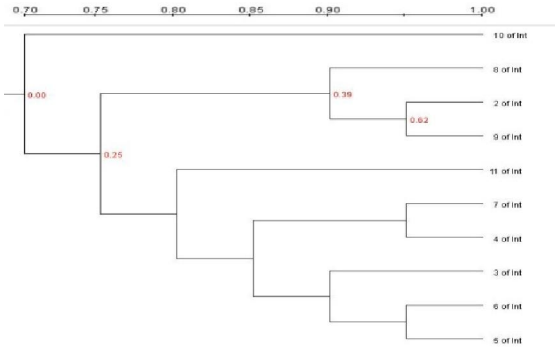

Figure 8. Dendrogram of PCR results for INT gene from 10 isolates of multi-antibiotic resistant *K. pneumoniae*. Strains were clustered by Dice Coefficient. The scales indicate the percentage of genetic similarity.

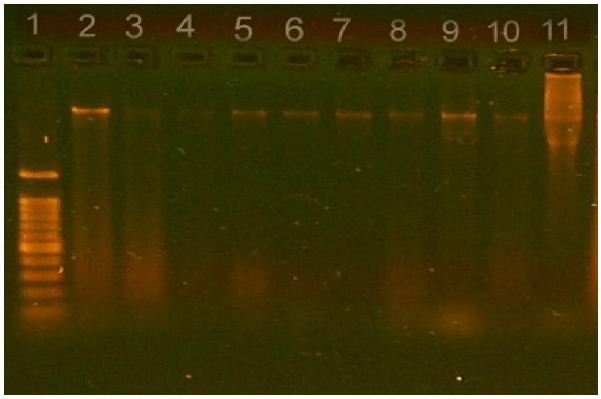

Figure 9. PCR patterns for Delta gene of *K. pneumoniae* strains.

A phenetic dendrogram in figure (10) represent a correlation similarity between bands of Delta gene in 10 tested *K. pneumoniae*.

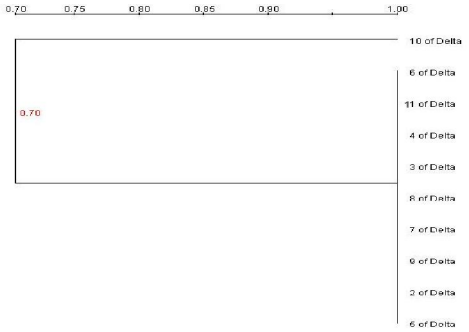

Figure (10): Dendrogram of PCR results for Delta gene from 10 isolates of multi-antibiotic resistant *K. pneumoniae*. Strains were clustered by Dice Coefficient. The scales indicate the percentage of genetic similarity.

The results of genotypic analysis by PCR for plasmid mutation of SHV and TEM enzymes and presence of INT and Delta T-cells virulence genes of the *K. pneumoniae* are shown in Table (5).

Table 5. Genotypic analysis for SHV and TEM enzymes and INT and Delta T-cells virulence genes by PCR.

Lane sequence	Code N$^{o.}$	SHV	INT	TEM	Delta
2	D-2	-ve	+ve	+ve	-ve
3	K-29	+ve	-ve	-ve	-ve
4	K-43	+ve	+ve	-ve	-ve
5	H-14	+ve	+ve	-ve	-ve
6	K-6	+ve	+ve	+ve	-ve
7	H-30	+ve	+ve	+ve	-ve
8	D-45	+ve	+ve	+ve	-ve
9	D-38	+ve	-ve	-ve	-ve
10	D-27	+ve	-ve	+ve	-ve
11	K-13	-ve	-ve	+ve	-ve
% of total positive genes		80%	60 %	60 %	0 %

* +ve, positive gene analysis; -ve, negative gene analysis.

3.4. Genotypic analysis relationships of ESBL-producing strains by using Pulsed-Field Gel Electrophoresis (PFGE):

The selected 10 strains from bacterial isolates of patients demonstrated different PFGE patterns. The patterns of the strains are illustrated in Figure (11) and the dendrogram of the same strains is shown in Figure (12). Four clusters of either two or three strains demonstrated more than 50% similarity in their PFGE patterns, i.e., strains N$^{o.}$ 3, 8, and 9; N$^{o.}$ 5 and 10; N$^{o.}$ 4 and 7; and N$^{o.}$ 2, 6, and 11. However, the strains with similar PFGE patterns did not have any relationship with each other in time and place of occurrence.

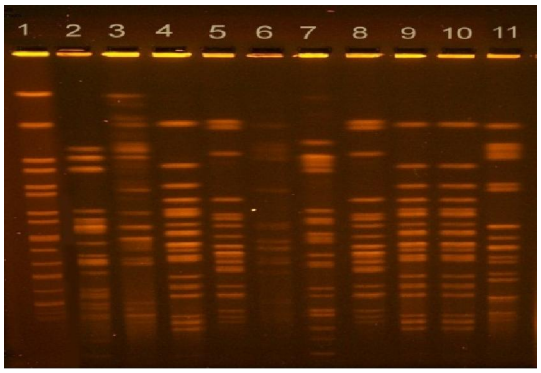

Figure 11. PFGE patterns of XbaI-digested genomic DNA from *K. pneumoniae* strains. Lane 1, lambda DNA concatemer standard.

A phenetic dendrogram in figure (12) represent a correlation similarity between bands of PFGE in 10 tested *K. pneumoniae*

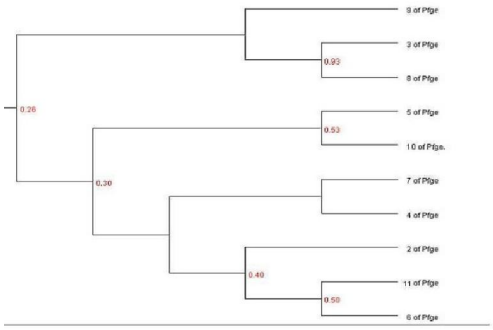

Figure 12. Dendrogram of PFGE results from 10 isolates of multi-antibiotic resistant *K. pneumoniae*. Strains were clustered by the unweighted-pair group method using Dice Coefficient. The scales indicate the percentage of genetic similarity.

4. Discussion

Screening of the production of ESBLs is done in most laboratories according to the CLSI guidelines that involve an initial screening with standard ceftazidime, aztreonam, cefotaxime, or ceftriaxone discs, followed by a confirmatory test with ceftazidime and cefotaxime disks alone and in combination with clavulanic acid (CLSI, 2008).

In this study, 43(38.4%) of strains were derpressed mutants while 10(8.93%) were plain ESBL producers and 26(23.2%) were plain for the

presence of Plasmid mediated AmpC β-Lactamase. The results were in agreement with Tan *et al.* (2009).

Plasmids play a major role in bacterial adaptation to environmental or man-made stress. The rapid dissemination of antibiotic resistance genes in bacterial populations as a consequence of the intensive use of antibiotics in medicine can be partly attributed to plasmid-mediated horizontal transfer. Plasmids capable of being transferred and stably maintained in a wide range of bacteria, the so-called broad-host-range plasmids, are of special interest with respect to interspecies gene exchange (Gotz *et al*., 1996). Most of the current knowledge comes from plasmids isolated from clinical material or from studies on plasmid-mediated dissemination of antibiotic and/or heavy metal resistance genes (Wittig *et al*., 1994), in which plasmids were usually isolated after selective cultivation of bacteria with subsequent screening for the presence of plasmids.

A variety of other ß-lactamases which are plasmid-mediated or integron-associated class A enzymes have been discovered (Giakkoupi *et al*., 2000; Mavroidi *et al*., 2001; Poirel *et al*., 1999; 2000 and 2001). They are not simple point mutant derivatives of any known ß-lactamases. They are remarkable for their geographic diversity. Novel chromosomally encoded ESBLs have also been described (Bellais *et al*., 2001). VEB-1 has greatest homology with PER-1 and PER-2 (38%) (Poirel *et al*., 1999). It confers high-level resistance to ceftazidime, cefotaxime, and aztreonam, which is reversed by clavulanic acid. In the present work PCR demonstrate different banding patterns of *K. pneumoniae* strains for genes of SHV and TEM enzymes, and INT and Delta T-cells virulence genes.

Also, results of PFGE analysis indicated there exist many different PFGE pattern for ESBL-producing of *K. pneumoniae* strains. Therefore, these strains not belonged to a single clone and not due to the spread of an epidemic strain of multidrug-resistant *K. pneumoniae*, in contrast of old epidemic studies reported in 1994 and 1996 (Arlet *et al*., 1994 and Rice *et al*., 2000) and similar to recent epidemic studies. We believed that the high percentage of antibiotic-resistance among nosocomial pathogens found in the present study were caused by the spread of several epidemic resistant strains within the hospital is an issue of interest. Our preliminary study of the PFGE patterns of nosocomial resistant strains did not indicate the presence of only a single or a few epidemic strains spreading but exhibited many different PFGE patterns as well.

Moreover, before the advent of molecular biologic techniques to assess the genetic relationships between nosocomially acquired organisms, typing methods that assessed phenotypic differences between organisms were widely used. At least seven phenotypic methods could potentially be used to type *K. pneumoniae* isolates harboring ESBLs. These include biotyping (assessing the potential clonal relationship between organisms by way of observing common biochemical reactions, colonial morphology, or environmental tolerances) (Arlet *et al*., 1990) and assessment of the antimicrobial susceptibility test pattern. Neither test has particularly good discriminatory power. Occasionally, stored isolates of organisms may lose transferrable genetic elements (for example, plasmids) which confer antibiotic resistance and appear to have a different antibiotic susceptibility pattern than when the isolate was examined fresh

Soumitesh *et al.* (2007) recorded sequence analysis of conserved "housekeeping" genes such as the bacterial 16_S rRNA gene are increasingly being used to identify bacterial species in clinical practice and scientific investigations (Petti *et al*., 2005; and Clarridge, 2007). In the case of 16S rRNA analysis, species identification is easiest when most or the entire gene can be sequenced. However, DNA sequencing is impractical in medical diagnostics where speed is often of the essence. Species-specific sequences can be identified very rapidly in assays that combine nucleic acid amplification and a sequence-specific probe of the amplified product. These approaches are usually only able to query short DNA sequences; therefore, it is important to identify the regions within the target gene that supply the most taxonomic information in the smallest stretch of nucleotides. Additional benefits of small amplicon size may include increased assay sensitivity and applicability to archival specimens (Chakravorty *et al*., 2006).

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11/11/2012