Biotyping and Molecular Characterization of *Klebsiella pneumoniae* Producing Extended-Spectrum Beta-Lactamase in Cairo, Egypt: A Multicentre Study.

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Abstract: Extended-spectrum beta-lactamase (ESBL) - producing *Klebsiella pneumoniae* have spread rapidly worldwide posing a serious threat in healthcare facilities. The epidemiology of *K. pneumoniae* producing ESBL is complex and varies among hospitals and countries. Objective: This study aimed to determine the biotype and molecular characterization of ESBL producing *K pneumonia* and their clonal relatedness by PFGE in Cairo, Egypt. Methods: Over 12-months, bacterial isolates were collected from clinical specimens of inpatients and outpatients of seven hospitals in Cairo, Egypt. *K pneumonia* isolates were identified and biotyped using API 20E. Antimicrobial susceptibility testing to 16 β-lactam and non-β-lactam agents was determined by disk diffusion test. *K. pneumoniae* isolates were confirmed phenotypically as ESBL producers by combined disc test. Selected *K. pneumoniae* isolates were further subjected to Polymerase Chain Reaction (PCR) and Pulsed-field gel electrophoresis (PFGE) tests to determine the ESBL genes (TEM and SHV) and their clonal relatedness. Results: A total of 50 out of 66 (67%) *K. pneumoniae* isolates were positive for ESBLs. bla_{TEM} and bla_{SHV} were detected in 70% and 80% of ESBLs positive *K pneumoniae* isolates respectively. All ESBLs producing *K pneumoniae* isolates were 100% susceptible to imipenem and 35 (53%) of them were resistant for equal or more than eight antibiotics of different groups. PFGE results showed diverse and unrelated clones. Conclusions: This study showed high prevalence of ESBL producing *K. pneumonia* (67%). Bla_{SHV} was found as a predominant gene responsible for ESBLs production. Imipenem is still the most reliable and effective antimicrobial treatment option. ESBL producing *K. pneumonia* spread through the dissemination of a plasmid rather than the occurrence of a clonal outbreak in this study.
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Key words: ESBL; *K. pneumonia*; PCR; TEM; SHV; PFGE

1. Introduction

Klebsiella pneumoniae, an opportunistic pathogen responsible for a wide range of nosocomial infections that include pneumonia, urinary tract infection, abdominal infection, intra-vascular device infection, surgical site infection, soft tissue infection, and subsequent bacteremia, is estimated to cause approximately 8% of hospital acquired infections. This Gram-negative bacterium can also be found in the environment in association with plants, as well as in soil and in water (Bachman et al., 2011; Russo et al., 2011; Boll et al., 2012; Cruz et al., 2012; Hennequin, et al., 2012; Wu et al., 2012). *K. pneumoniae* has been implicated in 7-12% of hospital-acquired pneumoniae in ICUs in the United States, accounting for 15, 32, and 34% of community-acquired pneumoniae in Singapore, Africa and Taiwan, respectively (Lin et al., 2011).

K. pneumonia strain isolates are frequently resistant to multiple antibiotics, which leads to a therapeutic dilemma (Hennequin and Forestier, 2009, Hennequin, et al., 2012). Antimicrobial resistance to fluoroquinolones, late-generation cephalosporins, and carbapenems among *K. pneumonia* isolates is increasing rapidly by >1% per year (Bachman et al., 2011). Production of β-lactamase enzymes is the main mechanism of resistance against β-lactam antibiotics.

Extended spectrum β lactamases (ESBLs) are group of enzymes which have the capability of hydrolyzing third-generation cephalosporins and aztreonam (but not cephamycins and carbapenems). However they are sensitive to inhibitors such as clavulanic acid, sulbactam and tazobactam. To date, 890 various ESBLs have been discovered worldwide (Numanovic et al., 2013). They are classified according to two basic schemes: the molecular classification scheme according to Ambler (1991) and the functional classification system according to Bush-Jacoby-Medieros (1995). Most of the ESBLs are mutants of the TEM and the SHV enzymes and they developed from point mutation of genes which code production of primordial TEM-1, TEM-2, or SHV-1 β

lactamases with replacement of the configuration of amino acids at an active site for these enzymes (Numanovic et al., 2013). *K. pneumoniae* is the most common bacteria producing ESBLs and the predominant types of ESBLs in *K. pneumoniae* are SHV type followed by TEM (Eftekhar et al., 2012; Zaniani et al., 2012). The Infectious Diseases Society of America has listed it as one out of six pathogens for which new drugs are urgently needed in order to combat resistance development (Isendahl et al., 2012).

ESBL-producing *K. pneumoniae* have spread rapidly worldwide and pose a serious threat in healthcare-associated infections in many regions of the world (Ghafourian et al., 2011). Carbapenems have been the treatment of last resort against *K. pneumoniae* isolates with ESBLs, plasmid-encoded enzymes that inactivate penicillins and cephalosporins (Bachman et al., 2011). The hospital outbreak of multidrug resistant *Klebsiella* spp., especially ESBL has drawn much attention in recent years (Ho et al., 2011). Clinical treatment failure occurs frequently, especially when inappropriate antimicrobial therapy is used to treat infections caused by ESBL- producing organisms. Therefore, if infections with ESBL-producing organisms can be predicted by the clinical characteristics of patients, this may lead to a better selection of antibiotics and may improve the outcome of infections (Boll et al., 2012; Ghafourian et al., 2011).

Taking all information above into consideration, antibiotic susceptibility test alone is not enough. Confirmation of phenotype characteristic of these β-lactamase enzymes is important to be done, not only for epidemiology matters, also for patient's safety (Saharman and Lestari, 2013). The detection of the common ESBL genes such as TEM and SHV by molecular methods in the ESBL producing *K. pneumonia* and their patterns of antimicrobial resistance can provide useful information about their epidemiology and can aid a rational antimicrobial therapy (Kaur and Aggarwal, 2013).

This study aimed to determine the biotype and molecular characterization of ESBL producing *K pneumonia* and their clonal relatedness by PFGE from seven hospitals in Cairo, Egypt.

2. Materials and Methods

2.1. Bacterial Isolates Identification and Biotyping by API-20E System

Over a period of 12 months bacterial isolates were collected from inpatients and outpatient of seven hospitals: Theodor Bilharz Research Institute (TBRI), Dar El- Fouad, El Manial, New Kaser Al Ainy, Cairo university pediatric hospital "Abu-El Reesh" and Al Helal Hospitals, Nasser Research Institute, Cairo, Egypt. Bacterial species identification was confirmed by using the analytical profile index for Enerobacteriaceae (API-20E) (Bio-Mérieux SA, France). *K. pneumoniae* isolates were classified into five biotype codes (B1-5) according to their numerical profiles (Table1).

2.2. Antimicrobial Susceptibility Testing

The antimicrobial susceptibilities were determined by the disk diffusion test using commercial disks (Mast Diagnostics, U.K.) according to the criteria recommended by the CLSI (2010) using *E. coli* ATCC 25922 as the control strain. Antibiotics tested were: amikacin (Ak; 30µg), ciprofloxacin (CIP; 5µg), ampicillin (AMP;10 µg), cefotaxime (CTX; 30 µg), ceftazidime (CAZ; 30 µg), ceftriaxone (CRO; 30 µg), aztreonam (ATM; 30 µg), ampicillin/ sulbactam (SAM; 10/10 µg), ticracycline/clavulanic acid (TIM;75/10 µg), trimethoprim/ sulphamethoxazole (SXT; 1.25/23.75 µg), tetracycline (TE; 30 µg), nalidixic acid (NA; 30 µg), imipenem (IPM; 10 µg), gentamycin (CN; 10 µg), chloramphinicol (C; 30 µg) and cephalothin (CF; 30 µg).

2. 3. Phenotypic Detection of ESBL

2.3.1. Initial Screening test

The disk-diffusion method for antibiotic susceptibility testing was used to screen ESBL production. *K. pneumoniae* isolate was considered a potential ESBL producer according to the CLSI (2010) recommendation, if the zone diameter result of the following antibiotics were as follows: ceftriaxone ≤ 25 mm, ceftazidime ≤ 22 mm, aztreonam ≤ 27 mm and cefotaxime ≤ 27 mm.

2.3.2. Confirmatory test (Combined disc method)

A standardized suspension of the isolate was plated onto Mueller-Hinton agar using the antimicrobial discs cefotaxime (CTX; 30µg), cefotaxime/clavulanic acid (30/10µg), ceftazidime (CAZ; 30µg) and ceftazidime/clavulanic acid (30/10 µg). After incubation, the zone of inhibition around each of the discs is measured. An increase of zone diameter ≥ 5mm for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone indicates positive for ESBL production (CLSI, 2010).

2.4. Genotypic analysis for TEM and SHV by PCR

Representative ESBL *K. pneumoniae* isolates were selected according to their antimicrobial resistance (resistant to equal or more than eight antibiotics of different groups) for molecular testing. Ten strains were chosen for PCR to determine the presence of TEM, SHV genes as described by Ayogi, (2003). Bacterial DNA was extracted using DNA QIAmp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Specific primers for the genes (forward primer 5′-CACTCAAGGATGTATTGTG -3′; Reverse primer 5′- TTAGCGTTGCCAGTGCTCG-3′ for *SHV* gene and forward primer 5′-TGAAGACGAAAG GGCCTCCTG-3′; Reverse primer 5′-TAATCAGT GAGGCACCTATCTC -3′ for *TEM* gene) were used for PCR amplification that produced 882 bp and 770 bp PCR espectively (Chen et al., 2004). Amplification reactions were performed in a Gene/amp PCR system 9700 (Applied Biosystem).

Table 1: Biotyping of *K. pneumoniae* isolates by API 20E System.

Biotyping codes	Numerical profiles	ONP	AHD	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
B1	**5005773**	+V	-V	+V	-V	+V	-V	+V	-V	-V	+V	-V	+V	+V	+V	+V	+V	+V	+V	+V	+V
B2	**5215773**	+V	-V	-V	-V	+V	-V	+V	-V	-V	+V	-V	+V	+V	+V	+V	+V	+V	+V	+V	+V
B3	**5015773**	+V	-V	+V	-V	-V	-V	+V	-V	-V	+V	-V	+V	+V	+V	+V	+V	+V	+V	+V	+V
B4	**5205773**	+V	-V	+V	-V	+V	-V	-V	-V	-V	+V	-V	+V	+V	+V	+V	+V	+V	+V	+V	+V
B5	**1205773**	+V	-V	+V	-V	-V	-V	+V	-V	-V	+V	-V	+V	+V	-V	+V	+V	+V	+V	+V	+V

ONPG (O-nitrophenyl-βgalactosidase), **ADH** (Arginine dehydrolase), **LDC** (Lysine decarboxylase), **ODC** (Ornithine decarboxylase), **CIT** (Citrate), **H_2S** (H_2S), **URE** (Urease), **TDA** (Tryptophan deaminase), **IND** (Indole), **VP** (Voges-Proskauer reaction), **GEL** (Gelatinase), **GLU** (Glucose), **MAN** (Mannitol), **INO** (Inositol), **SOR** (Sorbitol), **RHA (**Rhamnose), **SAC** (Saccharose), MEL (Melibiose), **AMY** (Amygdalin) and **ARA** (Arabinose) tests.

2.5. Genotypic analysis relationships of ESBL producing strains by PFGE

Ten ESBLs producers isolates were subtyped by PFGE in accordance with standardized *Klebsiella* protocol using restriction enzyme (*Xba I*) (New England Biolabs, Beverly, MA, USA) as described by Gautom (1997). PFGE was performed by means of the CHEF-DR II system (Bio-Rad, Richmond, CA, USA). The DNA was subjected to electrophoresis for 22 hours at 14°C in a 1% agarose gel at 6 V/cm with a linear gradient pulse time of 5-35 seconds. Clonal relatedness was interpreted by the criteria of Tenover et al. (1997).

2.6. Statistical Methods

Data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentages). A probability value (P value) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel 2007 (Microsoft Corporation, NY., USA), SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows and Quick Calcs online calculators for scientists (Graph pad software Inc., CA, USA).

3. Results

3.1. Bacterial isolates

Out of 104 bacterial isolates, which were morphologically or biochemically related to *klebsiella* spp., 66 *K. pneumoniae* isolates were collected in a period of 12 months, from inpatients and outpatient of seven hospitals: Theodor Bilharz Research Institute (TBRI) (n=2), Dar El- Fouad (n=39), El Manial (n=7), New Kaser Al Ainy (n=9) , Cairo university pediatric hospital "Abu-El Reesh" (n=1) and Al Helal Hospitals (n=3), Nasser Research Institute(n=5), Cairo, Egypt. *K. pneumoniae* were isolated from different clinical specimens including: urine (n=23; 34.9%), blood (n=17; 25.8%), pus (n=7; 10.6%), sputum (n=7; 10.6%), wound swab (n=5; 7.6%), endotracheal tube (n=3; 4.5%), stool (n=2; 3%), CFS (n=1; 1.5%) and ascitic fluid (n=1;1.5%).

3.2. Biotyping by API20E system

API 20E system biotyping showed the predominance of B1, as it represented 48 of the total isolates (73%), followed by B3 and B4 biotypes in 10 (15%) and 6 (9%) of the isolates respectively. The remaining two isolates had biotypes B2 and B5 (one each; 1.5%). The five biotypes profiles were represented in the isolates from the seven hospitals as follows: Dar Al Fouad hospital showed biotypes B1 (33), B3 (2) and B4 (5), El Manial hospital showed biotypes B1 (2), B3 (4) and B4 (1), New Kaser Al Ainy showed biotypes B1 (6), B3 (1) and B5 (1), Nasser research institute showed 3 isolates of B1 and 2 isolates of B3, Al Helal hospital showed 3 isolates of B1, TBRI showed 2 biotypes (B2 and B3) one isolate for each, Cairo university pediatric hospital " Abu –El Reesh", showed one isolate of B1.

3.3. Antibiotic susceptibility testing

Analysis of the susceptibility patterns of the isolates against different antibiotics showed that all tested strains were sensitive to imipenem, resistant to ampicilin and showed different susceptibility profiles for other antibiotics. Chloramphinicol, trimethoprim/ sulphamethoxazole and amikacin, revealed the highest efficiency against *K. pneumoniae* isolates (56%, 54.5% and 53% respectively). *K. pneumoniae* isolates were highly resistant to ampicillin/sulbactam (88%),

cephalothin (87.8%), ceftriaxone (80.3%), cefotaxime (77.3%), ticracycline/clavulanic acid (72.7%), tetracycline (68.1%) and aztreonam (67.6%). *K. pneumoniae* isolates were sensitive to, gentamycin, ciprofloxacin, nalidixic acid and ceftazidime, in 44%, 44%, 36.4% and 34.8% respectively.

The susceptibility patterns of ESBL producing *K. pneumoniae* isolates against different antibiotics showed that all tested strains were sensitive to imipenem and resistant to ampicilin. They were highly resistant to β-lactam antibiotics including cephalothin (100%), ampicillin/ sulbactam (80%), ceftriaxone (96%), cefotaxime (94%), ceftazidime (80%), ticracycline/ clavulanic acid (76%) and aztreonam (80%). Antimicrobial susceptibility testing to non-β-lactam agents, including chloramphinicol, amikacin, trimethoprim/sulphamethoxazole, ciprofloxacin, gentamycin, nalidixic acid and tetracycline revealed efficiency against ESBL producing *K. pneumoniae* isolates in 54%, 52%, 46%, 42%, 34% , 34% and 28% respectively.

3.4. Phenotypic Detection of ESBLs

A total of 50 out of 66 (67%) strains were positive for ESBLs by combined disc test; 35 (53%) of strains were resistant for equal or more than eight antibiotics of different groups. According to different clinical specimen's samples, ESBLs were detected in urine (65.2%; 15/23), blood (88.2%; 15/17), pus (85.7%; 6/7), sputum (85.7; % 6/7), endotracheal tube (33.3%; 1/3), wound swab (80%; 4/5), stool (100%; 2/2) and CSF (100%;1/1) specimens. Regarding to the distribution of ESBLs among different hospitals, ESBLs was detected in Dar Al Fouad hospital, El Manial hospital, New Kaser Al Ainy , Nasser research institute, Al Helal hospital, TBRI, Cairo university pediatric hospital "Abu-El Reesh" in 69.2 % (27/39), 100% (7/7), 66.7% (6/9), 80% (4/5), 100% (3/3), 100% (2/2) and 100 % (1/1) respectively. Regarding the biotyping, 68.8 % of B1, 90% of B3 and 100% of B2, B4 and B5 were ESBLs positive *K. pneumoniae* isolates.

3.5. Genotypic analysis for TEM and SHV

Among the selected 10 ESBLs positive *K. pneumoniae,* 80% was positive for *SHV* gene and 70% was *TEM* gene positive. The most prominent biotype B1 showed positivity in 85.7% (6/7) for both *SHV* and *TEM* genes. In Dar El Fouad hospital; 3 isolates K-1, K-44 and K-47 of B1 showed positive results for SHV and TEM while K-10 of B4 was negative for both SHV and TEM. Two genetic patterns were detected in El Manial hospital; 2 isolates K-3 and K-15 of B1 were positive for both genes and K-6 of B3 was negative for TEM and positive SHV. Also, New Kaser Al Ainy showed 2 different genetic patterns; one K-19 of B3 was positive and another one K-66 of B1 was negative for both genes. Nasser research institute showed only one isolate of B1 positive for both genes (figure1, table 3).

3.6. Genotypic analysis relationships of ESBL producing strains by PFGE

The PFGE typing of the ESBL-producing isolates revealed seven different patterns among the isolates. Relatedness was detected between the genomes of 2 isolates from Dar El Fouad and one isolate from El Manial hospital in 93.2% and 88.7% respectively. However, one isolate was from one hospital and the other two related isolates presented from the same hospital showed different antimicrobial susceptibility pattern. PFGE pattern similarity was also detected between 2 isolates with 98.2 % relatedness, although these isolates were isolated from two different hospitals El-Manial and Nasser research institute (figure 2)

4. Discussion

The increasing incidence of antibiotic resistance is getting more global attention. Antibiotic multi-resistant Gram negative bacteria pose a risk to public health (Saharman and Lestari, 2013). Extended spectrum ß-lactam antibiotics have been widely used for treatment of serious Gram-negative infections since the 1980s. However, bacterial resistance has emerged quickly due to the production of ESBLs (Eftekhar et al., 2012).

Enterobacteriaceae producing ESBL are emerging as a threatening cause of both hospital and community acquired infection as they are often resistant to standard antimicrobial choices (Weisenberg et al., 2012; Rakotonirina et al., 2013). It is generally thought that patients infected by an ESBL-producing organism are at an increased risk of treatment failure (Ghafourian et al., 2011). Recently, an increasing percentage of ESBL-producing *K. pneumoniae* strains have been detected worldwide with a significant impact on the clinical course of disease and expenditure of resources (Wang et al., 2012). As it is shown in a meta-analysis study that infections with ESBL-producing bacteria are associated with nearly twice the mortality rate compared to that of non-ESBL producers (Isendahl et al., 2012).

Extended spectrum ß-lactamases are enzymes produced by some *K. pneumoniae* species that inactivate penicillins, expanded- spectrum cephalosporins, monobactams including older beta-lactam antimicrobial agents and are inhibited by clavulanic acids, sulbactam or tazobactam (Akpaka et al., 2010; Kaur and Aggarwal, 2013). The first ESBLs have evolved by genetic mutation from native β-lactamases TEM and SHV. The epidemiology of ESBL-producing *K pneumonia* is complex and their

prevalence among clinical isolates varies from country to country, institution to institution and therapeutic choices for infections caused by ESBL-producing bacterial strains remain limited (Akpaka et al., 2010). In Egypt, a high prevalence of infections caused by ESBL-producing *E. coli* and *Klebsiella* spp. has been reported (Fam et al., 2011; Al-Agamy, 2012).

In our study, A total of 50 out of 66 (67%) of *K . pneumonia* isolates were positive for ESBLs.

Table 2. Data of *K. pneumoniae* isolates were included in the study.

Code No.	Hospital	Sample	API Typing Code	AK	AM	AMP	ATM	CTX	CAZ	CRO	CF	C	CIP	CN	IPM	NA	TE	TIM	SXT	Combined Disc
K-1	Dar Al Fouad	Wound swab	B1	I	R	R	R	R	I	R	R	S	R	R	S	R	R	R	S	+ ve
K-2	Dar Al Fouad	Pus	B1	S	R	R	R	R	R	R	R	S	S	R	S	S	R	R	S	+ ve
K-3	El-Manial	Pus	B1	S	R	R	R	R	R	R	R	S	S	R	S	S	R	R	S	+ ve
K-4	New Kaser	Urine	B1	S	R	R	R	R	R	R	R	S	S	R	S	S	R	R	S	+ ve
K-5	El-Manial	Pus	B4	I	R	R	R	R	I	R	R	S	R	R	S	R	R	R	S	+ ve
K-6	El-Manial	Sputum	B3	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve
K-7	New Kaser	Urine	B1	S	R	R	R	R	I	R	R	S	R	R	S	R	R	R	S	+ ve
K-8	New Kaser	Urine	B1	I	R	R	R	R	R	R	R	S	R	R	S	R	R	R	S	+ ve
K-9	Dar Al Fouad	Urine	B4	S	R	R	R	R	R	R	R	S	R	S	S	R	R	R	R	+ ve
K-10	Dar Al Fouad	Blood	B4	S	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve
K-11	Abu-El Reesh	Urine	B1	S	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve
K-12	Nasser institute	Blood	B3	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve
K-13	Nasser institute	CSF	B3	S	R	R	R	R	R	R	R	S	S	R	S	S	S	R	S	+ ve
K-14	El-Manial	Blood	B3	S	I	R	R	R	I	R	R	S	R	R	S	R	R	R	S	+ ve
K-15	El-Manial	Blood	B1	S	R	R	R	R	R	R	R	S	S	R	S	S	R	R	S	+ ve
K-16	Dar Al Fouad	Blood	B1	S	I	R	R	R	R	R	R	S	S	S	S	S	S	R	S	+ ve
K-17	Dar Al Fouad	Urine	B3	I	R	R	R	R	S	R	R	R	R	R	S	R	R	R	S	- ve
K-18	New Kaser	Pus	B5	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	I	+ ve
K-19	New Kaser	Urine	B3	S	R	R	R	R	R	R	R	S	R	S	S	R	R	R	R	+ ve
K-20	El-Manial	Pus	B3	I	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve
K-21	El-Manial	Urine	B3	S	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve
K-22	Dar Al Fouad	ETT	B4	S	R	R	R	R	R	R	R	R	R	R	S	S	R	S	R	+ ve
K-23	Dar Al Fouad	Sputum	B1	S	R	R	R	R	I	R	R	S	R	S	S	R	R	R	R	+ ve
K-24	TBRI	Sputum	B3	R	R	R	R	R	R	R	R	S	S	S	S	S	I	R	S	+ ve
K-25	TBRI	Pus	B2	S	R	R	R	R	R	R	R	S	I	S	S	S	I	S	S	+ ve
K-26	Dar Al Fouad	Sputum	B1	I	I	R	R	R	R	R	R	R	**S**	R	S	R	S	I	S	+ ve
K-27	Dar Al Fouad	Wound swab	B1	R	I	R	I	R	S	R	R	S	S	S	S	S	S	I	S	+ ve
K-28	Dar Al Fouad	Urine	B1	R	R	R	R	R	R	R	R	R	S	R	S	R	I	R	S	+ ve
K-29	Dar Al Fouad	Urine	B1	R	R	R	R	R	R	R	R	S	S	S	S	I	S	R	S	+ ve
K-30	Dar Al Fouad	Sputum	B1	R	R	R	R	R	S	R	R	S	S	S	S	R	I	I	S	+ ve
K-31	Dar Al Fouad	Blood	B1	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve
K-32	Dar Al Fouad	Blood	B1	S	I	R	I	R	S	R	R	S	S	S	S	S	S	I	S	+ ve
K-33	Dar Al Fouad	Ascitic fluid	B1	I	R	R	I	I	R	R	R	R	R	S	S	R	R	I	R	- ve
K-34	Dar Al Fouad	Sputum	B1	I	I	R	R	R	I	R	R	R	R	S	S	R	R	I	R	+ ve
K-35	Dar Al Fouad	Blood	B3	I	R	R	I	I	S	R	R	S	S	I	S	I	R	R	R	+ ve
K-36	Dar Al Fouad	Urine	B4	S	R	R	I	R	S	R	R	R	S	R	S	S	R	S	R	+ ve
K-37	Dar Al Fouad	Stool	B4	S	R	R	I	R	S	R	R	S	S	R	S	S	S	S	S	+ ve
K-38	Dar Al Fouad	Blood	B1	S	S	R	I	I	S	I	R	S	R	S	S	R	S	S	R	+ ve
K-39	Dar Al Fouad	Blood	B1	R	R	R	R	R	R	R	R	R	I	R	S	R	R	R	S	+ ve
K-40	Al-Helal	Wound swab	B1	R	I	R	I	R	S	R	R	S	S	S	S	S	I	R	S	+ ve

K: *Klebsiella* isolate **; ETT:** endotracheal tube; **CSF:** cerebral spinal fluid ; **B:** Biotype; **S:** sensitive, **I:** intermediate, **R:** resistant; **Ak:** amikacin, CIP: ciprofloxacin,AMP: ampicillin, CTX: cefotaxime, CAZ: ceftazidime, CRO: ceftriaxone, ATM: aztreonam, SAM: ampicillin/ sulbactam, TIM: ticracycline/clavulanic acid, SXT: trimethoprim/ sulphamethoxazole, TE: tetracycline, NA: nalidixic acid, IPM: imipenem, CN: gentamycin , C: chloramphinicol and CF: cephalothin.

Table 2 (continue). Data of *K. pneumoniae* isolates were included in the study.

Code No.	Hospital	Sample	API Typing Code	AK	SAM	AMP	ATM	CTX	CAZ	CRO	CF	C	CIP	CN	IPM	NA	TE	TIM	SXT	Combined Disc
K-41	Al- Helal	Wound swab	B1	R	R	R	I	R	R	R	R	R	S	R	S	S	R	R	R	+ ve
K-42	Al –Helal	Blood	B1	S	S	R	R	I	R	I	R	S	S	S	S	S	I	R	R	+ ve
K-43	Dar Al Fouad	Urine	B1	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve
K-44	Dar Al Fouad	Urine	B1	R	R	R	I	R	S	R	R	R	S	S	S	S	R	I	I	+ ve
K-45	Dar Al Fouad	Sputum	B1	S	S	R	S	S	S	S	S	S	S	S	S	S	I	S	S	- ve
K-46	Dar Al Fouad	Urine	B1	S	R	R	S	S	S	S	R	R	R	S	S	R	R	R	R	- ve
K-47	Dar Al Fouad	Blood	B1	S	R	R	R	R	I	R	R	R	R	R	S	R	R	R	R	+ ve
K-48	Dar Al Fouad	Urine	B1	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	- ve
K-49	Dar Al Fouad	ETT	B1	R	R	R	R	R	I	R	R	S	S	S	S	S	I	R	S	- ve
K-50	Dar Al Fouad	Urine	B1	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve
K-51	Dar Al Fouad	Urine	B1	S	S	R	I	I	S	S	S	S	S	S	S	S	S	S	S	-ve
K-52	New Kaser	Blood	B1	S	R	R	S	S	S	S	S	S	S	S	S	S	S	R	R	-ve
K-53	New Kaser	ETT	B1	S	R	R	S	S	S	S	S	R	R	S	S	R	R	R	R	- ve
K-54	Dar Al Fouad	Blood	B1	S	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve
K-55	Dar Al Fouad	Wound swab	B1	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	- ve
K-56	Nasser institute	Urine	B1	S	R	R	I	R	S	R	R	S	R	S	S	R	R	R	R	+ ve
K-57	Nasser institute	Urine	B1	S	R	R	S	S	S	S	S	S	S	S	S	S	R	R	R	- ve
K-58	Nasser institute	Stool	B1	S	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve
K-59	Dar Al Fouad	Urine	B1	I	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve
K-60	Dar Al Fouad	Blood	B1	R	I	R	S	I	S	I	R	S	S	S	S	I	I	S	S	- ve
K-61	Dar Al Fouad	Urine	B1	R	R	R	R	R	R	R	R	R	I	R	S	R	R	R	R	-ve
K-62	Dar Al Fouad	Urine	B1	S	R	R	S	S	S	S	R	R	R	R	S	R	R	R	R	- ve
K-63	Dar Al Fouad	Pus	B1	S	S	R	S	S	S	S	R	S	R	S	S	I	R	R	R	-ve
K-64	Dar Al Fouad	Blood	B1	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve
K-65	New Kaser	Urine	B1	R	R	R	R	R	S	R	R	S	I	R	S	R	R	S	R	- ve
K-66	New Kaser	Blood	B1	S	S	R	R	R	R	R	R	R	S	S	S	I	R	I	S	+ ve

K: *Klebsiella* isolate**; ETT:** endotracheal tube; **B:** Biotype; **S:** sensitive, **I:** intermediate, **R:** resistant; **Ak:** amikacin, CIP: ciprofloxacin, **AMP:** ampicillin, **CTX :** cefotaxime, **CAZ:** ceftazidime, **CRO:** ceftriaxone, **ATM:** aztreonam, **SAM:** ampicillin/ sulbactam, **TIM:** ticracycline/clavulanic acid, **SXT:** trimethoprim/ sulphamethoxazole, **TE:** tetracycline, **NA:** nalidixic acid, **IPM:** imipenem, **CN:** gentamycin **, C:** chloramphinicol and **CF:** cephalothin.

Table 3. Data of ESBLs positive *K. pneumoniae* strains that selected for genotypic analysis.

Code No.	Hospital	Sample	API Typing Code	AK	SAM	AMP	ATM	CTX	CAZ	CRO	CF	C	CIP	CN	IPM	NA	TE	TIM	SXT	ESBL	SHV	TEM
K-1	Dar Al Fouad	Wound swab	B1	I	R	R	R	R	I	R	R	S	R	R	S	R	R	R	S	+ ve	+ve	+ ve
K-3	El-Manial	Pus	B1	S	R	R	R	R	R	R	R	S	S	R	S	S	R	R	S	+ ve	+ ve	+ ve
K-6	El-Manial	Sputum	B3	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve	+ve	- ve
K-10	Dar Al Fouad	Blood	B4	S	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	+ ve	- ve	- ve
K-15	El-Manial	Blood	B1	S	R	R	R	R	R	R	R	S	S	R	S	S	R	R	S	+ ve	+ ve	+ ve
K-19	New Kaser	Urine	B3	S	R	R	R	R	R	R	R	S	R	S	S	R	R	R	R	+ ve	+ ve	+ ve
K-44	Dar Al Fouad	Urine	B1	R	R	R	I	R	S	R	R	R	S	S	S	S	R	I	I	+ ve	+ ve	+ ve
K-47	Dar Al Fouad	Blood	B1	S	R	R	R	R	I	R	R	R	R	R	S	R	R	R	R	+ ve	+ ve	+ ve
K-56	Nasser institute	Urine	B1	S	R	R	I	R	S	R	R	S	R	S	S	R	R	R	R	+ ve	+ ve	+ ve
K-66	New Kaser	Blood	B1	S	S	R	R	R	R	R	R	R	S	S	S	I	R	I	S	+ ve	- ve	- ve

K: *Klebsiella* isolate **; ETT:** endotracheal tube; **B:** Biotype; **S:** sensitive, **I:** intermediate, **R:** resistant; **Ak:** amikacin, CIP: ciprofloxacin, **AMP:** ampicillin, **CTX :** cefotaxime, **CAZ:** ceftazidime, **CRO:** ceftriaxone, **ATM:** aztreonam, **SAM:** ampicillin/ sulbactam, **TIM:** ticracycline/clavulanic acid, **SXT:** trimethoprim/ sulphamethoxazole, **TE:** tetracycline, **NA:** nalidixic acid, **IPM:** imipenem, **CN:** gentamycin **, C:** chloramphinicol and **CF:** cephalothin.

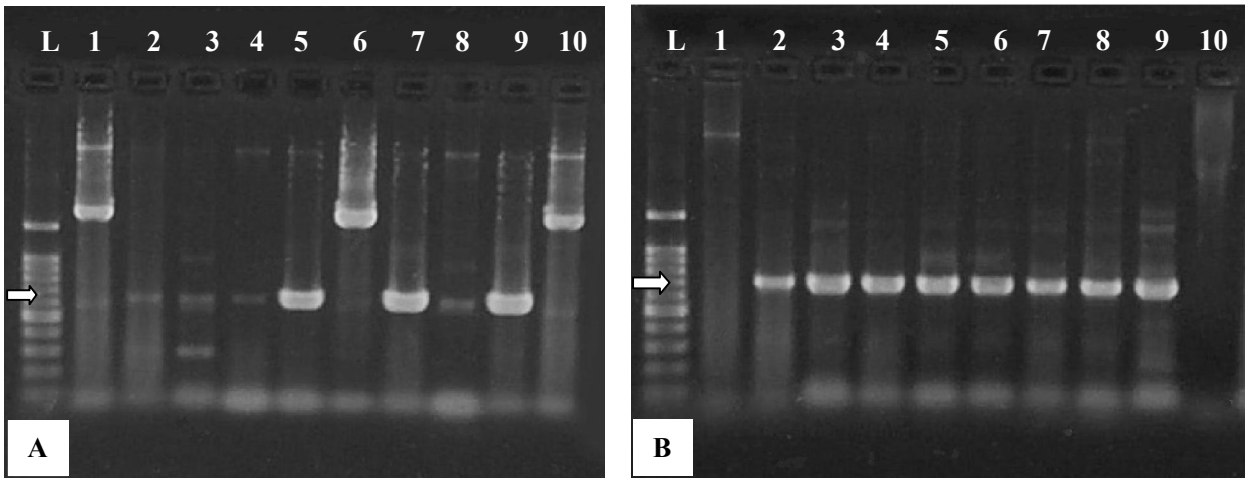

Figure 1:Agarose gel electrophoresis (2%) of a) TEM gene and b) SHV gene PCR reactions ten *K. pneumoniae* strains; L: Ladder, lanes from 1-10 *K. pneumoniae* strains k-10, k-19, k-44, k-15, k-1, k-6, k-56, k-3,k-47, k-66.

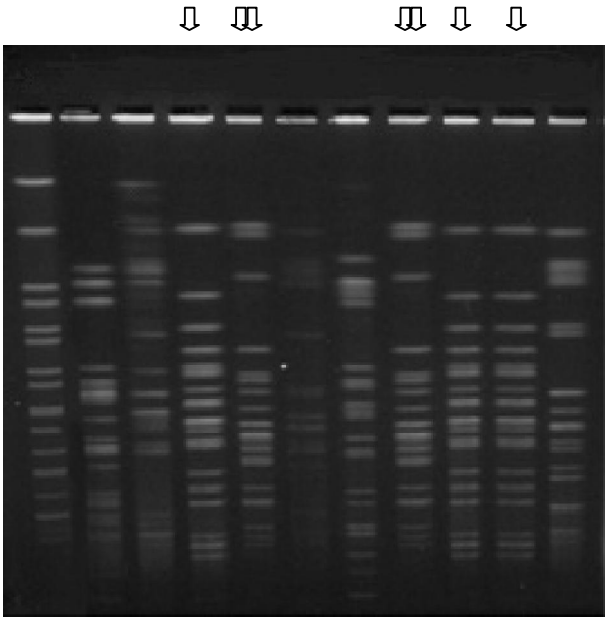

Figure 2.PFGE patterns of Xbal-digested genomic DNA from *K. pneumonia* strains. Lane L, Lambda DNA concatemer standard; lane 1, K-10 Dar El Fouad (pattern I); lanes 2 and 3, K-19 New Kaser Al Ainy and K-44 Dar El Fouad (pattern II and III); lane 4, K-15 El-Manial (pattern IV); lane 5, K-1 Dar El Fouad (pattern V); lane 6, K-6 El-Manial (pattern VI); lane 7; K-56 Nasser institute (pattern IV); lane 8, K-3 El-Manial (pattern III); lanes 9 and 10, k-47 Dar El Fouad, k-66 New Kaser Al Ainy (pattern III and VII). The one arrow indicates isolates of same profile (III), whereas the two arrows indicate same profile (IV).

The prevalence of ESBL producing isolates of *K. pneumonia* varies in different countries (Ghafourian et al., 2011). This high prevalence of ESBL-producing *K. pneumonia* found in this study is nearly similar with the result of Abdel-Hady et al. (2008) who noted that the prevalence rate of ESBL-producing *K pneumonia* was 67% in neonatal care unit, Mansoura University Children's Hospital and El Kholy et al. (2011) who reported a prevalence rate of 63.8% in Pediatric Teaching Hospital, Cairo University. High prevalence rate were also recorded globally. Reinert et al. (2007) in global multicentre study reported that the highest prevalence rates of ESBL-producing *K pneumonia* were observed in India (72%) and Mexico (71.4%). Many Indian studies reported that the incidence of ESBLs among the isolates of *K pneumoniae* in the major hospitals of India range from 44.4%, 52.27% to 80% (Mathur et al., 2002; Varaiya et al., 2008; Kaur and Aggarwal, 2013). Other studies showed different frequency of ESBLs production in *K. pneumonia* in different region in Iran where ESBLs production was variable from 61% in Mashhad (Fazly Bazzaz et al., 2009), 69.7% in Tehran (Feizabadi et al., 2010), 70% in Isfahan, (Masjedian Jazi et al., 2007) to 74% in Milad hospitals, Tehran (Ghafourian et al., 2011). In Indonesia, Saharman and Lestari (2013) noted high number of ESBL-producing *K pneumonia* (58.42%) and this result was similar with that of SENTRY Asia Pacific surveillance program study conducted during 1998 to 2002 in which the prevalence rate was around 60% in several Asian countries (Jean and Hsueh, 2011). Also in a study in Thailand, the prevalence of the ESBLs producing *K. pneumoniae* isolates was 64% in children (Chaikittisuk and Munsrichoom, 2007).

However, lower prevalence rates of ESBLs producing *K. pneumoniae* was reported by other studies. The prevalence was in the range of 7.5% to 22.8% and 8.5% to 29.8% in *K. pneumonia* in Taiwan

and Korea respectively (Pai, 1998; Yu et al., 2006). A multicenter study in mainland of China showed that the prevalence of ESBL production in *K. pneumonia* isolates increased from 11% in 1994 to 34% in 2001 (Lagamayo, 2008). In Pakistan, the prevalence of the ESBLs producing *K. pneumoniae* was 36% (Jabeen et al., 2005).

Based on the results of this study, our molecular study revealed the ESBLs producing K. pneumonia contained SHV (80%), and TEM (70%) genes by PCR. The SHV gene has high frequency compared to TEM gene; a fact which is similar to previous studies by Taşli and Bahar (2005), Ramazanzadeh (2010) and Ghafourian et al (2011) but it was different compared to other studies (Hernandez et al., 2005; Zaniani, et al., 2012). In Egyptian study by Abdel-Hady et al. (2008) observed that the PCR amplicons revealed the presence of SHV-2 in 100%, and SHV-1 gene in 44%. Some ESBLs producing *K. pneumonia* showed negative results in PCR method for SHV and TEM genes in our study, therefore other beta-lactamases genes may be involved in ESBLs resistance. However, further studies are required for finding the other genes in ESBLs producing *K. pneumoniae* bacteria (Zaniani, et al., 2012). Despite the fact that a small number of isolates were tested in some hospitals in our study, ESBL production among our isolates was high and showed different frequency in different hospitals ranging from 66.7% (in New Kaser Al Ainy) to 100% (in Al Helal, TBRI and Abu-El Reesh hospitals). Therefore the pattern of ESBLs producing bacteria varies in different parts of Cairo, Egypt and separate studies of the ESBLs producing *K. pneumonia* is necessary in various parts to estimate the antibiotic resistance correctly for taking steps for reducing these resistances. These results was in agreement with that of a study carried by Ghafourian et al (2011) who showed different frequency of ESBLs producing *K. pneumonia* in different region in Iran with ESBLs production was variable from 43.6% in Ilam to 74% in Tehran hospitals.

In our study, 35 (53%) of ESBLs producing *K. pneumoniae* strains were resistant for equal or more than eight antibiotics of different groups. Imipenem was found as an effective antibiotic. Other studies reported that all ESBLs producing *K. pneumoniae* isolates were also susceptible to imipenem (Abdel-Hady et al., 2008; Bazzaz et al., 2009; Ghafourian et al., 2011). In this study, susceptibility testing of ESBL producing *K.pneumonie* strains showed that the highest resistance rate among 3rd generation of cephalosporins and aztreonam and these results were similar to observation reported by Akpaka et al. (2010). Antimicrobial susceptibility testing to non-β-lactam agents, including chloramphinicol, amikacin, trimethoprim/ sulphamethoxazole, ciprofloxacin, gentamycin, nalidixic acid and tetracycline revealed efficiency against ESBL producing *K. pneumoniae* isolates in 54%, 52%, 46%, 42%, 34% , 34% and 28% respectively. El Kholy et al. (2011) found comparable results regarding the β-lactam and the non-β-lactams agents against ESBL- producing *K.pneumonie*. This is expected as plasmid-mediated transfer of ESBLs may also result in increasing resistance to non-β-lactams since plasmids can harbour genes that confer resistance to various classes of antibiotics such as quinolones, aminoglycosides and chloramphenicol (Eftekhar et al., 2012; Rakotonirina et al., 2013).

In the present study, the PFGE typing of the ESBL-producing *K. pneumonia* revealed seven different patterns among the isolates. The genomes of K-44 and K-47 from Dar El Fouad and K-3 from El Manial hospital showed relatedness in 93.2% and 88.7% respectively. However, one isolate was from one hospital and the other two related isolates presented from the same hospital showed different antimicrobial susceptibility patterns. Two isolates, K-15 and K-56, isolated from two different hospitals showed PFGE pattern similarity with 98.2 % relatedness with different antimicrobial susceptibility patterns. This clonal diversity suggests that most of the strains have been unable to be maintained or spread in the different facilities of the hospital and they did not significantly share patient demographics and occurrence periods. The spread of ESBL-producing *K. pneumonia* appeared to be polyclonal and none of the bacterial strains were clonally related. Similar results were reported by Akpaka et al. (2010). In another study, PFGE analysis carried out by Kiratisin et al. (2008) demonstrated that there was no major clonal relationship among the typed ESBL producers in their hospital. However, in Egyptian study performed by El Kholy et al. (2011) PFGE revealed that the clonal isolates contributed to 79.3% of ESBL- producing *K. pneumonia*. In agreement with El Kholy et al (2011) our results showed that phenotypic methods such as biotyping and antimicrobial susceptibility patterns are useful, simple and cost beneficial tests but with a weak discriminatory power and molecular typing techniques consider more powerful tools in epidemiological studies.

In Conclusion: This study showed high prevalence of ESBL producing *K. pneumonia* (67%). The prevalence varies in different countries or different parts of each country and is dependent upon antibiotic policies in every region. Bla_{SHV} was found as a predominant gene responsible for ESBLs production. Imipenem is still the most reliable and effective antimicrobial treatment option. ESBL producing *K. pneumonia* spread through the dissemination of a plasmid rather than the occurrence of a clonal outbreak during this study. By

understanding the resistance pattern and prevalence of the ESBL producing *K. pneumonia,* a rational use of antibiotic and appropriate methods of screening ESBL in routine laboratories to avoid treatment failure. More epidemiological studies are needed to identify sources and reservoirs of ESBL-producing *K. pneumoniae* to differentiate between sporadic and epidemic cases, in addition to application of suitable infection control measures.

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