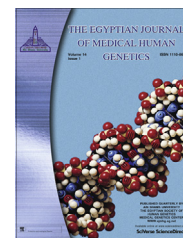




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ORIGINAL ARTICLE

Detection of bla SHV and bla CTX-M genes in ESBL producing *Klebsiella pneumoniae* isolated from Egyptian patients with suspected nosocomial infections

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KEYWORDS

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Abstract The correct identification of the genes involved in ESBL mediated resistance is necessary for the surveillance and epidemiological studies of their transmission in hospitals. The aim of the present study was to find the prevalence of ESBL producing *Klebsiella pneumoniae* among *K. pneumoniae* isolates separated from Egyptian patients with suspected nosocomial infections, to detect their drug resistance pattern and to look for bla SHV and bla CTX-M genes in such organisms. **Subjects and methods:** 138 *K. pneumoniae* isolates from Egyptian patients with suspected nosocomial infections were screened for ESBL production by the pattern of antimicrobial susceptibilities. Phenotypic identification for ESBL production was confirmed by double disc synergy test, phenotypic confirmatory double disc test and by MicroScan panel system. bla SHV and bla CTX-M genes in ESBL producing *K. pneumoniae* were detected using multiplex PCR. **Results:** The prevalence of ESBL producing *K. pneumoniae* was 21% (30/138). Their pattern of antimicrobial susceptibility showed that 90% was resistant to (Sulphamethoxazole/Trimethoprim), 70% was resistant to (Amoxicillin/Clavulanate), 63.3% was resistant to Cefotaxime and Ceftazidime, 60% was resistant to Amikacin, 46.7% was resistant to Doxycycline, Cefoxitin, Ceftriaxone and Levofloxacin, 40% was resistant to Cefepime, 20% was resistant to Ertapenem and (Sulbactam/Cefoperazone), 13.3% was resistant to (Piperacillin/Tazobactam), 10% was resistant to (Imipenem/Cilastatin) and Gentamycin and 6.7% was resistant to Meropenem and Ciprofloxacin. Among the ESBL producing *K. pneumoniae*, three out of 30 (10%) and 16 out of 30 (53.3%) were positive for bla SHV and bla CTX-M genes respectively.

It could be concluded that ESBL producing isolates of *K. pneumoniae* have been increasingly recognized in the hospital settings in Egypt and are associated with multiple drug resistance. Thus,

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molecular identification of the genes encoding beta lactamases would be essential for a reliable epidemiological investigation of their transmission in hospitals and antimicrobial resistance.

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1. Introduction

Extended spectrum or third generation cephalosporins e.g. Cefotaxime, Ceftazidime, and Ceftriaxone were introduced into clinical use in the early 1980s to provide effective therapy largely for nosocomial infections caused by multi-resistant and especially Gentamycin resistant *Enterobacteriaceae*, particularly *Klebsiella* spp., *Enterobacter* spp. and *Pseudomonas aeruginosa* [1].

Extended-spectrum beta-lactamases (ESBLs) are a heterogeneous group of plasmid-mediated bacterial enzymes that confer significant resistance to oxyimino-cephalosporin and monobactam antimicrobials [2]. Resistance to cephalosporins has become widespread throughout the world, and numerous types of ESBLs have been detected in various bacterial organisms [3]. Various authors have reported the prevalence of ESBLs to be in the range of 6–88% in various hospitals [4], especially among *Klebsiella pneumoniae* and *Escherichia coli*.

Systemic infections with ESBL producing *Enterobacteriaceae* are associated with severe adverse clinical outcomes [5]. It is thus essential for a diagnostic microbiology laboratory to have updated methods for the detection of ESBL-producing strains, taking into account the local epidemiology of ESBL genotypes and their various expression profiles [6].

Extended-spectrum beta-lactamases arise mainly due to mutations in β -lactamases encoded by the bla SHV, bla TEM, and bla CTX-M genes. More than 300 different ESBL variants have been described [7]. Though TEM and SHV variants are the most common ESBLs, during the past decade, strains expressing CTX-M ESBLs have begun to emerge in many countries [8] and are now the most frequent non-TEM, non-SHV ESBL type. CTX-M β -lactamases are characterized by selective hydrolysis of cefotaxime rather than Ceftazidime, though some CTX-M types, such as CTX-M-15, may actually hydrolyse Ceftazidime [9].

Detection of ESBLs is primarily based on phenotypic testing, such as evidencing a synergy image using the double-disc synergy test performed with expanded-spectrum cephalosporins and ticarcillin-clavulanic acid discs [10,11]. This test is not always obvious and is usually time-consuming since it requires subculturing or the use of cloxacillin-containing plates to inhibit the naturally occurring and plasmid-mediated cephalosporinases [12]. Over the last 20 years, alternative strategies aimed at replacing or complementing traditional phenotypic methods have been proposed. Standard PCR and gene sequencing is still the most widely used technique [13,14].

The aim of the present study was to find the prevalence of ESBL producing *K. pneumoniae* among *K. pneumoniae* isolates separated from Egyptian patients with suspected nosocomial infections, to detect their drug resistance pattern and to look for bla SHV and bla CTX-M genes in such organisms.

2. Patients and methods

One hundred and thirty-eight *K. pneumoniae* isolates from different clinical specimens (blood, sputum, urine, pus aspirates and endotracheal secretions) were collected over three months from patients with suspected nosocomial infections in a tertiary care hospital. Specimens were collected and cultured on blood agar (Oxoid Ltd., Basingstoke, UK) and MacConkey agar (Difco/BD Diagnostics Systems, Sparks, MI, USA) except for urine samples which were plated on Cysteine Lactose Electrolytes Deficient (CLED) agar. All isolated *K. pneumoniae* strains were identified in the clinical samples by conventional microbiological methods (colonial morphology, Gram staining, and biochemical tests) according to Collee et al. [15].

Initial screening for ESBL was done by the pattern of antimicrobial susceptibilities. Phenotypic identification of ESBL production was confirmed by double disc synergy test, and phenotypic confirmatory double disc test according to CLSI guidelines [16] and by MicroScan panel system.

2.1. Antimicrobial susceptibility testing and initial ESBL identification

Isolates were tested for the pattern of antimicrobial susceptibilities by an agar disc diffusion method using paper discs (AB Biodisk, Solna, Sweden) on Mueller–Hinton (MH) agar including susceptibility to the following antibiotics: Amikacin, (Amoxicillin/Clavulanate), Cefepime, (Cefoperazone/Sulbactam), Cefotaxime, Cefoxitin, Ceftazidime, Ceftriaxone, Ciprofloxacin, Doxycycline, Ertapenem, Gentamycin, (Imipenem/Cilastatin), levofloxacin, Meropenem, (Piperacillin/Tazobactam) and (Sulfamethoxazole/Trimethoprim). After overnight incubation at 37 °C, inhibition zone diameters were read. The results of a disc diffusion test are interpreted by comparing the measured zone diameter with the interpretive criteria recommended by CLSI guidelines [16].

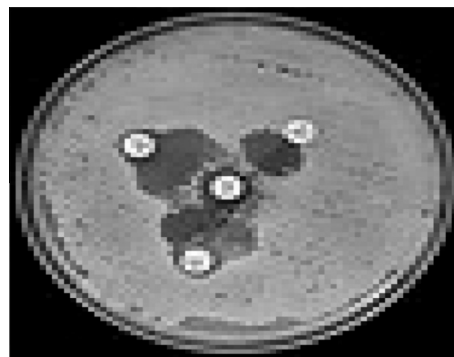


Figure 1 Double disc synergy test using discs of CTX30 = Cefotaxime 30 μ g, CAZ30 = Ceftazidime 30 μ g, CRO30 = Ceftriaxone 30 μ g, AMC30 = Amoxyclovan 30 μ g.

2.1.1. Double disc synergy test [17]

The organism to be tested was spread onto a Mueller–Hinton agar plate. The antibiotic discs used were Ceftriaxone (30 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), and Amoxicillin/Clavulanic acid (20/10 µg). The three antibiotics were placed at distances of 30 mm (edge to edge) from the Amoxicillin/Clavulanic acid disc that was placed in the middle of the plate. After 24-h incubation, if an enhanced zone of inhibition between either of the Cephalosporin antibiotics and the Amoxicillin/Clavulanic acid disc occurred, the test was considered positive. This indicated synergistic activity with Clavulanic acid and the presence of an ESBL (Fig. 1).

2.1.2. Phenotypic confirmatory disc diffusion test (combined disc method) [18]

A Ceftazidime (30 µg) disc was used alone and in combination with Clavulanic acid (30 µg/10 µg) for phenotypic confirmation of the presence of ESBLs. A ≥ 5 mm increase in zone diameter for either of the Cephalosporin discs and their respective Cephalosporin/Clavulanate disc was interpreted as ESBL producer (Fig. 2)

2.1.3. MicroScan panel identification (ID) and antimicrobial susceptibility testing (AST)

The MicroScan WalkAway 96 SI System (Dade Behring, CA) identification and antimicrobial susceptibility testing were performed with Neg BP combo panel type 30. All procedures were performed according to the manufacturer's recommendations. The screening of ESBL-producing microorganisms and interpretation were based on rules contained within the LabPro Expert system (version 2.0), which uses growth in the presence of Cefpodoxime (4 µg/ml) and Ceftazidime (1 µg/ml) at concentrations recommended by the CLSI [16] for ESBL screening. The MicroScan panel MIC-based Clavulanate synergy test was used for confirmation of the presence of ESBLs.

Antibiotic susceptibilities were determined by overnight microdilution, with commercial dehydrated panels provided by (Dade Behring, CA). The ESBL plus panels were prepared and inoculated according to the manufacturer's recommended procedures. The inoculated panels were then placed into the MicroScan WalkAway instrument for incubation, final read-



Figure 2 Phenotypic confirmatory disc diffusion test using Ceftazidime disc 30 µg and Ceftazidime/Clavulanate disc 30/10 µg. ≥ 5 mm increase in zone diameter for antimicrobial agent tested in combination with Clavulanate versus its zone when tested alone = ESBL.

ing and interpretation of the results, which was conducted according to CLSI guidelines [16]. The following antimicrobial agents (concentration ranges in mg/L) were used in the MicroScan ESBL plus panel: Aztreonam, 0.5–16; Cefepime, 1–32; Cefotetan, 1–32; Cefpodoxime, 0.5–64; Cefotaxime, 0.5–128; Cefotaxime/Clavulanate, 0.125/4–16/4; Cefoxitin, 2–32; Ceftazidime, 0.5–128; Ceftazidime/Clavulanate, 0.125/4–16/4; Ceftriaxone, 1–64; Imipenem, 0.5–16; Meropenem, 0.5–16; Piperacillin, 16–64.

2.2. Multiplex PCR for detection of bla SHV and bla CTX-M genes

2.2.1. Preparation of plasmid DNA

Plasmid DNA was isolated from a single colony of each isolate according to Jemima and Verghese [19] using Gen Jet Plasmid Miniprep Kit Cat. No. K0502 (Fermentas, Thermo scientific, France).

2.2.2. Multiplex PCR

The Multiplex PCR was done to identify bla SHV and bla CTX-M genes simultaneously. The sequences of primers used for detection of bla SHV gene were: 5'ATT TGT CGC TTC TTT ACT CGC-3' as a forward primer and 5' TTT ATG GCG TTA CCT TTG ACC-3' as a reverse primer. The two primers included a 1018 bp fragment. For detection of bla CTX-M gene, the sequences of primers used were 5'-ATG TGC AGY ACC AGT AAR GT 3' as a forward primer and 5'-TGG GTR AAR TAR GTS ACC AGA 3' as a reverse primer. The two primers included a 544 bp fragment [19].

PCR was performed in a DNA thermal cycler (Biometra, Germany) using the kit Maxima Hot Start PCR Master Mix (2 \times), Cat. No. K1051 (Fermentas, Thermo scientific, France), according to the manufacturer's instructions. It is a ready-to-use mix containing Maxima hot start Taq DNA polymerase, optimized hot start PCR buffer, Mg Cl₂, and dNTPs. The reaction was performed in a 50-µl volume containing 25 µl Maxima hot start PCR master mix (2 \times), the primers (1 µM each), together with one µg of the extracted DNA. Nuclease free water was used to complete 50 µl volumes.

Amplification profile was done according Jemima and Verghese [19]. Positive and negative controls were amplified with each run. After gel electrophoresis, the ethidium bromide stained PCR products were visualized under UV light. The size of the bands were compared to the molecular weight marker, Gene Ruler 1 Kb plus DNA ladder Cat. No. SM1331 (Fermentas, Thermo scientific, France).

3. Results

From the 138 *K. pneumoniae* isolates, 30 were ESBL producing (21%) that were obtained from 30 clinical samples as follows: 11 (36.7%) from sputum, 3 (10%) from urine, 6 (20%) from blood, 5 (16.7%) from pus aspirates, and 5 (16.7%) from endotracheal secretions.

The pattern of antimicrobial susceptibility among the 30 ESBL producing *K. pneumoniae* showed that 90% was resistant to (Sulfamethoxazole/Trimethoprim), 70% was resistant to (Amoxicillin/Clavulanate), 63.3% was resistant to Cefotaxime and Ceftazidime, 60% was resistant to Amikacin, 46.7%

Table 1 Pattern of antimicrobial susceptibility in ESBL producing *Klebsiella pneumoniae* isolates separated from patients ($N = 30$).

Sensitivity Name of antibiotic disc	Sensitive		Intermediate		Resistant	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
Amikacin	12	40	–		18	60
Amoxicillin/Clavulanate	4	13.3	5	16.7	21	70
Cefepime	12	40	6	20	12	40
Cefoperazone/Sulbactam	17	56.7	7	23.3	6	20
Cefotaxime	5	16.7	6	20	19	63.3
Cefoxitin	12	40	4	13.3	14	46.7
Ceftazidime	5	16.7	6	20	19	63.3
Ceftriaxone	5	16.7	11	36.6	14	46.7
Ciprofloxacin	24	80	4	13.3	2	6.7
Doxycycline	11	36.6	5	16.7	14	46.7
Ertapenem	20	66.7	4	13.3	6	20
Gentamycin	18	60	9	30	3	10
Imipenem/Cilastatin	24	80	3	10	3	10
Levofloxacin	15	50	1	3.3	14	46.7
Meropenem	24	80	4	13.3	2	6.7
Piperacillin/Tazobactam	15	50	11	36.7	4	13.3
Sulfamethoxazole/Trimethoprim	2	6.7	1	3.3	27	90

was resistant to Doxycycline, Cefoxitin, Ceftriaxone and Levofloxacin, 40% was resistant to Cefepime, 20% was resistant to Ertapenem and (Cefoperazone/Sulbactam), 13.3% was resistant to (Piperacillin/Tazobactam), 10% was resistant to (Imipenem/Cilastatin), and Gentamycin and 6.7% was resistant to Meropenem and Ciprofloxacin (Table 1).

Multiplex PCR detection of bla SHV and bla CTX-M genes in ESBL producing *K. pneumoniae* showed that among the clinical isolates, 63.3% of isolates carried one of the two genes. Three out of 30 (10%) isolates were positive for bla SHV gene, giving a 1018 bp band, while 16 out of 30 (53.3%) isolates were positive for bla CTX-M gene. Isolates that were positive to bla SHV gene were different from those that were positive to bla CTX-M gene (Fig. 3).

4. Discussion

The spread of ESBL-producing bacteria has been strikingly rapid worldwide, indicating that continuous monitoring systems and effective infection control measures are absolutely required. Therapeutic options for infections due to ESBL producers have also become increasingly limited. Health care interactions including the use of antibiotics, particularly oxyminocephalosporins and hospital transfer are among well-

defined risk factors for acquisition of ESBL-producing bacteria [20].

Molecular detection and identification of beta lactamases would be essential for a reliable epidemiological investigation of antimicrobial resistance. These enzymes can be chromosomal or plasmid mediated. The gene code for the enzymes may be carried on integrons. Integrons help in the dissemination of antimicrobial drug resistance in health care settings. Therefore, ESBL producing organisms should be identified quickly so that appropriate antibiotic usage and infection control measures can be implemented [21].

The present study was conducted on 30 ESBL producing *K. pneumoniae* isolates that were obtained from 30 clinical samples isolated from patients admitted to a tertiary care hospital and have suspected nosocomial infections. The clinical samples were 11 (36.3%) sputum, 6 (20%) blood, 5 (16.7%) pus aspirates, 5 (16.7%) endotracheal secretions and 3 (10%) urine. El sherif and Maamoun [22] performed a study on isolates with the ESBL phenotype in a hospital in Egypt, that were obtained from various clinical specimens: 34% was isolated from urine cultures, 21.5% from sputum, 17% from blood culture, 13% from wounds, 10% from stool of healthy carrier and 4.5% from bronchoalveolar lavages. However, in that study, 41.5% of isolates

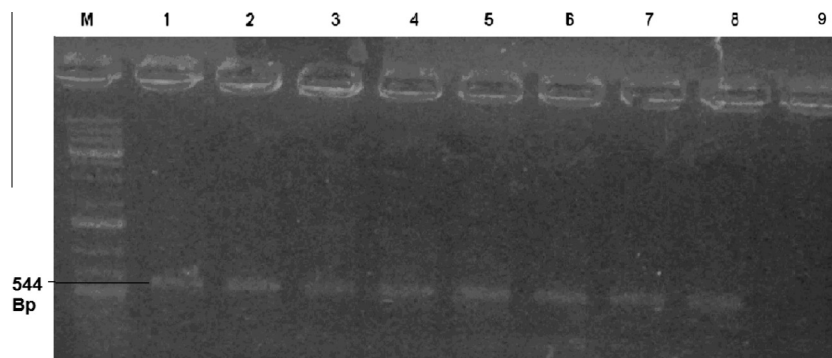


Figure 3 Gel electrophoresis of the PCR products of bla CTX-M gene. First lane, molecular weight marker; Gene Ruler 1Kbp DNA ladder, lanes numbered 1, 2, 3, 4, 5, 6, 7, 8 show 544 bp bands of PCR products while lane number 9 shows negative result.

were from outpatients (urine and stool specimens only), and the remaining 58.5% was from inpatients.

In the present study, the ESBL producing *K. pneumoniae* isolates were 21% (30/138) of all *K. pneumoniae* collected over a period of three months. In comparison with the rest of the world, there is generally a lack of comprehensive data regarding ESBL-producing *Enterobacteriaceae* in African countries. However, there is sufficient evidence to highlight the prevalence of ESBLs in Africa. It is recognized that Egypt has an extremely high rate of ESBL producers, with up to 70% of isolates producing the enzyme [23]. An outbreak was reported in a neonatal intensive care unit in Cairo, Egypt, in which 80% of the isolates were *K. pneumoniae*, of which 58% was ESBL producers [24].

Some studies reported high percentages of ESBL producing organisms, Feizabadi et al. [25] found that among 89 *K. pneumoniae* isolates, the phenotypic confirmatory test detected 62 isolates (69.7%) as ESBL-producing *K. pneumoniae*. Also, Lal et al. [26] in India found that 86% of *Klebsiella* isolates were found to be resistant to at least one of the third generation cephalosporins, of which 97.1% was confirmed to be positive for ESBL. Another study from north India reported the prevalence of ESBL as 66.7% in *K. pneumoniae* isolates [27]. And, Rodrigues et al. [28] have reported that 53% of the nosocomial Gram-negatives were found to be ESBL producers.

On the other hand, other studies reported low percentages of ESBL producing organisms, Chong et al. [29] found that *K. pneumoniae* with ESBL production were 4.9% of total *K. pneumoniae* isolated in a Japanese hospital. And, Kumar et al. [30] from Hyderabad, India, reported that 19.8% of *Enterobacteriaceae* species isolated over a period of one year were ESBL producers.

The prevalence of ESBL-expressing bacteria varies across different geographical regions. Fewer than 10% of isolated strains express ESBLs in Sweden, Japan and Singapore, compared to rates higher than 30% in Portugal, Italy, New York and some Latin American countries. In Turkey, as many as, 58% of isolated strains express ESBLs [7].

In the present study, the pattern of antimicrobial susceptibilities was studied in all ESBL producing *K. pneumoniae* isolates and showed that 90% was resistant to (Sulfamethoxazole/Trimethoprim), 70% was resistant to (Amoxicillin/Clavulanate), 63.3% was resistant to Cefotaxime and Ceftazidime, 60% was resistant to Amikacin, 46.7% was resistant to Doxycycline, Cefoxitin, Ceftriaxone and levofloxacin, 40% was resistant to Cefepime, 20% was resistant to Ertapenem and (Cefoperazone/Sulbactam), 13.3% was resistant to (Piperacillin/Tazobactam), 10% was resistant to (Imipenem/Cilastatin), and Gentamycin and 6.7% was resistant to Meropenem and Ciprofloxacin. In agreement with the present study, Chong et al. [29] found that the ESBL-producing bacteria were resistant to almost all generations of cephalosporins but remained highly susceptible to carbapenems which is consistent with the resistance pattern of organisms with ESBLs [31].

Feizabadi et al. [25] studied 89 *K. pneumoniae* isolates and found that all strains were susceptible to imipenem and the rates of resistance to different antibiotics were in the following order: Aztronam (79.7%), Cefexime (67.4%), Cefpodoxime (66.2%), Cefotaxime (65.1%), Ceftazidime (61.7%) while, Morosini et al. [32] mentioned that most ESBL-producing strains, particularly those with the TEM, SHV, and CTX-M genotypes, are resistant to aminoglycosides.

In the present study, 63.3% of ESBL producing *K. pneumoniae* carried one of the two genes. Among the clinical isolates, 3 out of 30 (10%) isolates were positive for bla SHV gene while 16 out of 30 (53.3%) isolates were positive for bla CTX-M gene. Isolates that were positive to bla SHV gene were different from those that were positive to bla CTX-M gene.

El sherif and Maamoun [22] studied 77 isolates of *Klebsiella* spp. with the ESBL phenotype for detection of the four genes of bla (TEM, SHV, CTX-M, and OXA) using multiplex PCR. SHV gene was found in 45.5% of *Klebsiella* spp. and CTX-M was found in 44% of *Klebsiella* spp. And, Chong et al. [29] found that among 20 ESBL *K. pneumoniae*, TEM/SHV were found in 4 (20%), CTX-M 0 (0%) and TEM/SHV + CTX-M 16 (80%) while, Khalaf et al. [33] mentioned that the CTX-M genotype appeared to be the most common type in North Africa. And, it has been known for their rapid spread in Europe and Asia [34].

Kiratisin et al. [35] noticed increasing trends in the prevalence of CTX-M ESBLs. They found that a total of 87.3% of ESBL-producing isolates carried several bla genes. The prevalence of bla CTX-M was strikingly high: 99.2% for ESBL-producing *K. pneumoniae* which carried bla SHV at 87.4%. Thus, they concluded that CTX-M ESBL is highly endemic in Thailand. Previous survey in Thailand from 1994 to 1996 revealed that, among 32 isolates of ESBL-producing *E. coli* and ESBL-producing *K. pneumoniae*, bla SHV was the most predominant while bla CTX-M was not detected [36]. The first detection of bla CTX-M in Thailand was documented from a study of 48 *Enterobacteriaceae* isolates recovered during 1998 and 1999, where the prevalence of bla CTX-M was 52% and subsequently increased to 65% among 52 isolates collected in 2003 [37].

Jemima and Verghese [19] performed multiplex PCR for detection of bla CTX-M and bla SHV in 62 ESBL positive *Klebsiella* spp. Results showed that bla SHV like genes were found in 45% and bla CTX-M genes were found in 40% of *Klebsiella* spp. Also, Sekar et al. [38] reported the prevalence of bla CTX-M gene in 14/39 selected clinical Gram negative isolates in India.

However, low percentage of CTX-M gene was reported in other study. Tofteland et al. [6] studied 25 clinical isolates of *K. pneumoniae* in Norway with reduced susceptibilities to oxyimino-cephalosporins for bla TEM/SHV/CTX-M ESBL genes. An ESBL phenotype was recognized in 84% of *K. pneumoniae* isolates expressing reduced susceptibility to an expanded-spectrum cephalosporin by the E test and combined disc method. Bla CTX-M was reported in three cases, bla SHV in 15 cases and bla TEM was detected in only one case.

Higher percentages of SHV gene were reported in other studies, Feizabadi et al. [25] detected the genes encoding the ESBLs including bla SHV and bla CTX-M among 89 *K. pneumoniae* isolates by PCR. The prevalence of bla SHV was 67.4% and bla CTX-M-I was 46.51% and bla CTX-M-III was 29%. Lal et al. [26] randomly selected 95 *Klebsiella* isolates for PCR of TEM and SHV genes. Isolates having both TEM and SHV genes were common (67.3%) whereas only 20% isolates possessed TEM gene and 8.4% SHV gene alone. And, Grover et al. [39] in India reported that bla TEM and bla SHV alone or together were present in 88.8% of the *Klebsiella* isolates in their study.

It could be concluded that ESBL producing isolates of *K. pneumoniae* have been increasingly recognized in the hospital

settings in Egypt as well as throughout the world. Moreover, multiple resistances to antimicrobials are apparent. Thus, molecular identification of the genes encoding beta lactamases would be essential for a reliable epidemiological investigation of their transmission in hospitals and antimicrobial resistance.

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